Bacterial diversity in hydrothermal sediment and epsilonproteobacterial dominance in experimental microcolonizers at the Mid-Atlantic Ridge

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Summary

We report here a molecular survey based on 16S rRNA genes of the bacterial diversity found in two deep-sea vent niches at the Mid-Atlantic Ridge: hydrothermal sediment (Rainbow site), and microcolonizers made of three different substrates (organic-rich, iron-rich and pumice) that were exposed for 15 days to a vent emission. Bacterial diversity in sediment samples was scattered through many bacterial divisions. The most abundant and diverse environmental sequences (phylogenotypes) in our libraries corresponded to the Gammaproteobacteria, followed by the Acidobacteria. We detected members of all the subdivisions within the Proteobacteria. Myxobacterial lineages were the most represented within the delta subdivision. Phylotypes ascribing to the Cytophaga-Flavobacterium-Bacteroides, Planctomycetales, high and low G+C Gram-positives, Nitrospirae, and the candidate division TM7 were also identified. Compared to this broad taxonomic coverage, microcolonizers were almost exclusively colonized by epsilonproteobacteria, although these exhibited considerable morphological and phylogenetic in-group diversity. No specificity for any of the substrates tested was seen. This observation further supports the idea of the ecological dominance of epsilonproteobacteria in the fluid–seawater interface environment. Because oxidation of reduced S species and/or sulphur-reduction is thought to be essential for their energetic metabolism in these areas, we mapped different oxidation states of S in individual bacterial filaments from the iron-rich microcolonizer. For this, we used high-resolution, non-destructive synchrotron micro-X-ray Absorption Near-Edge Spectroscopy (micro-XANES), which revealed the co-existence of different S oxidation states, from sulphide to sulphate, at the level of individual cells. This suggests that these cells were metabolizing sulphur in situ.

Introduction

Deep-sea hydrothermal systems have attracted much attention during the last 30 years for a number of reasons. They constitute ecosystems: (i) with a rich animal and microbial diversity founded basically on chemolithoautotrophic primary production; (ii) where the most hyperthermophilic organisms have been isolated from, and (iii) that are considered modern analogues of ancient and more widespread hydrothermal systems (for reviews, see Prieur, 1997; Stetter, 1999; Jeanthon, 2000; Nisbet and Sleep, 2001; Reysenbach and Cady, 2001; Reysenbach and Shock, 2002). Hydrothermal emissions are hot (the temperature varies from a few degrees above seawater temperature in diffusers to more than 300°C in black smokers) and enriched in various metals from mantle regions as well as hydrogen sulphide and other reduced species. Their encounter with the cold (2°C on average) and highly oxygenated deep seawater generates steep physicochemical gradients. As a consequence, a variety of ecological niches form in these systems that are characterized by different temperatures, nature of substrate and availability of electron donors and acceptors for energy metabolism.

In addition to the study of strict hyperthermophiles from chimney fragments and hot fluids, the prokaryotic diversity of warm to hot fluid-seawater-mixing regions has been focus of intense research. Most microorganisms in
areas exposed to the fluid–seawater interface are thought to be chemolithoautotrophs or chemoorganotrophs oxidizing reduced forms of sulphur and iron, as was proposed long ago (Jannasch and Mott, 1985). Although a relatively large bacterial diversity is found in these areas, members of the epsilon subdivision of the Proteobacteria appear to predominate, regardless the geographic provenance of samples. This trend has been observed in vent-cap chambers incubated in situ at the Mid-Atlantic Ridge (Reysenbach et al., 2000; Corre et al., 2001), mat samples from the South-east Pacific Rise (Longnecker and Reysenbach, 2001), and in the epibiotic microbiota of many deep-sea vent animal teguments or tubes. These include the polychaetes Alvinella pompejana and Paralvinella palmiformis (Haddad et al., 1995; Alain et al., 2002a) and the vestimentiferan Riftia pachyptila (López-García et al., 2002), endemic from the Eastern Pacific, and the Atlantic shrimp Rimicaris exoculata (Polz and Cavanaugh, 1995).

The Epsilonproteobacteria group some S-oxidizers, such as Thiovulum spp. which oxidize hydrogen sulphide and displays chemotaxis along oxygen gradients (Thar and Fenchel, 2001) or Arcobacter spp., some of which produce filamentous sulphur (Wirsen et al., 2002). In addition, several epsilonproteobacterial isolates from deep-sea vents reduce sulphur (Campbell et al., 2001; Alain et al., 2002b; Miroshnichenko et al., 2002). Very recently, Takai et al. (2003) have reported the isolation of epsilonproteobacteria from hydrothermal vents at the Okinawa Trough (Western Pacific) and the Central Indian Ridge that cluster with environmental groups lacking cultivated members so far. These are able to grow using H₂ or reduced sulphur compounds (elemental sulphur or thiosulphate) as electron donors, and O₂, nitrate or elemental sulphur as electron acceptors. Deep-sea vent sulphur or thiosulphate oxidizers are also frequent within the alpha and gamma subdivisions of the Proteobacteria (Jannash et al., 1985; Distel et al., 1988; Muyzer et al., 1995), and within members of the Flavobacteria (Teske et al., 2000).

Sulphur-based metabolisms thus play a major role in this ecosystem, which is particularly substantiated by the development of mats of sulphur oxidizers sometimes associated with apparent filamentous sulphur structures (Jannash et al., 1989; Moyer et al., 1995; Taylor et al., 1999).

