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Characterization of bacterial symbioses in *Myrtea* sp. (*Bivalvia*: *Lucinidae*) and *Thyasira* sp. (*Bivalvia*: *Thyasiridae*) from a cold seep in the Eastern Mediterranean

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Abstract

Cold seeps have recently been discovered in the Nile deep-sea fan (Eastern Mediterranean), and data regarding associated fauna are still scarce. In this study, two bivalve species associated with carbonate crusts and reduced sediment are identified based on sequence analysis of their 18S and 28S rRNA-encoding genes, and associated bacterial symbioses are investigated using 16S rRNA gene sequencing and microscopy-based approaches. The specimens are closely related to *Myrtea spinifera* and *Thyasira flexuosa*, two species previously documented at various depths from other regions but not yet reported from the Eastern Mediterranean. Both species harbour abundant gammaproteobacterial endosymbionts in specialized gill epithelial cells. The *Myrtea*-associated bacterium is closely related to lucinid symbionts from both deep-sea and coastal species, whereas the *Thyasira*-associated bacterium is closely related to the symbiont of a *T. flexuosa* from coastal waters off the U.K. An epsilonproteobacterial sequence has also been identified in *Thyasira* which could correspond to a helicoid-shaped morphotype observed by electron microscopy, but this was not confirmed using fluorescent *in situ* hybridization. Virus-like particles were observed within some symbionts in *Thyasira*, mostly in bacteriocytes localized close to the ciliated zone of the gill filament. Overall, results indicate that very close relatives of shallow species *M. spinifera* and *T. flexuosa* occur at cold seeps in the Eastern Mediterranean and harbour chemoautotrophic symbioses similar to those found in their coastal relatives.

Introduction

Cold seeps recently discovered around the Nile deep-sea fan in the Eastern Mediterranean display some original features such as temperatures of 14 °C from bottom waters. Although a diversity of habitats is reported, including brine pools, methane-emitting mud volcanoes and pockmarks with authigenic carbonate crust pavement, macrofauna is not as abundant as in seeps of the

gulf of Mexico or the gulf of Guinea (Olu-Le Roy *et al.* 2004; Bayon *et al.* 2009). In that respect, seep fauna follows the general 'low biomass' trend observed in the Eastern Mediterranean (Olu-Le Roy *et al.* 2004). Yet, the diversity of symbiont-bearing species collected from the Anaximander and Olimpi mud volcano areas, with at least seven and four species, respectively, is high compared to other seeps at similar depths (Olu-Le Roy *et al.* 2004). Siboglinid annelids associated with sulphur-

oxidizing symbionts (*Lamellibrachia* sp.) occur attached to carbonate crusts, along with small mytilid bivalves (*Idas* sp.), which have been shown to harbour six distinct bacterial 16S rRNA gene phylotypes within their gills, including methane-oxidizing bacteria (Duperron *et al.* 2008, 2009). Other documented chemosynthetic fauna include the bivalve *Lucinoma* aff. *kazani*, a thiotroph-associated lucinid living in reduced sediment (Duperron *et al.* 2007a). Representatives of the thyasirid (*Thyasira striata* Sturany, 1896), lucinid (*Myrtea amorpha* Sturany, 1896) and vesicomysid (*Isorropodon perplexum* Sturany, 1896) bivalve families have been documented from the Olimpi and Anaximander areas (Olu-Le Roy *et al.* 2004). Lucinids and thyasirids are frequently occurring bivalves at cold seeps and mud volcanoes (Salas & Woodside 2002; Carney 1994; Callender & Powell 1997; Olu-Le Roy *et al.* 2004; Rodrigues *et al.* 2008; Taylor & Glover 2009).

The Lucinidae, in which sulphide-oxidizing symbiosis is likely obligate, is by far the most disparate and species-rich chemosymbiotic family, occupying a great variety of habitats over a broad geographical range (60° N to 55° S) (Taylor & Glover 2000, 2006). In this highly diverse family (373 species) symbiosis has been studied in <2% of the species (Appeltans *et al.* 2010). Phylogenetic characterization of lucinid symbionts has shown that they belong to the Gammaproteobacteria, and are related to symbionts from thyasirid and solemyid bivalves as well as to symbionts from siboglinid tubeworms (Stewart *et al.* 2005).

