Chemosynthetic bacteria found in bivalve species from mud volcanoes of the Gulf of Cadiz

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Abstract

As in other cold seeps, the dominant bivalves in mud volcanoes (MV) from the Gulf of Cadiz are macrofauna belonging to the families Solemyidae (Acharax sp., Petrasma sp.), Lucinidae (Lucinoma sp.), Thyasiridae (Thyasira vulcoulotre) and Mytilidae (Bathymodiolus mauritanicus). The δ13C values measured in solemyid, lucinid and thyasirid specimens support the hypothesis of thiotrophic nutrition, whereas isotopic signatures of B. mauritanicus suggest methanotrophic nutrition. The indication by stable isotope analysis that chemosynthetic bacteria make a substantial contribution to the nutrition of the bivalves led us to investigate their associated bacteria and their phylogenetic relationships based on comparative 16S rRNA gene sequence analysis. PCR-denaturing gradient gel electrophoresis analysis and cloning of bacterial 16S rRNA-encoding genes confirmed the presence of sulfide-oxidizing symbionts within gill tissues of many of the studied specimens. Phylogenetic analysis of bacterial 16S rRNA gene sequences demonstrated that most bacteria were related to known sulfide-oxidizing endosymbionts found in other deep-sea chemosynthetic environments, with the co-occurrence of methane-oxidizing symbionts in B. mauritanicus specimens. This study confirms the presence of several chemosynthetic bivalves in the Gulf of Cadiz and further highlights the importance of sulfide- and methane-oxidizing symbionts in the trophic ecology of macrobenthic communities in MV.

Introduction

Symbiotic relationships between bacteria and marine invertebrates in deep-sea environments derive all or part of their nutrition from symbiont metabolism (Fisher, 1990; Cavanaugh, 1994; Lee et al., 1999; Petersen & Dubilier, 2009). Symbiotic associations with thiotrophic (sulfur- and sulfide-oxidizing) and methanotrophic (methane-oxidizing) bacteria occur in a wide range of animal species that live in reducing environments, such as hydrothermal vents, whale falls, sunken wood, cold seeps and sediments (Cavanaugh et al., 2006; Duperron et al., 2009). Sulfur-based symbioses are by far the most commonly reported within these systems (Distel et al., 1988; Imhoff et al., 2003; Stewart et al., 2005). In particular, bivalves with sulfide-oxidizing gill symbionts are frequently found in environments that are inhospitable to other invertebrates due to a low oxygen (O2) content and the presence of free hydrogen sulfide (H2S) (reviewed in Southward, 1986). Sulfide-oxidizing bacteria are involved in symbioses with members of at least five bivalve families, occurring intracellularly in Vesicomyidae, Lucinidae, Mytilidae and Solemyidae, and mostly extracellularly in Thyasiridae. Bivalve symbioses have been studied using molecular methods and sequences of many host species and their associated symbionts have been published (e.g. Distel et al., 1988; Eisen et al., 1992; Imhoff et al., 2003; Duperron et al., 2007a, b). Bivalve symbionts belong to several related clades within the Gammaproteobacteria (Dubilier et al., 2008). Besides sulfide oxidizers, some members of the Mytilidae have methane-oxidizing gammaproteobacterial symbionts, and others have multiple symbioses with both sulfide- and methane-oxidizing bacteria within their gill tissues (Fisher,
The dominant bivalves, so far, identified at MV in the Gulf of Cadiz are large chemosymbiotic species belonging to the families Solemyidae (Acharax sp. and Petrasma sp.), Lucinidae (Lucinoma sp.; Rodrigues, 2009), Thyasiridae (Thyasira vulcolutre; Rodrigues et al., 2008) and Mytilidae (Bathymodiolus mauritianicus; Génio et al., 2008). In this study, the stable isotopic signature ($^{13}$C, $^{15}$N and $^{34}$S) of a number of the bivalves collected from these Gulf of Cadiz MV was determined to provide information on the nature of their nutritional source. The indication that the activities of autotrophic and methanotrophic bacteria make a substantial contribution to the nutrition of these bivalves led us to examine the diversity of their bacterial symbionts and further investigate the phylogenetic relationship with other bivalve endosymbionts based on a comparative analysis of 16S rRNA genes.