The prokaryotic diversity in hydrothermal sediments has been considerably less studied, but it appears to be much more varied than that of exposed surfaces. To date, most reports relate to Pacific areas. A recent 16S rRNA-based molecular survey of archaea and bacteria in Guaymas basin hydrothermal sediment revealed a variety of archaea, including ANME-1 lineages that could participate in anaerobic methane oxidation, and a wide diversity of lineages scattered in various bacterial divisions (Teske et al., 2002). This high level of prokaryotic diversity in sediments is paralleled by a considerable diversity of microbial eukaryotes, as deduced from 18S rRNA genes retrieved from the same site (Edgcomb et al., 2002). In the case of eukaryotes, a variety of typical photosynthesizing lineages (e.g. green algae) was identified, what led to the conclusion that contamination from communities sedimenting from upper layers in the water column existed and could not be distinguished from autochthonous members. Likewise, although most bacterial lineages were affiliated with typical deep-sea or anaerobic bacteria, the presence of cyanobacterial-like sequences suggests that part of the bacterial diversity observed in the Guaymas system is not original from the sediment. We recently carried out a molecular survey of 18S rRNA genes present in Mid-Atlantic Ridge hydrothermal sediment. Our results showed, in contrast, the existence of a wide diversity of eukaryotic lineages that were in no case related to photosynthesisers, thus excluding the possibility of contamination from surface water in this site (López-García et al., 2003). The bacterial diversity in these samples should therefore correspond to autochthonous bacteria in these deep-sea sediments as well.

Here, we present a 16S rRNA-based molecular survey of the bacterial diversity found in this hydrothermal sediment from the Rainbow site (Mid-Atlantic Ridge) presumably lacking contaminants from surface waters, and in three experimental devices for microbial colonization (microcolonizers) that were exposed for two weeks to a fluid emission (Mid-Atlantic Ridge). This should allow us, first, comparing Atlantic and Pacific hydrothermal sediment communities, second, comparing sediment and substrates directly exposed to the fluid–seawater interface in the same oceanic ridge, and finally, testing whether there is any specificity in the colonization of the different substrates, namely inert mineral, organic-rich, and iron-rich, that were used in the microcolonizers. In addition, using a synchrotron-based approach (see Philippot et al., 2002 and Foriel et al., 2003 for details), we show the coexistence of different S oxidation states in individual cells from microcolonizers, suggesting that they were actively metabolizing sulphur.

Results

Bacterial diversity in hydrothermal sediment

The hydrothermal sediment from the Rainbow site studied here is characterized by record enrichments in different metals and rare earth elements (Cave et al., 2002; Douville et al., 2002). Levels of Fe, Cu, Mn, V, P and As are especially high, even when compared to those of other hydrothermal sediments. CaCO₃ accounted for ~32% of the sediment (dry weight), and were contributed
mostly by haptophyte coccoliths and foraminifer shells (López-García et al., 2003). After DNA extraction, we amplified 16S rRNA genes present in the sample by polymerase chain reaction (PCR) using different combinations of bacterial and prokaryotic specific primers (see Experimental procedures). We then constructed a total of four different 16S rDNA libraries, from which we sequenced partially more than one hundred clones. This allowed us to have a first insight on the diversity of bacterial groups represented in the sediment by BLAST sequence comparison with sequences in databanks. As can be seen in Fig. 1, we detected a variety of bacterial lineages. The most diverse and abundant clones in our libraries affiliated to the Gammaproteobacteria and to the Acidobacteria. The rest of phylotypes appear scattered over a broad taxonomic distribution including the additional four subdivisions of the Proteobacteria (alpha, beta, delta and epsilon), the Cytophaga-Flavobacterium-Bacteroides (CFB) group, the High G+C (Actinobacteria) and Low G+C (Firmicutes) Gram-positives, the Planctomycetales, the Nitrospirae and the candidate division TM7 (Fig. 1).

We then selected a number of representative clones covering the whole diversity observed, which were completely sequenced and subjected to more detailed phylogenetic analysis (Table 1). In order to handle a reasonable taxonomic diversity encompassing the closest environmental sequences to our clones and also representatives of major bacterial groups compatible with maximum likelihood (ML) analyses, we constructed five different data sets. Four of these, including all but one sediment sequence, are shown in Fig. 2. The fifth data set corresponds to the Epsilonproteobacteria (see below). In general, our clones ascribed to groups that have been already identified in deep-sea vents or sediments, and their closest relatives are environmental sequences from these environments, or otherwise, from anaerobic sludge or metal-contaminated sites (Fig. 2 and Table 1). One exception is clone AT-s2–18 (Fig. 2A), which clearly branches within the candidate division TM7 (Hugenholtz et al., 2001). Members of this group had not been previously identified in deep-sea vents. Among the most represented bacterial divisions were the CFB and the Acidobacteria, the latter being particularly diverse. In both cases, our sequences defined clusters with other environmental phylotypes (Fig. 2B).

We found representatives of all Proteobacteria subdivisions (Fig. 2C and D and see below). Among the Deltaproteobacteria, most phylotypes branch within the Myxobacteria, gliding bacteria with complex social behaviour and developmental cycles that are not uncommon in deep-sea vent areas (Moyer et al., 1995; Teske et al., 2002). The clone AT-s3–57 forms a well-defined cluster with environmental sequences and with Bdellovibrio spp., fast swimmer bacterial predators common in different environments (Martin, 2002; Snyder et al., 2002). Among the sequences belonging to the Betaproteobacteria, we identified a sequence (AT-s3–41) that can be ascribed to the genus Hydrogenophilus (Fig. 2C), whose members are thermophilic (Hayashi et al., 1999; Stohr et al., 2001). We also detected some phylotypes within the Alphaproteobacteria, but most of the sediment diversity was found within the Gammaproteobacteria (Fig. 2D). Some of these phylotypes clustered with other environmental sequences, many of which (clones MERTZ) have been retrieved from Antarctic sediments (Bowman and McCuaig, 2003). However, several sequences did not have clear close relatives among sequences in databases, particularly those of...
Table 1. Phylogenetic affiliation of bacterial clones obtained from hydrothermal sediment at the Mid-Atlantic Ridge (Rainbow site) as deduced from BLAST searches.

<table>
<thead>
<tr>
<th>Clone</th>
<th>Sequence length (bp)</th>
<th>% GC</th>
<th>% identity</th>
<th>Phylum</th>
<th>Closest 16S-rDNA match in database, nature of habitat where it was retrieved (accession number)</th>
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<tbody>
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<td>AT-s16</td>
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<td>54.35</td>
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<td>Gammaproteobacteria</td>
<td>Sulphur-oxidising bacterium OAlII2, shallow vent (AF170423)</td>
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<tr>
<td>AT-s26</td>
<td>1484</td>
<td>56.46</td>
<td>2</td>
<td>Gammaproteobacteria</td>
<td>Unc. gammaproteobacterium clone MERTZ_2 CM_24, continental shelf sediments collected off Antarctica (AF424060)</td>
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<td>AT-s43</td>
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<td>Unc. bacterium clone BPC036, hydrocarbon seep sediment (AF154089)</td>
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<tr>
<td>AT-s68</td>
<td>1456</td>
<td>54.61</td>
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<td>Gammaproteobacteria</td>
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<td>Gammaproteobacteria</td>
<td>Unc. gammaproteobacterium BD6-6, deep-sea sediments (AB015576)</td>
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<td>Sulphur-oxidizing chemosymbiotic endosymbiont of the bivalve Codakia costata (L25712)</td>
</tr>
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<td>1452</td>
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<td>Sulphur-oxidizing bacterium NDII-1, shallow water hydrothermal vent (AF170424)</td>
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<td>AT-s3–57</td>
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<td>50.98</td>
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</tr>
<tr>
<td>Clone</td>
<td>Sequence Length (bp)</td>
<td>% GC</td>
<td>Total Number of Similar Sequences</td>
<td>Phylogenetic Ascription</td>
<td>Closest 16S-rDNA Match in Database, Nature of Habitat Where It Was Retrieved (Accession Number)</td>
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<td>AT-s2</td>
<td>1468</td>
<td>59.33</td>
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<td>Acidobacteria</td>
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<td>1477</td>
<td>57.9</td>
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<tr>
<td>AT-s65</td>
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<td>AT-s2–57</td>
<td>1474</td>
<td>59.38</td>
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<td>CFB group</td>
<td>Unc. bacterium clone ML1218M-14, alkaline, hypersaline Mono Lake, California (AF452599)</td>
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<td>1498</td>
<td>51.04</td>
<td>1</td>
<td>TM7 candidate division</td>
<td>Unc. bacterium Noosa AW44, sludge from enhanced biological phosphorous removal reactor (AF269023)</td>
</tr>
</tbody>
</table>

a. Sequences 98–100% identical, including partial sequences.

CFB, Cytophaga-Flavobacterium-Bacteroides; Unc., uncultured.
clones AT-s3–26, AT-s2–13, AT-s16, and AT-s3–1. The inspection of the alignment and the fact that we detected in most cases other, closely related, sequences in our libraries (Table 1) suggest that these are not chimeras. Therefore, they could be representatives of novel groups or families within this subdivision.

Bacterial diversity in microcolonizers

The three substrates that had been exposed for 15 days to a fluid emission (organic-rich, iron-rich and inert mineral–pumice fragments) were densely colonized during that time, as can be seen in scanning electron microscopy
photographs (Fig. 3). The morphologies observed included many different types of filaments, and individual cells with different diameters and lengths, often forming biofilms embedded in a matrix. We could not observe a morphological specificity of cell types on the different substrates, as a wide range of forms was always observed. The only possible exception could be constituted by the observation of filaments surrounded by rigid sheaths that were observed exclusively on the iron-rich substrate (Fig. 3C).

From the morphological diversity observed, the finding of a large microbial diversity by molecular methods was to be expected. To test this and whether there was a differential colonization of the different substrates, we constructed environmental 16S rDNA libraries of DNA extracted from each one of the three substrates (see Experimental procedures). We partially sequenced ~60 clones, from which we selected 16 representatives that were sequenced completely. With the only exception of clone AT-co1, a CFB member (Fig. 2B and Table 2), all microcolonizer phylotypes affiliated to the Epsilonproteobacteria (Figs 1 and 4; Table 2). Despite this, phylotypes were diverse within the group, which correlates with the morphological variety observed. Our sequences branched within three different clusters, from which clusters B/II and F/I (Fig. 4 and see below) are very often found in deep-sea vent environments. A proportion of the clones, represented by AT-pp13, belongs to the Arcobacter group. Some marine Arcobacter spp. are autotrophic sulphide oxidizers (Wirsen et al., 2002). Several sequences belonged to the group B defined by Corre et al. (2001), which is equivalent to the group II by Teske et al. (2002). It includes members of Thiomicrospira, a genus including thiosulphate oxidizers (Kuenen et al., 1992). Takai et al. (2003) have recently isolated members of this cluster which are able to live on a mixture of hydrogen, thiosulphate and sulphur using nitrate or oxygen (1%) as electron acceptors. Most of our sequences branched within the cluster F/I, which includes many deep-sea vent and deep-sea sediment lineages as well as, although not very closely related to our sequences, epibionts of vent polychaetes and shrimp (Fig. 4). Takai et al. (2003) also isolated several members of this group able of growing on hydrogen-thiosulphate-sulphur mixtures using nitrate or oxygen as electron acceptors, but in this case most grew better with 10% oxygen in the medium. This could suggest this lineage may occupy a more oxygen-enriched, likely colder, part of the physico-chemical gradient at hydrothermal vents.

In general, most clones have as closest relatives environmental sequences retrieved from deep-sea sediment, deep-sea vent environments, and epibionts on Riftia pachyptila tubes (R76 and R103 clones) (López-García et al., 2002). The sequences of the three substrates being interspersed in the epsilonproteobacterial tree, a specificity of colonization appears to be rejected by our data.

Imaging sulphur-metabolizing activities in bacterial filaments from microcolonizers

Many epsilonproteobacteria isolated from deep-sea vents have sulphur oxidizing and/or reducing capabilities, as can be deduced from their growth in laboratory conditions (Campbell et al., 2001; Alain et al., 2002b; Miroshnichenko et al., 2002; Takai et al., 2003). Given the availability of large amounts of both, hydrogen sulphide and oxygen at the places colonized by the epsilonproteobacteria, the oxidation of reduced sulphur states with oxygen could be hypothesised as an obvious reaction to obtain energy from.