**Materials and methods**

**Study sites and bivalve collection**

Bivalve species belonging to the families Solemyidae, Lucinidae, Thyasiridae and Mytilidae have been found at a number of MV in the Gulf of Cadiz (Fig. 1). In summary, Petrasma sp. have mostly been found in shallower MV (Mercator MV, Gemini MV, Kidd MV, Yuma MV, Ginsburg MV and Darwin MV) from 358 to 1105 m depth, while Acharax sp. have a deeper bathymetric range (960–3902 m, occurring in Yuma MV, Ginsburg MV, Jesus Baraza MV, Captain Arutyunov MV, Carlos Ribeiro MV and Porto MV; Rodrigues, 2009). Both these species of solemyids are found buried deep in the sediment. In contrast, live specimens of Lucinoma sp. (Lucinidae) and B. mauritanicus (Mytilidae) have only been recorded at single MV (C.F. Rodrigues, unpublished data). For instance, Lucinoma sp. were identified at the crater of Mercator MV (358 m) where active methane bubbling was observed and B. mauritanicus in the fissures of carbonate slabs covering the crater at Darwin MV (1115 m). Thyasira vulcolutre (Thyasiridae) was recorded in the craters of Captain Aruryunov MV (1320 m, where it reached high densities) and Carlos Ribeiro MV (2200 m) (Génio et al., 2008; Rodrigues et al., 2008).

In this study, specimens were collected using a TV-assisted grab or a USNEL box-corer from seven MV located in the Gulf of Cadiz (Fig. 1): Mercator MV, Gemini MV (El Arraiache field; Van Rensbergen et al., 2005), Darwin MV, Ginsburg MV, Meknès MV, Yuma MV (Western Moroccan field) and Carlos Ribeiro MV (Deep water field) during several TTR cruises (IOC-UNESCO) and the Microsystems 2007 (NIOZ) cruise onboard the RV Prof. Logachev and the RV Pelagia, respectively (Table 1, also listing grid references of sampling sites). Bivalve species were examined.
and species were determined with the aid of the taxonomic expert Graham Oliver (National Museum of Wales, Cardiff, UK). One to three specimens of each species from different MV were dissected and prepared separately for DNA extraction. The soft tissues (gills and foot) were removed from the shells, dissected, washed three times in sterile water and stored at −80°C for symbiont characterization studies and stable isotope measurements.

Fig. 1. Gulf of Cadiz sampling sites showing (a) the distribution and (b) morphology of Acharax sp. (1), Petrasma sp. (2), Lucinoma sp. (3), Thyasira vulcunlutra (4) and Bathymodiolus mauritanicus (5). *Indicates bivalve specimens collected for analysis. CA, Captain Arutyunov; CR, Carlos Ribeiro; Dar, Darwin; Fiu, Fiuza; Gem, Gemini; Gin, Ginsburg; JB, Jesus Baraza; Kid, Kidd; Mek, Meknes; Mer, Mercator; Por, Porto; Yum, Yuma. Photographs by Dr Graham Oliver (National Museum of Wales, Cardiff, UK).
**Stable isotope analysis**

In the laboratory, samples were lyophilized and homogenized in a mortar and pestle, and then separated into batches for $^{13}$C, $^{15}$N and $^{34}$S analyses. The ground sample for carbon analysis was acidified with HCl (1 M) until no further bubbling occurred; the sample was then resuspended in distilled water, shaken for 5 min., centrifuged and the supernatant was discarded; this procedure was repeated three times; finally, the sample was dried at 60 °C. The ground sample for sulfur analysis was resuspended in distilled water, shaken for 5 min., centrifuged and the supernatant was discarded; this procedure was repeated and the sample was dried at 60 °C. All samples were analyzed at ISO-Analytical Laboratory (Cheshire, UK) using the elemental analysis-isotope ratio MS method.

The isotope compositions are reported relative to standard material and follow the same procedure for all stable isotopic measurements as follows:

$$
\delta^x E = \left[ \left( \frac{E}{E_{\text{sample}}} \right) \left/ \left( \frac{E}{E_{\text{standard}}} \right) \right. \right] - 1 \times 100
$$

where $E$ is the element analyzed (C, N or S), $x$ is the molecular weight of the heavier isotope and $y$ the lighter isotope ($x = 13, 15$ and $y = 12, 14$ and 32 for C, N and S, respectively). The standard materials with which the samples are compared are PDB (Pee Dee Belemnite) for carbon; air N$_2$ for nitrogen; and CDT (Canon Diablo toilite) for sulfur.

**DNA extraction**

DNA was extracted from freeze-dried gill tissue of bivalves using the DNeasy® Blood and Tissue kit (Qiagen) using the manufacturer’s protocol, with some modifications. In summary, to facilitate DNA extraction, the tissue was initially placed in a FastPrep Lysing Matrix Tubes E (MP Biomedicals) and homogenized for 40 s at speed 6 using a FastPrep instrument (QBiogene) in lysis buffer ATL and proteinase K supplied with the DNeasy® Blood and Tissue kit (Qiagen). Subsequently, samples were then incubated at 56 °C until the tissue was completely lysed and the remaining procedure was followed as recommended by the manufacturer. DNA extracts were visualized by standard agarose gel electrophoresis, and the DNA was quantified against the Hyperladder I DNA marker (Bioline) using the Gene Genus gel electrophoresis, and the DNA was quantified against the manufacturer. DNA extracts were visualized by standard agarose gel electrophoresis, and the DNA was quantified against the Hyperladder I DNA marker (Bioline) using the Gene Genus Imaging System (Syngene). It should be noted that before the above DNA extraction procedure was performed, preliminary experiments carried out on ethanol-preserved tissue were not successful. This was thought to be due to DNA degradation during ethanol storage as reported previously for mammalian tissues (Kilpatrick, 2002).