In order to test if sulphur oxido-reduction activities were taking place in situ, we analysed single filaments recovered from the microcolonies by micro-X-ray Absorption Near-Edge Structure (micro-XANES) at the European Synchrotron Radiation Facility (ESRF). Synchrotron-based analyses have been used recently to image the spatial distribution at a μm-scale of diverse potential
Table 2. Phylogenetic affiliation of bacterial clones from substrates recovered after 15 days exposure to a fluid source at the Mid-Atlantic Ridge (Lucky Strike).

<table>
<thead>
<tr>
<th>Clone</th>
<th>Sequence length (bp)</th>
<th>% GC</th>
<th>Total number of similar sequences a</th>
<th>Phylogenetic ascription</th>
<th>Closer 16S-rDNA match in database (accession number)</th>
<th>% Identity</th>
</tr>
</thead>
<tbody>
<tr>
<td>AT-co1</td>
<td>1435</td>
<td>49.76</td>
<td>1</td>
<td>CFB Group</td>
<td>Unc. CFB bacterium BD3-6, deep-sea sediments (AB015532)</td>
<td>89</td>
</tr>
<tr>
<td>AT-co11</td>
<td>1431</td>
<td>50.94</td>
<td>1</td>
<td>Epsilonproteobacteria</td>
<td>Unc. epsilonproteobacterium clone R76-B47, tubes of the vestimentiferan Riftia pachyptila (AF449245)</td>
<td>97</td>
</tr>
<tr>
<td>AT-co12</td>
<td>1427</td>
<td>50.18</td>
<td>3</td>
<td>Epsilonproteobacteria</td>
<td>Unc. epsilonproteobacterium clone R76-B49, tubes of the vestimentiferan Riftia pachyptila (AF449242)</td>
<td>96</td>
</tr>
<tr>
<td>AT-co13</td>
<td>1431</td>
<td>50.49</td>
<td>9</td>
<td>Epsilonproteobacteria</td>
<td>Unc. epsilonproteobacterium clone R103-B19, tubes of the vestimentiferan Riftia pachyptila (AF449233)</td>
<td>97</td>
</tr>
<tr>
<td>AT-co15</td>
<td>1437</td>
<td>51.43</td>
<td>2</td>
<td>Epsilonproteobacteria</td>
<td>Unc. bacterium PVB_OTU_6, microbial mat from hydrothermal vent system, Hawaii (U15106)</td>
<td>92</td>
</tr>
<tr>
<td>AT-co16</td>
<td>1428</td>
<td>51.22</td>
<td>3</td>
<td>Epsilonproteobacteria</td>
<td>Unc. epsilonproteobacterium clone BD2-1, deep-sea sediments (AB015531)</td>
<td>98</td>
</tr>
<tr>
<td>AT-co23</td>
<td>1430</td>
<td>50.59</td>
<td>2</td>
<td>Epsilonproteobacteria</td>
<td>Unc. epsilonproteobacterium clone NKB11, Nankai Trough sediments at 3843 m deep (U15104)</td>
<td>98</td>
</tr>
<tr>
<td>AT-cs3</td>
<td>1437</td>
<td>51.25</td>
<td>1</td>
<td>Epsilonproteobacteria</td>
<td>Unc. epsilonproteobacterium clone R103-B19, tubes of the vestimentiferan Riftia pachyptila (AF449233)</td>
<td>95</td>
</tr>
<tr>
<td>AT-cs7</td>
<td>1429</td>
<td>50.91</td>
<td>12</td>
<td>Epsilonproteobacteria</td>
<td>Unc. epsilonproteobacterium clone R103-B19, tubes of the vestimentiferan Riftia pachyptila (AF449233)</td>
<td>97</td>
</tr>
<tr>
<td>AT-cs8</td>
<td>1433</td>
<td>50.84</td>
<td>1</td>
<td>Epsilonproteobacteria</td>
<td>Unc. epsilonproteobacterium clone R103-B46, tubes of the vestimentiferan Riftia pachyptila (AF449238)</td>
<td>98</td>
</tr>
<tr>
<td>AT-cs10</td>
<td>1429</td>
<td>50.95</td>
<td>12</td>
<td>Epsilonproteobacteria</td>
<td>Unc. bacterium PVB_OTU_3 clone PVB_12, microbial mat from hydrothermal vent system, Hawaii (U15104)</td>
<td>96</td>
</tr>
<tr>
<td>AT-cs15</td>
<td>1428</td>
<td>49.3</td>
<td>5</td>
<td>Epsilonproteobacteria</td>
<td>Unc. epsilonproteobacterium clone R76-B78, tubes of the vestimentiferan Riftia pachyptila (AF449251)</td>
<td>97</td>
</tr>
<tr>
<td>AT-pp6</td>
<td>1434</td>
<td>51.26</td>
<td>2</td>
<td>Epsilonproteobacteria</td>
<td>Unc. bacterium PVB_OTU_2 clone PVB_7, microbial mat from hydrothermal vent system, Hawaii (U15100)</td>
<td>95</td>
</tr>
<tr>
<td>AT-pp13</td>
<td>1434</td>
<td>47.56</td>
<td>7</td>
<td>Epsilonproteobacteria</td>
<td>Unc. bacterium from vestimentiferan tubeworm (D83061)</td>
<td>94</td>
</tr>
<tr>
<td>AT-pp26</td>
<td>1422</td>
<td>51.02</td>
<td>9</td>
<td>Epsilonproteobacteria</td>
<td>Unc. epsilonproteobacterium clone BD2-5, deep-sea sediments (AB015535)</td>
<td>95</td>
</tr>
<tr>
<td>AT-pp27</td>
<td>1428</td>
<td>49.02</td>
<td>3</td>
<td>Epsilonproteobacteria</td>
<td>Unc. epsilonproteobacterium clone R76-B78, tubes of the vestimentiferan Riftia pachyptila (AF449251)</td>
<td>97</td>
</tr>
<tr>
<td>AT-pp46</td>
<td>1433</td>
<td>49.93</td>
<td>2</td>
<td>Epsilonproteobacteria</td>
<td>Unc. epsilonproteobacterium clone R76-B129, tubes of the vestimentiferan Riftia pachyptila (AF449252)</td>
<td>97</td>
</tr>
</tbody>
</table>