**PCR amplification of bacterial 16S rRNA genes**

Bacterial 16S rRNA genes were amplified from tissue DNA samples using the primer combinations 27F-1492R (Lane, 1991) under the following PCR conditions. PCR mixtures contained (total 50 μL, molecular-grade water) 0.4 pmol μL$^{-1}$ of primers, 1 μL of tissue DNA template, 1 x reaction buffer (Bioline), 1.5 mM MgCl$_2$, 1.5 U BioTaq DNA polymerase (Bioline), 0.25 mM each dNTP and 10 μg bovine serum albumin (BSA). Reaction mixtures were held at 95 °C for 2 min, followed by 30 cycles of 94 °C for 30 s, 52 °C for 30 s and 72 °C for 90 s plus 1 s per cycle, with a final extension step of 5 min at 72 °C. PCR products analyzed by
denaturing gradient gel electrophoresis (DGGE) were then reamplified in a nested PCR reaction using the reaction mixture described above without BSA. This reamplification step was added after initial observations that some samples did not amplify by direct PCR and products were only observed after nested PCR. Therefore, for consistency, all samples were amplified by nested PCR before DGGE, which had the advantage that the same PCR products that could be cloned were also screened by PCR-DGGE. Bacterial 16S rRNA genes were reamplified with the primers 357FGC-518R (Muyzer et al., 1993) at 95 °C for 5 min, followed by 10 cycles of 94 °C for 30 s, 55 °C for 30 s, 72 °C for 60 s, and 20 cycles of 92 °C for 30 s, 52 °C for 30 s and 72 °C for 60 s, with a final step of 10 min at 72 °C.

**DGGE analysis**

DGGE was performed using a DCode™ Universal Mutation Detection System (Bio-Rad Laboratories). The PCR samples were loaded onto 8% w/v polyacrylamide (37.5 : 1 acrylamide : bisacrylamide) gels in a 1 × Tris–acetate–EDTA (TAE) buffer with a denaturing gradient ranging from 30% to 60% (denaturation of 100% corresponds to 7 M urea and 40% v/v deionized formamide). Electrophoresis was run for 10 min at 80 V and then 290 min at 200 V in 1 × TAE buffer at 60 °C (Webster et al., 2003, 2006). The gel was stained with SYBR-Gold (Molecular Probes). Dominant DGGE bands were excised and sequenced with the 518R primer, and partial bacterial 16S rRNA gene sequences using NCBI BLAST (http://www.ncbi.nlm.nih.gov) to identify sequences with highest sequence identity.

**Bacterial 16S rRNA gene libraries**

Only products assessed by DGGE and without a PCR product in the negative controls were cloned. Each 16S rRNA gene library was constructed from five independent PCR products that were pooled and cleaned using the Wizard PCR Preps DNA Purification System (Promega) according to the manufacturer’s instructions. Cloning was with pGEM-T Easy (Promega) according to the manufacturer’s instructions, using optimized insert : vector ratios and overnight ligation at 4 °C. Libraries were screened by PCR with M13 primers. All 16S rRNA gene clones with verified inserts were randomly selected and sequenced using an ABI 3130xl 16 capillary Genetic Analyzer.

**Phylogenetic analysis**

Sequence chromatographs were analyzed using the CHROMAS software package version 1.45 (http://www.techneylsium.com.au/chromas.html). Partial sequences were checked for chimeras with CHIMERA CHECK from the Ribosomal Database Project II (http://rdp.cme.msu.edu/), and their closest relatives were identified by NCBI BLAST (http://www.ncbi.nlm.nih.gov). All nucleotide sequences were aligned using CLUSTAL (Thompson et al., 1997) with sequences retrieved from the database. Alignments were edited manually using BIOEDIT SEQUENCE ALIGNMENT EDITOR version 5.0.9 (Hall, 1999) and regions of ambiguous alignment were removed. The phylogenetic relationships between pairs of 16S rRNA gene sequences were determined using both distance and maximum parsimony and implemented in MEGA4 (Tamura et al., 2007). The LogDet distance analysis (Lockhart et al., 1994) was used as the primary tool for estimating phylogenetic relationships, but other methods including p-distance and Jukes–Cantor were also carried out, which yielded similar tree topologies. All LogDet distances trees were constructed using the minimum evolution criterion and the data were bootstrapped 1000 times to assess support for nodes.