a. Sequences 98–100% identical, including partial sequences.
Clones named co were obtained from an organic substrate, pp from pumice, and cs from an iron-rich substrate. Unc., uncultured.
biogenic markers in fossil microfilaments from the East Pacific Rise (Philippot et al., 2002; 2003). The data presented here are part of a general study devoted to imaging metabolic processes at the scale of individual microbial filaments using high resolution and non-destructive synchrotron techniques. In this paper, we focus on the micro-XANES results. All other results are presented in a complementary paper (Foriel et al., 2003). Micro-XANES allows studying the relative amount and distribution of the different oxidation states of sulphur (S\textsuperscript{2-} to S\textsuperscript{6+}) at μm-scale, being therefore appropriate to obtain chemical redox S maps in single microbial filaments. Figure 5 shows the S redox maps obtained in a portion of a single filament taken from our iron-rich microcolonizer. We could detect three main sulphur species with X-ray peaks at 2471 eV, 2478 eV and 2482 eV. The marked peak at 2482

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eV is characteristic of sulphate. The peaks at 2471 eV and 2478 eV are typical of sulphide, -SH radicals or both. This suggests that S-metabolizing activities were taking place within cells. Although the technique does not allow discrimination between cell surface and cytoplasmic content, the distribution of S species in cells does not appear completely homogeneous (Fig. 5). This could indicate that redox reactions involving changes in S states occur preferentially at certain places, for instance at cellular poles or discretely in metabolically more active membrane areas.

Discussion

Bacterial diversity in Mid-Atlantic Ridge sediments

Our results show the existence of a variety of bacterial phylotypes in hydrothermal sediment and virgin substrates exposed for two weeks to a fluid emission at the Mid Atlantic Ridge. The diversity of the metal enriched hydrothermal sediment was comparatively larger, covering a wide spectrum of bacterial divisions as is the case in Pacific hydrothermal sediment (Teske et al., 2002). The phylogenetic diversity of bacteria was not only large, but some phylotypes are very divergent and could represent previously undetected lineages within bacterial divisions. This is accompanied by a large diversity, and a considerable degree of novelty, of microbial eukaryotes on the same Pacific and Atlantic hydrothermal sediments (Edgcomb et al., 2002; López-García et al., 2003). However, as 16S rRNA-based surveys of bacterial diversity have been carried out for more than 15 years (Olsen et al., 1986), the chances to find out novelty at great scale are logically decreasing. It is interesting to note the detection of the candidate division TM7 in deep-sea hydrothermal sediments, which extends the observation of its members to this habitat as well. Although no TM7 representative has ever been isolated in pure culture, there is a certain amount of information about their morphology and ultrastructure after their enrichment and identification by fluorescent in situ hybridization in wastewater treatment sludge. They possess typical Gram positive walls and, at least some of the species visualized, have sheaths (Hugenholtz et al., 2001). They have been detected in a variety of habitats, including terrestrial, freshwater and marine environments, being frequently identified in anaerobic environments (activated sludge, peat bogs) or in metal-contaminated soils or aquifers.

Also divergent are several gammaproteobacterial phylotypes detected in the Rainbow sediment without clear close neighbours in databases (Fig. 2). Gammaproteobacteria are very diverse in marine plankton and sediments, and might be dominant in deep-sea plankton (Fuhrman and Davis, 1997; López-García et al., 2001). They appear also diverse in deep-sea vents. Mats of the sulphur-oxidizing *Beggiatoa* spp. and *Thiomicrospira* spp. are frequent in these areas (Jannash et al., 1985; 1989; Muyzer et al., 1995), and many deep-sea vent animal endosymbionts belong to this group as well (Distel et al., 1988). Gammaproteobacteria not only accounted for the largest proportion of clones in our sediment libraries (44%) but they were also very diverse. Being metabolically
Neisseria branches at the base of a group comprising the parasitic (Madigan et al., 1997). In addition to the Gammaproteobacteria, we identified members of all the remaining subdivisions of the Proteobacteria in the Rainbow sediment (Figs 2 and 4). The Alphaproteobacteria, represented in our case by two sequences (Fig. 2C), are also versatile and frequent in marine samples, being particularly diverse and abundant in surface waters (Morris et al., 2002). We do not have any clue about the physiology of these microorganisms detected by their 16S rRNA and phylogenetically distant from their closest cultivated relatives. However, it is interesting to note that sulphate-producing thiosulphate-oxidizing alphaproteobacteria were frequently isolated from slope sediments and hydrothermal sediments in the Galapagos (Teske et al., 2000). We detected two phylotypes ascribing to the Betaproteobacteria. Members of this subdivision have not been detected in hydrothermal sediment from the Guaymas basin in the Pacific (Teske et al., 2002), but they have been identified in cold seep sediments at the Nankai Trough (nearly 4000 m depth) (Li et al., 1999) and in a vent-cap system displayed in the Mid-Atlantic Ridge (Reysenbach et al., 2000). One of the betaproteobacterial sequences branches at the base of a group comprising the parasitic Neisseria spp. and its lifestyle is unknown, but the other phylotype corresponds to a member of the genus Hydrogenophilus (Fig. 2C). Hydrogenophilus spp. are thermophilic and aerobic or facultative anaerobic, growing chemolithoautotrophically oxidizing hydrogen (Hayashi et al., 1999; Stohr et al., 2001). They have been identified in continental geothermal regions, but this is the first time that members of this genus are detected in deep-sea vents. The presence of one Hydrogenophilus phylotype attests for the hydrothermal influence on the sediment, although temperature measurements were not taken during sampling. Only one sequence of epsilonproteobacteria was detected, and it clustered with the Group F/I (Fig. 4) that was highly represented in microcolonizers. Finally, as is frequently observed in anoxic sediments, members of the Deltaproteobacteria were relatively abundant. Most deltaproteobacteria are sulphate reducers and strictly anaerobic. However, in our case, we failed to identify bona fide lineages of typical sulphate reducers, although they are most likely present (the diversity detected in the sediment is still far from saturation). Clone AT-s3–57 is very distantly related to Bdellovibrio spp., bacterial predators that are widely distributed (Martin, 2002; Snyder et al., 2002), and the rest of our clones belonged to the Myxobacteria, gliding microorganisms displaying complex social and developmental cycles. Myxobacteria are heterotrophs that were thought to be exclusively aerobic some time ago, but anaerobic species have been recently isolated (Reichenbach, 1999; Sanford et al., 2002). Myxobacteria are frequent in cold marine sediments (Ravenschlag et al., 1999), but they have also been detected in hydrothermal sediments at Loihi seamount (Hawaii) and Guaymas (Moyer et al., 1995; Teske et al., 2002).