All 16S rRNA gene sequences retrieved during this study were deposited in the EMBL database (http://www.ebi.ac.uk/emb/ under accession numbers FM213420–FM213421 and FN822778–FN822779.

**Results**

**Stable isotope analysis**

In this study, different bivalve tissues (gills, foot and mantle) were analyzed separately, but isotopic values from different tissue types showed no significant difference. However, clear differences in the average δ13C, δ15N and δ34S isotopic values of tissues from different bivalve species were observed. The carbon isotopic signature of *B. mauritanicus* specimens was significantly depleted (−52.4 ± 1.0‰) than solemyid, lucinid and thyasirid species (average δ13C values between −35.6 ± 1.3‰ and −28.8 ± 0.6‰) (Fig. 2).

The average δ15N values (Fig. 2) varied between −3.4 ± 0.3‰ (*Acharax* sp. from Ginsburg MV) and +6.1 ± 0.5‰ (*Petrasma* sp. from Gemini MV). The average δ34S results showed a wide range of values from −16.5 ± 3.1‰ (*Petrasma* sp. from Meknès MV) to +16.8 ± 0.2‰ (*B. mauritanicus* from Darwin MV). Specifically for the thiotrophic species of solemyids, lucinids and thyasirids, the values varied between −16.5 ± 3.1‰ (*Petrasma* sp. from Meknès MV) and +1.5 ± 0.9‰ (*T. vulcolute*).

**Molecular analysis**

DNA from foot and gill tissues were analyzed separately, and no bacterial symbiont sequences were amplified in any of the foot tissue samples analyzed. DGGE profiles of bacterial 16S rRNA genes derived from the gill tissue from several bivalves are shown in Fig. 3, and a summary of the DGGE bands excised and sequenced is presented in Table 2. High 16S rRNA gene sequence similarity (> 98%) was found between the bacteria identified in this study and endosymbionts from
other similar bivalve species. Most of the excised DGGE band sequences belonged to the Gammaproteobacteria, although some sequences were related to members of other bacterial phyla including Alphaproteobacteria, Betaproteobacteria, Epsilonproteobacteria, Chlamydiae and Spirochaetes.

Bacterial 16S rRNA gene libraries constructed from the same gill tissue samples as analyzed by DGGE were in agreement. The majority of bacterial sequences obtained by PCR cloning from different specimens [e.g. Acharax sp. (from Yuma MV and Ginsburg MV), Petrasma sp. (from Gemini MV, Mercator MV and Yuma MV), Lucinoma sp. (from Mercator MV) and B. mauritanicus (from Darwin MV)] were assigned within a Gammaproteobacteria clade (Fig. 4a) in which many thiotrophic and methanotrophic symbionts of marine invertebrates are included. Similarly, bacterial phylotypes from other phylogenetic groups were also obtained and are shown in Fig. 4b.

Interestingly, despite the evidence for methanotrophic nutrition provided by stable isotopic data, the first molecular analysis of the mytilid specimen (B. mauritanicus Darwin MV_1) only revealed bacterial 16S rRNA gene sequences related to Bathymodiolus puteoserpentis thioautotrophic symbionts (Figs 3 and 4a, Table 2). However, further PCR and cloning analysis on another specimen (B. mauritanicus Darwin MV TTR17_1) identified the co-occurrence of thiotrophic and methanotrophic sequences in this species (Fig. 4a; C.F. Rodrigues & S. Duperron, ongoing work), and methanotropic sequences were related to other Bathymodiolus sp. symbionts. In addition, Fig. 3 also suggests that more bacterial phylotypes (DGGE bands) were present within the B. mauritanicus specimens and it is possible that some of these bands may represent methanotrophic endosymbionts.

Discussion

Analysis of the natural stable isotopic compositions of tissues (δ¹³C, δ¹⁵N and δ³⁴S) has been widely used as a means to determine food sources in organisms inhabiting reducing environments (Levin et al., 2000; MacAvoy et al., 2003; Van Dover et al., 2003). The isotopic values reported here are within the range of values described previously for bivalves harboring symbionts (Fisher, 1990; Petersen & Dubilier, 2009). Solemyid, lucinid and thyasirid species in the Gulf of Cadiz exhibited δ¹³C values typical of seep and vent symbiotic bivalves that only bear sulfur-oxidizing bacteria and use a carbon source less depleted in ¹³C, such as inorganic carbon (Brooks et al., 1987). Stable isotope values are known to be influenced by the RubisCO form used by the symbiont (Robinson & Cavanaugh, 1995), and the values presented here would fit with the use of a type I RubisCO, as documented in most chemoautotrophic bivalves (Elsaied & Naganuma, 2001; Scott et al., 2004).

The δ¹³C value determined for the mytilid species was within the range reported previously in cold seep methanotrophic mussels (−40‰ to −70‰) (Kennicutt et al., 1992; Conway et al., 1994; MacAvoy et al., 2005). Previous studies suggest that tissue carbon isotopic values of mytilids reflect the relative input of thermogenic and biogenic methane seepage at a given location (Kennicutt et al., 1992; Duperron et al., 2007b), and the values reported here seem to indicate the use of thermogenic methane by mytilid mussels at Darwin MV, although no δ¹³C values for methane have been published for this site. In practice, however, the δ¹³C values for animals having symbiotic methane oxidizers can be difficult to interpret, as their tissues rarely have δ¹³C values that would be expected through fractionation of the source methane or inorganic carbon (Petersen & Dubilier, 2009). Other factors can influence the δ¹³C values in B. mauritanicus, such as the co-occurrence of sulfide- and methane-oxidizing symbionts, which will be sensitive to the relative activities of each of the symbionts.

The observed δ¹⁵N discrepancy between bivalve species from the Gulf of Cadiz suggests that they are either using
different chemical species of nitrogen, tapping into different pools of nitrogen or discriminating differently after acquisition of their nitrogen source (MacAvoy et al., 2005). The interspecific $\delta^{15}$N variations may be due to the species-specific types of symbionts characterized by different fractionation factors occurring during the assimilation of dissolved inorganic nitrogen and/or due to the location of the symbionts. Another hypothesis could be the relative availability of reduced compounds for these co-occurring bivalves depending on the depth at which they live in the sediment. The relatively high $\delta^{15}$N values (>$5\%$) measured for Petrasma specimens from Meknès MV and Gemini MV may indicate symbiont sparseness, since Levin & Michener (2002) suggested that this value may be at the limit for species with symbionts, although it also could be due to the shallower location of these sites and the utilization of organic matter from photosynthetic origin as was shown for Bathymodiolus azoricus at different depths (Riou et al., 2010).

The $\delta^{34}$S values (<5\%) in solemyids, lucinids and thyasirids clearly indicate a thiotrophic mode of nutrition with reliance on sulfide from seeps because such depleted $\delta^{34}$S values of tissues indicate a sulfur source other than seawater sulfate (seawater sulfate $\delta^{34}$S = +21\%; Trust & Fry, 1992; Vetter & Fry, 1998). Intraspecies differences may be caused by varying dependence on their symbionts with changing sulfide content in the sediment as was noted for Thyasira sarsi and Thyasira equalis (Dando & Spiro, 1993). Moreover, the highly depleted $\delta^{34}$S value found in Petrasma sp. from Mercator MV and Meknès MV, and Lucinoma sp. from Mercator MV could be derived from the utilization of biogenically produced H$_2$S. On the other hand, the less depleted $\delta^{34}$S value of the mytilid (B. mauritanicus) tissue, combined with the highly depleted carbon signature, reinforces the evidence that these organisms rely on methane oxidation for their energy production, but does not exclude the possibility of some reliance on the assimilatory uptake of inorganic sulfur from seep fluids for the biosynthesis of organic sulfur compounds (Vetter & Fry, 1998). Because the isotopic fractionation associated with sulfate reduction to H$_2$S by bacteria is large and variable, the primary sulfur source is often difficult to identify based on $\delta^{34}$S values alone (Kenneccut et al., 1992). Nevertheless, the analysis of stable isotope ratios does support the hypothesis for chemoautotrophic-sourced host carbon nutrition for several species in the Gulf of Cadiz and provides strong evidence to warrant further investigation using molecular microbiological methods.

DGGE analysis of bacterial 16S rRNA genes was used initially to obtain an overview of the bacterial diversity present in the gill tissues of all the bivalve species. For most of the specimens, a dominant brightly stained band was found and this was considered to represent the main bacterial symbiont. This was further supported by sequence
analysis, as dominant DGGE bands and sequences obtained by PCR cloning from the gills of bivalves (Petrasma sp., Acharax sp., Lucinoma sp. and B. mauritanicus) were often related to known thiotrophic symbionts belonging to the Gammaproteobacteria (Figs 3 and 4; Table 2), and in all cases, grouped phylogenetically with bacterial symbionts from related bivalve species. However, in some instances, other bacterial taxa were also identified including members of the Spirochaetes, Epsilonproteobacteria and Alphaproteobacteria, inferring the possibility of other novel symbioses.