In addition to the Proteobacteria, and several lineages scattered in various bacterial divisions (Nitrospirae, low GC and high GC Gram-positives, TM7 and Planctomycetes), we found in sediment samples a variety of Cytophaga-Flavobacterium-Bacteroides and, most particularly, of the Acidobacteria/Holophaga (Fig. 2B). Most members of the Acidobacteria are not yet cultivated, but have been detected by molecular tools in activated sludge or in marine sediments (Ludwig et al., 1997; Ravenschlag et al., 1999). Their presence has not been previously reported in deep-sea vents, and they have been detected neither in Guaymas nor in the Loihi seamount area (Moyer et al., 1995; Teske et al., 2002). However, they appear to be quantitatively very abundant in shallow submarine vents near to Milos (Greece) (Sievert et al., 2000). This abundance was interpreted as a consequence of an allochthonous input of organic matter to the system, since cultivated members of the group are heterotrophs. Acidobacteria were also found to be very abundant in soils following a geothermal heating event at Yellowstone (Norr is et al., 2002). Their amount increased in soils incubated at 50°C indicating that various members of this group are thermophilic. This opens the possibility for the Rainbow lineages being also thermophilic.

A first comparison of the bacterial diversity in Atlantic and Pacific hydrothermal sediments reveals, on the one hand, some similarities, as sediment samples show a diversity much larger than that observed in fluid–seawater interface but, on the other hand, there are some differences. Thus, neither alpha- and beta-proteobacterial nor acidobacterial sequences were retrieved from the Guaymas sediment (Teske et al., 2002). Conversely, some bacterial divisions were not detected in our Rainbow sediment (e.g. candidate divisions OP11, OP5 or OP3). This could reflect the fact that diversity has not been fully explored in both cases and/or the introduction of different biases during library construction. However, there could also be true differences owing to the particular composition of the sediments and nature of the hydrothermal influence. Rainbow sediment exhibits a mixed diversity. Close relatives to some of our sequences have been detected in cold seeps or cold deep-sea sediments (Li et al., 1999; Bowman and McCuaig, 2003), but others are closely related to lineages found in deep-sea vents or typical thermophiles such as the Hydrogenophilus-like sequences or the presumably thermophilic Acidobacteria (Sievert et al., 2000). Therefore, the Rainbow sediment studied here might constitute a mid-point or a transition area between hot hydrothermal regions and the cold, deep-sea.
Ecological importance of the Epsilonproteobacteria and S-oxidizing metabolisms at the fluid–seawater interface

The bacterial diversity that we observed in the three substrates exposed to an emission source for two weeks at the Mid-Atlantic Ridge shows three things. First, the colonization process is very fast; in only two weeks the exposed surfaces were completely covered by microorganisms showing a variety of morphologies (Fig. 3). Second, there is an overwhelming predominance of diverse epsilonproteobacteria in the clone libraries derived from the microcolonizers and third, there is no particular specificity of colonization among the different substrates assayed, as all are almost exclusively colonized by epsilonproteobacteria. Only one clone belonging to the CFB group was found in the organic-rich substrate but, even if this could be explained by the fact these microorganisms are heterotrophs capable of degrading complex organics, the finding of this clone is probably not significant when compared to the vast diversity of epsilonproteobacteria detected.

Our results thus confirm previous observations showing the prevalence of epsilonproteobacteria in these habitats, and that these organisms colonize all the available surfaces regardless their nature (animal teguments, mineral precipitates or artificial substrates). In the Rainbow sediment, we identified one epsilonproteobacterial sequence, and several sequences of this group were also identified in the Guaymas sediment. In both cases, these phylotypes were related to those found at the fluid–seawater interface, but they were a minor fraction of the overall diversity. This suggests that epsilonproteobacteria are present in deep-sea sediment and when the conditions are particularly appropriate for them, as is the case in vent surroundings where hydrogen sulphide is easily supplied, they bloom. Our data also show that epsilonproteobacteria are the first colonizers occupying virgin exposed surfaces to the fluid–seawater interface. One can speculate that young communities on any exposed surface in this ecological niche are formed basically by fast-developing epsilonproteobacteria and that, as communities evolve, they diversify and allow the creation of heterogeneous microniche suitable for the subsequent colonization by other bacteria. For instance, the creation of anoxic pockets and the production of sulphate as one abundant secondary metabolite (see below) would favour the settlement of sulphate-reducing deltaproteobacteria that would close the S cycle. Deltaproteobacteria are indeed observed in mature surfaces where epsilonproteobacteria dominate, such as tubes of big specimens of Riftia pacifica, or mucous tubes of Paralvinella palmiformis (Alain et al., 2002a; López-García et al., 2002).