Table 2. Closest bacterial 16S rRNA gene sequence matches to excised DGGE bands using the nucleotide BLAST program

<table>
<thead>
<tr>
<th>DGGE band* (accession number)</th>
<th>Closest sequence match by NCBI nucleotide BLAST (accession number)</th>
<th>Phylogenetic group</th>
<th>% Sequence similarity (alignment length, bp)</th>
<th>Environmental location of the closest sequence match</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (FM213436)</td>
<td>Lucinoma annulata symbiont (M99449)</td>
<td>Gammaproteobacteria</td>
<td>98 (172)</td>
<td>Lucinoma annulata gill tissue, sediment, Santa Monica Basin, CA</td>
</tr>
<tr>
<td>2 (FM213438–FM213441)</td>
<td>Solemya velum symbiont (M90415)</td>
<td>Gammaproteobacteria</td>
<td>98–99 (165)</td>
<td>Solemya velum gill tissue, eelgrass beds, Woods Hole, MA</td>
</tr>
<tr>
<td>3 (FM213442)</td>
<td>Solemya velum symbiont (M90415)</td>
<td>Gammaproteobacteria</td>
<td>98 (169)</td>
<td>Solemya velum gill tissue, eelgrass beds, Woods Hole, MA</td>
</tr>
<tr>
<td>4 (FM213443)</td>
<td>Uncultured bacterium clone TAG_368_2CF3 (FN393030)</td>
<td>Epsilonproteobacteria</td>
<td>99 (150)</td>
<td>Rimicaris exoculata Hydrothermal vent field, Mid-Atlantic Ridge</td>
</tr>
<tr>
<td>5 (FM213444)</td>
<td>Uncultured bacterium clone FW1023-058 (EF693294)</td>
<td>Chlamydiae</td>
<td>91 (174)</td>
<td>Groundwater, Oak Ridge, TN</td>
</tr>
<tr>
<td>6 (FM213445)</td>
<td>Acharax sp. endosymbiont ‘Oregon 56’ (AJ441197)</td>
<td>Gammaproteobacteria</td>
<td>100 (194)</td>
<td>Acharax sp. gill tissue, Hydrate Ridge cold seep sediment, Cascadia Margin</td>
</tr>
<tr>
<td>7 (FM213446, FM213447)</td>
<td>Solemya velum symbiont (M90415)</td>
<td>Gammaproteobacteria</td>
<td>98–99 (194)</td>
<td>Solemya velum gill tissue, eelgrass beds, Woods Hole, MA</td>
</tr>
<tr>
<td>8 (FM213448)</td>
<td>Solemya velum symbiont (M90415)</td>
<td>Gammaproteobacteria</td>
<td>98 (194)</td>
<td>Solemya velum gill tissue, eelgrass beds, Woods Hole, MA</td>
</tr>
<tr>
<td>9 (FM213449)</td>
<td>Uncultured bacterium clone C2-E05 (FJ930196)</td>
<td>Betaproteobacteria</td>
<td>100 (194)</td>
<td>Poreites compressa coral mucus, Hawaii</td>
</tr>
<tr>
<td>10 (FM213450)</td>
<td>Acharax sp. endosymbiont ‘Oregon 56’ (AJ441197)</td>
<td>Gammaproteobacteria</td>
<td>100 (194)</td>
<td>Acharax sp. gill tissue, Hydrate Ridge cold seep sediment, Cascadia Margin</td>
</tr>
<tr>
<td>11 (FM213451)</td>
<td>Uncultured bacterium clone C2-E05 (FJ930196)</td>
<td>Betaproteobacteria</td>
<td>99 (94)</td>
<td>Poreites compressa coral mucus, Hawaii</td>
</tr>
<tr>
<td>12 (FM213452)</td>
<td>Bradyrhizobium elkanii strain NBRC 14791 (ABS09378)</td>
<td>Alphaproteobacteria</td>
<td>100 (169)</td>
<td>Glycine max root nodule</td>
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<td>13 (FM213453)</td>
<td>Bathymodiolus puteoserpentis thioautotrophic gill symbiont (DQ321712)</td>
<td>Gammaproteobacteria</td>
<td>98 (194)</td>
<td>Bathymodiolus puteoserpentis gill tissue, Snake Pit hydrothermal vent, Atlantic Ocean</td>
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<tr>
<td>14 (FM213455)</td>
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<td>85 (198)</td>
<td>Seawater from a deep-sea coral reef, Norway</td>
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<tr>
<td>15 (FM213454)</td>
<td>Uncultured bacterium clone SPG12_461_471_B37 (FJ746172)</td>
<td>Alphaproteobacteria</td>
<td>94 (170)</td>
<td>Deep-sea sediment, South Pacific Gyre</td>
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<td>X (FM213437)*</td>
<td>Uncultured bacterium clone GHI10 (EUB577749)</td>
<td>Spirochaetes</td>
<td>95 (165)</td>
<td>Cerastoderma edule crystalline style</td>
</tr>
</tbody>
</table>

*DGGE bands were excised from the DGGE gels shown in Fig. 3.