A prominent role of S-dependent metabolisms, and most particularly, the oxidation of reduced forms of sulphur in warm areas of deep-sea vents is deduced from the availability of hydrogen sulphide and other reduced S species in combination with geochemical constraints (McCollom and Shock, 1997). This is further supported by the isolation of many microbial species that metabolize sulphur to gain energy. As mentioned above, many gammaproteobacteria oxidize sulphur or thiosulphate, but also others, including alphaproteobacterial or flavobacterial species from vents (Teske et al., 2000). Sulphate-reducing deltaproteobacteria would assure the completion of the S cycle in anoxic sediments and anoxic microniche. Sulphur-based energy reactions appear to be crucial for the metabolism of deep-sea vent epsilonproteobacteria, either as electron acceptor or as electron donor. Hydrogen sulphide oxidation would be a readily energy source for these organisms. However, most cultivation attempts use either hydrogen (electron donor) or sulphur (electron donor or acceptor), and therefore, there is generally no information about the capacity to directly oxidize hydrogen sulphide by the few strains of epsilonproteobacteria that have been isolated from the most represented groups around vents (Takai et al., 2003). Unfortunately, metabolic activities displayed in situ are difficult to measure, and those shown in the laboratory do not necessarily reflect what microorganisms do in nature. Trying to investigate if S metabolism could be visualized in cells having grown in deep-sea vents, we used high-resolution synchrotron-based techniques to map different S oxidation states at a single filament-level (Philippot et al., 2002; 2003). The filament shown here (Fig. 5) was retrieved from the iron-rich substrate of our microcolonizers. As all phylotypes retrieved from this substrate belonged to this phylogenetic group, the filament most probably corresponded to an epsilonproteobacterium. Our results showing the co-occurrence in the filament of different S species, including sulphide and sulphate, suggest that cells were actively metabolizing sulphur (see Foriel et al., 2003 for further details). Furthermore, they were likely oxidizing sulphide to sulphate and not the opposite, as the presence of sulphate reducing bacteria (typically deltaproteobacteria) was not detected by 16S rDNA sequencing, and as no epsilonproteobacteria is known to reduce sulphate. Additionally, the filament morphology favours the exposure to the fluid–seawater interface where oxygen is present (sulphate reducing bacteria are strict anaerobes). Coupling micro-XANES analysis with in situ fluorescent hybridization using phylogenetic probes could be useful to link metabolic information to community structure in these environments.

Experimental procedures

Sampling and scanning electron microscopy

Samples were taken with the aid of the Remote Operated
Vehicle (ROV) Victor during the French cruise ATOS 2001 to the Mid-Atlantic Ridge hydrothermal area, as previously described (López-García et al., 2003). A sediment core was obtained from Rainbow hydrothermal sediment (36°6’N, 33°11’W, depth 2264 m). A fraction of the sediment corresponding to the first cm upper part was removed in a laminar flux chamber and frozen in liquid nitrogen until use. Homemade sterile microcolonizers consisting of different substrates placed into extensively perforated 50 ml polypropylene Corning tubes were deployed adjacent to a fluid emission at the Tour Eiffel chimney (Lucky Strike site, 37°17’N, 32°16’W, depth 1695 m) for 15 days (see Fig. 2 in López-García et al., 2003), and collected in a close sterile container by the ROV Victor. Two of them consisted of an inert nylon mesh embedding, respectively, a meat-based substrate (autoclaved ground ham) and several pieces of 0.5-mm diameter iron wire. A third one was made of pumice fragments. The container was opened in a laminar flux chamber on board, and the microcolonizers were stored at 4°C in 75% ethanol, 2% NaCl. For scanning electron microscopy observation, samples were dehydrated in increasing ethanol concentrations (50%, 70%, 90% and 100%), critical-point dried and gold coated. Observation was carried out with a JEOL (JSM 840 A) scanning electron microscope (SEM) operating at 17 kV at the Service de Microscopie Electronique de l’Institut Fédératif de Recherche Biologie Intégrative (Paris, France).

**Synchrotron micro-X-ray absorption near-edge spectroscopy (micro-XANES)**

Micro-XANES spectra of the different oxidation states of sulphur (S²⁻ to S⁶⁺) were obtained using beamline ID21 (Susini, 2002) at the European Synchrotron Radiation Facility (Grenoble, France). Analyses were performed following the procedure described by Philippot et al. (2002, 2003) and Foriel et al. (2003). For this, microbial filaments scratched out from different microcolonizer substrates were deposited over a Mylar® polyester film, and placed on a metallic grid. Samples were then subjected to incident beam energies from 20 eV below the main absorption edge energy of sulphate (2482 eV for S⁶⁺) to 20 eV above this value. The structure of the S K absorption edge was scanned in the near edge region. The microscope used a Fresnel zone-plate as a focusing lens and delivered a microbeam of 0.8 x 0.8 μm² with a measured photon flux of about 10⁶ photons/s (David et al., 2000). Recorded micro-XANES spectra were compared with spectra of pure standard products that were analyzed during the same experimental session to optimize the correction procedure and interpretation of the X-ray spectra.

**Nucleic acid extraction**

Nucleic acids were purified from small fragments of the different microcolonizer substrates (~200–300 μl of organic and iron rich plastic mesh, and ~100 μl of pumice) and from approximately 50–100 μl of hydrothermal sediment. The respective substrate volumes were separated from mother samples in Eppendorf tubes in a laminar flux chamber. Microcolonizer samples were rehydrated with phosphate saline buffer (130 mM NaCl, 10 mM phosphate buffer, pH 7.7, PBS). Phosphate-buffered saline was also added to the sediment to a same final volume of 0.5 ml. Samples were then subjected to six freezing/thawing cycles in liquid nitrogen to facilitate cell lysis. Subsequently, 80 μg ml⁻¹ proteinase K, 1% SDS, 1.4 M NaCl, 0.2 β-mercaptoethanol and 2% CTAB (final concentrations) were added sequentially. Lysis suspensions were incubated overnight at 55°C. Lysates were extracted once with hot phenol (65°C), once with phenol-chloroform-isoamylalcohol, and once with chloroform-isoamyl-alcohol. Nucleic acids were concentrated by ethanol precipitation.