DGGE band X was excised from replicate PCR-DGGE analysis of 16S rRNA genes derived from Lucinoma sp. specimen 1 found at Mercator MV (analysis not shown in Fig. 3).
Fig. 4. Phylogenetic trees of bacterial 16S rRNA gene sequences associated with the gills of some bivalve species from the Gulf of Cadiz belonging to (a) Gammaproteobacteria and (b) other bacterial taxa. Minimum evolution trees derived from LogDet distance analysis. The trees were constructed from 1030 (a) and 754 (b) bases of aligned 16S rRNA gene sequences. Bootstrap support values over 50% (1000 replicates) are shown. First value, bootstrap derived by LogDet distance; second value, derived by maximum parsimony. Representative sequences of Deltaproteobacteria (Desulfosarcina variabilis M34407, Desulfovobacter postgatei AF418180 and Desulfuromonas variabilis AF192153) and Deinococcus-Thermus (Thermus aquaticus L09663, Meiothermus ruber L09672 and Deinococcus radiodurans M21413) were used as outgroups in (a) and (b), respectively. For comparison, preliminary data from a more extensive study on Bathymodiolus mauritanicus (Cruise TTR17 station AT664GR; 35°12′33.520N, 7°11′11.485W) are also included to confirm the presence of methanotrophic symbionts in these specimens (C.F. Rodrigues & S. Duperron, unpublished data).
In contrast to other symbiotic thyasirids (Distel & Wood, 1992), no known thiotrophic Gammaproteobacteria symbionts were found in the gills of T. vulcolutre. Instead, bacterial phylotypes belonged to uncultured members of the Alphaproteobacteria and a distantly related group of Gammaproteobacteria with unknown metabolisms. Because only a fraction of thyasirid species are symbiotic, and the symbionts are mostly extracellular, this group of bivalves has
been described as representative of an early stage in the evolution of the bacterium–bivalve symbiosis (Dufour, 2005). It may be that *T. vulcolutre* is asymbiotic and the observed stable isotope values suggestive of thiotrophy could be due to ingestion of free-living sulfur-oxidizing bacteria.

Consistent with the DGGE analysis, a number of other bacterial phylotypes were also present in the 16S rRNA gene libraries (Fig. 4b), supporting the possibility that members of the genera *Petrasma*, *Acharax*, *Lucinoma* and *Thyasira* may have more than one known symbiont as reported previously (Distel & Wood, 1992; Krueger & Cavanaugh, 1997; Imhoff et al., 2003; Duperron et al., 2007a,b). This is further supported by multiple symbioses in *Bathymodiolus* sp. that have two metabolically distinct symbionts (Duperron et al., 2009) and up to six bacterial phylotypes in *Idas* sp. (Duperron et al., 2008). It is possible that, as more work is undertaken to understand bacterium–bivalve symbioses, many more bacterial phylotypes with symbiotic properties will be discovered. In our study, one 16S rRNA gene clone from *Lucinoma* sp. was related to *Spirochaetes* symbionts found in deep-sea octocorals (Penn et al., 2006); corals are known to harbor several bacterial groups (Webster & Bourne, 2007). *Spirochaetes* have been found in a number of other marine invertebrates as symbionts (e.g. gutless marine oligochaetes, Blazekaj et al., 2005; Ruehland et al., 2008), but also not necessarily as symbionts (e.g. *Lucinoma kazani*, Duperron et al., 2007a). Phylotypes belonging to *Betaproteobacteria*, *Epsilonproteobacteria* and *Actinobacteria* were also found in *Petrasma* sp. specimens and, again, suggest greater symbiont diversity than thought previously. *Epsilonproteobacteria* are increasingly being recognized as an ecologically significant group of bacteria in deep-sea hydrothermal environments (Nakagawa et al., 2005) and cold seeps, where they occur as sulfur-oxidizing species (Takai et al., 2005). Alternatively, it has also been suggested that *Epsilonproteobacteria* may act as sulfide detoxifiers, as was shown for an epsilonproteobacterial phylotype found in two endemic hydrothermal vent fauna (Campbell et al., 2006).