**16S ribosomal RNA gene libraries and sequencing**

16S rDNA genes were amplified by PCR using different combinations of the bacteria-specific forward primers B-27F (AGAGTTTGATCCTGCTAGC), ANT-1 (AGAGTTTGATCCTGCTAGC) and the prokaryote-specific reverse primer 1492R (GGT ACCCTTGTTAGACT). PCR reactions were normally performed under the following conditions: 30 cycles (denaturation at 94°C for 15 s, annealing at 55°C for 1 min, extension at 72°C for 2 min) preceded by 2 min denaturation at 94°C, and followed by 10 min extension at 72°C. For some amplification reactions, dimethyl sulphoxide was added to a final concentration of 3–5%. A total of seven bacterial rDNA clone libraries (four for sediment DNA and three for microcolonizers) were constructed using the Topo TA Cloning system (Invitrogen) following the instructions provided by the manufacturers. After plating, positive transformants were screened by PCR amplification of inserts using flanking vector primers. A total of 181 expected-size amplicons from these libraries was partially sequenced (Genome Express) with the primer 1492R. After preliminary phylogenetic analysis, 61 clones representative of the phylogenetic diversity found were chosen for complete sequencing using primer B-27F. Two of these clones were discarded from subsequent phylogenetic analyses as possible chimeras after sequence checking by the Chimera Detection program of the Ribosomal Database Project II (Cole et al., 2003) and visual inspection of the sequence alignment.

**Phylogenetic analysis**

Closest relatives to our sequences were identified in databases by BLAST (Altschul et al., 1997) and retrieved from GenBank (http://www.ncbi.nlm.nih.gov). Sequences were automatically aligned using the program BABA (H. Philippe, personal communication) to a 16S rRNA gene alignment containing ~16 600 sequences. The multiple alignment was then manually edited using the program ED from the MUST package (Philippe, 1993). A preliminary phylogenetic analysis of all partial sequences was done by distance methods (neighbour-joining, NJ) using the program MUST, allowing the identification of identical or nearly identical sequences and the selection of clones for complete sequencing. For more detailed phylogenetic analyses of the 59 complete bacterial sequences, we selected five subsets of sequences including the closest relatives to our clones in databases as well as cultivated representatives. Gaps and ambiguously aligned
positions were excluded from our analyses. The relatively low number of positions used as compared to full-length sequences is due to the inclusion of environmental sequences shorter than those determined in this work, but that were closely related. The five data sets, and the number of positions used in phylogenetic reconstruction, were as follows: 38 sequences covering the major bacterial divisions and including an archaeal outgroup (988 positions, Fig. 1A), 46 sequences covering Planctomycetes, CFB and Acidobacteria divisions (861 positions, Fig. 1B), 41 sequences corresponding to the α-, β- and deltaproteobacteria (929 positions, Fig. 1C), 44 sequences covering the gammaproteobacteria and including a betaproteobacterial outgroup (968 positions, Fig. 1D), and 52 sequences covering the epsilonproteobacteria and including a deltaproteobacterial outgroup (944 positions, Fig. 1E). Maximum likelihood (ML) trees were done using TREEFINDER (Jobb, 2002) applying a general time reversible model of sequence evolution (GTR), taking among-site rate variation into account by using an eight-category discrete approximation of a Γ distribution (invariable sites are included in one of the categories). The parameters of the Γ distribution estimated from the different sequence sets were: 0.35 (set of sequences in Fig. 1A), 0.26 (Fig. 1B), 0.25 (Fig. 1C), 0.19 (Fig. 1D) and 0.21 (Fig. 1E). Bootstrap proportions were inferred using 1000 replicates by NJ applying a ζ law using the estimated ζ values for each data set.

Nucleotide sequence accession numbers

The sequences reported in this study were submitted to GenBank with accession numbers AY225602 (AT-s63), AY225603 (AT-s3–44), AY225604 (AT-s71), AY225605 (AT-s3–41), AY225606 (AT-s3–57), AY225607 (AT-s3–34), AY225608 (AT-s3–60), AY225609 (AT-s3–66), AY225610 (AT-pp13), AY225611 (AT-co15), AY225612 (AT-co23), AY225613 (AT-pp6), AY225614 (AT-cs3), AY225615 (AT-cs10), AY225616 (AT-co11), AY225617 (AT-co16), AY225618 (AT-co12), AY225619 (AT-s3–19), AY225620 (AT-pp26), AY225621 (AT-pp46), AY225622 (AT-cs8), AY225623 (AT-ct5), AY225624 (AT-pp27), AY225625 (AT-cs7), AY225626 (AT-co13), AY225627 (AT-s68), AY225628 (AT-s3–68), AY225629 (AT-s3–26), AY225630 (AT-s3–43), AY225631 (AT-s2–13), AY225632 (AT-s3–1), AY225633 (AT-s16), AY225634 (AT-s26), AY225635 (AT-s80), AY225636 (AT-s2–59), AY225637 (AT-s75), AY225638 (AT-s3–48), AY225639 (AT-s3–25), AY225640 (AT-s2), AY225641 (AT-s3–24), AY225642 (AT-s3–59), AY225643 (AT-s3–37), AY225644 (AT-s65), AY225645 (AT-s36), AY225646 (AT-s3–28), AY225647 (AT-s3–23), AY225648 (AT-s3–42), AY225649 (AT-s54), AY225650 (AT-s2–57), AY225651 (AT-s3–56), AY225652 (AT-s2–38), AY225653 (AT-s2–18), AY225654 (AT-s83), AY225655 (AT-s2–33), AY225656 (AT-s3–3), AY225657 (AT-s30), AY225658 (AT-s92), AY225659 (AT-s98) and AY225660 (AT-co1).

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References