In addition to symbiotic relationships, the presence of other bacteria such as *Chlamydia*-like bacteria in a *Petrasma* sp. from Mercator MV (Fig. 3 and Table 2) may indicate the occurrence of parasitism, similar to the *Chlamydia*-like inclusions described in the digestive tissue of *Bathymodiolus heckerae* from Blake Ridge (Ward et al., 2004). Parasitic infections can impair the growth, reproduction, competitive ability, stress tolerance and survival of host species (reviewed by Ward et al., 2004). Similarly, the sparseness of symbionts suggested by the 15N values in some *Petrasma* sp. specimens is reinforced by the absence of a recognized symbiont in the *Petrasma* sp. from Meknès MV. This observation may indicate that this species of *Petrasma* does not rely exclusively on chemosulfotrophic nutrition and may be mixotrophic. However, suspension feeding in the congener clam species, *Solemya velum*, is limited (Scott et al., 2004). It may also be possible that symbiont density may vary in these bivalve species according to the availability of metabolic substrates, such as H2S.

The occurrence of almost identical symbiont phylotypes in specimens of *Acharax* sp. from geographically very distant localities (Gulf of Cadiz, NE Pacific Ocean and Indian Ocean; Imhoff et al., 2003) is quite remarkable, although identical sulfur-oxidizing bacterial ectosymbionts have been reported for geographically distinct ciliates (Rinke et al., 2009). This high sequence similarity between *Acharax* sp. symbionts may indicate that these bacteria are relatively modern descendents of a much more ancient phylogenetic line (Imhoff et al., 2003). The large phylogenetic distances between symbionts of different *Solemyidae* species support an ancient evolutionary history because all solemyid genera *Petrasma*, *Solemya* and *Acharax* have a particularly deep branching point compared with other clusters of symbiotic *Gammaproteobacteria* (Fig. 4a) as suggested previously (Imhoff et al., 2003). In fact, the association between endosymbionts and host animals can be highly specific. Because none of these phylotypes show a close relationship with free-living sulfur bacteria, it can be concluded that symbiotic and free-living sulfur bacteria represented separate lineages that have undergone a long period of divergence (Imhoff et al., 2003).

The majority of the analyses on the bacteria associated with mytilid specimens in the present study revealed phylotypes with 100% sequence similarity to *B. puteoserpentis* thioautotrophic symbionts (Won et al., 2003). However, preliminary results from a more extensive study (included in Fig. 4a for comparison) have identified the presence of a bacterial phylotype closely related to methanotrophic endosymbionts of *Bathymodiolus* sp. (C.F. Rodrigues & S. Duperron, unpublished data), confirming a dual symbiosis with sulfur and methane oxidizers as known for other species (e.g. *B. azoricus* and *B. puteoserpentis*, Distel et al., 1995; Fiala-Médioni et al., 2002). Nevertheless, more investigation needs to be carried out using FISH and/or electron microscopy techniques to confirm the presence, localization and relative abundance of methanotrophic bacteria in the gills of *B. mauritanicus*.

**Chemosynthetic bacteria found in bivalves from the Gulf of Cadiz – an overview**

Molecular analysis (PCR-DGGE and cloning of 16S rRNA genes) of the bacterial symbionts supported assumptions based on stable isotope data that *Petrasma* sp., *Acharax* sp. and *Lucinoma* sp. have dominant thiotrophic bacterial symbionts. However, in *T. vulcolutre*, no known thiotrophic symbionts were detected despite stable isotope evidence.
suggesting thiotrophy. *Bathymodiolus mauritanicus* was shown to possess a dual symbiosis with thio- and methanotrophic symbionts, although methanotrophs were only detected in new specimens after further investigation. Therefore, the Gulf of Cadiz MV symbiotic community appears to be similar to other vent and seeps symbiosis-dominated communities and that thiotrophy or sulfur oxidation is the dominant bacterial chemosynthetic activity in most bivalves (Fisher, 1990), although, based on stable isotope data, methane does seem to be the main source of carbon for *Bathymodiolus*.

The solemyids, *Acharax* sp. and *Petrasma* sp., have a widespread distribution in the Gulf of Cadiz, and despite the range of δ13C, δ15N and δ34S values suggesting different uptake and fractionation processes, their symbionts are highly specific. The sparseness of symbionts in some *Petrasma* sp. specimens (Meknes MV) points to a status of nonobligatory symbiosis for these species. However, of the five bivalve species studied in the Gulf of Cadiz, *Acharax* sp. and *Petrasma* sp. are the only hosts whose family is known to contain vertically transmitted endosymbionts (e.g. *Solomya*, Krueger & Cavanaugh, 1997) and, therefore, the apparent worldwide distribution of these symbionts may result from an ancestral distribution of their primordial hosts (Imhoff et al., 2003). In addition, this study also suggests the possibility that additional symbionts occur in some bivalve species, which needs further investigation.

This study clearly confirms the occurrence of several chemosynthetic species in the Gulf of Cadiz. However, the possible benefits to the partners in chemosynthetic symbioses seem to be of varying importance in these symbioses and more studies are needed to understand the role of the symbionts in these bivalves in detail. FISH techniques should be applied to confirm the symbiont presence, localization and abundances.

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**References**