As opposed to many other bivalves with chemoautotrophic symbionts, thyasirids are small (most specimens are under 10 mm in length) (Dufour 2005). Thyasirids show a varying nutritional dependence on symbiosis (Dando & Spiro 1993; Dufour & Felbeck 2006) and not all thyasirids possess a symbiosis with sulphide-oxidizing bacteria. In fact, 15 of the 26 species examined by Dufour (2005) were asymbiotic. At the moment, only one bacterial symbiont 16S rRNA-encoding gene sequence is available for this family (Distel & Wood 1992).

Following the collection of two bivalve species (genera *Myrtea* and *Thyasira*) from a large pockmark area with carbonates in the Central Nile Deep Sea Fan Province, north of the Nile delta, during the MEDECO cruise (2007), this study aimed to: (i) determine whether the species found are the same species documented by Olu-Le Roy *et al.* (2004); (ii) characterize their symbiotic associations using different approaches (16S rRNA gene sequence analysis, fluorescence *in situ* hybridization and electron microscopy); and (iii) provide data regarding deep-sea representatives of the Lucinidae and Thyasiridae, which are poorly documented compared to other symbiont-associated metazoan groups.

Material and Methods

Specimen sampling and storage

Specimens were collected from a large pockmark area named 'Central Zone' located in the Eastern Mediterranean during the 2007 MEDECO cruise aboard the RV *Pourquoi Pas?* (chief scientist: C. Pierre). Four lucinid bivalves, genus *Myrtea* (ML specimens, Fig. 1A,B), were found in sediment stuck to pieces of carbonate crusts collected at site 2B during the ROV *Victor 6000* dive 336-15 (32°30.07' N, 30°15.6' E; 1687 m depth). Four thyasirid bivalves (MT specimens, Fig. 1C), genus *Thyasira*, were recovered from sediment collected at site 2A during the dive 338-17 (32°31.97' N; 30°21.18' E; 1693 m depth). Upon recovery, specimens were dissected. One gill was stored in 100% ethanol for molecular work. The other gill was cut into two pieces, one half fixed for fluorescence *in situ* hybridization and the other for electron microscopy (see below). Shells were kept for morphological comparison with documented species.

DNA extraction and gene amplification

DNA was extracted from the gill tissue of all ML and MT specimens using a standard chloroform : isoamyl-alcohol extraction protocol, and genes encoding bacterial 16S rRNA gene were amplified using primers 27F and 1492R during 26 PCR cycles as described previously (Duperron *et al.* 2005). Three reactions per specimen were run in parallel and pooled prior to purification of the product using a QIAquick kit (Qiagen, CA). Products were cloned using a TA cloning kit (Invitrogen, CA). Positive clones were selected and full-length inserts were sequenced (GATC-biotech, Germany).

Host genes encoding 18S and 28S rRNA genes were amplified using primer pairs 18S-5'F/18S-1100r for 18S rRNA gene, and LSU-900f/LSU-1600r for 28S rRNA gene (Winnepenninckx *et al.* 1998; Olson *et al.* 2003; Williams *et al.* 2003). Amplifications were performed in a 50- μ L volume using 50 ng of DNA template, 5 μ L of 10 \times Taq buffer, 3 mM MgCl₂, 0.4 mM of each dNTP, 1 mM of each primer and 1 unit of Taq DNA polymerase. Target DNA was amplified for 30 cycles (94 °C for 1 min, 52 °C for 1 min, 72 °C for 1 min) with an initial denaturation step at 94 °C for 10 min and a final elongation step at 72 °C for 10 min. PCR products were sequenced (GATC-biotech).

Phylogenetic analyses

Sequences were checked against the GenBank database using BLAST and aligned with relatives using CLUSTALX

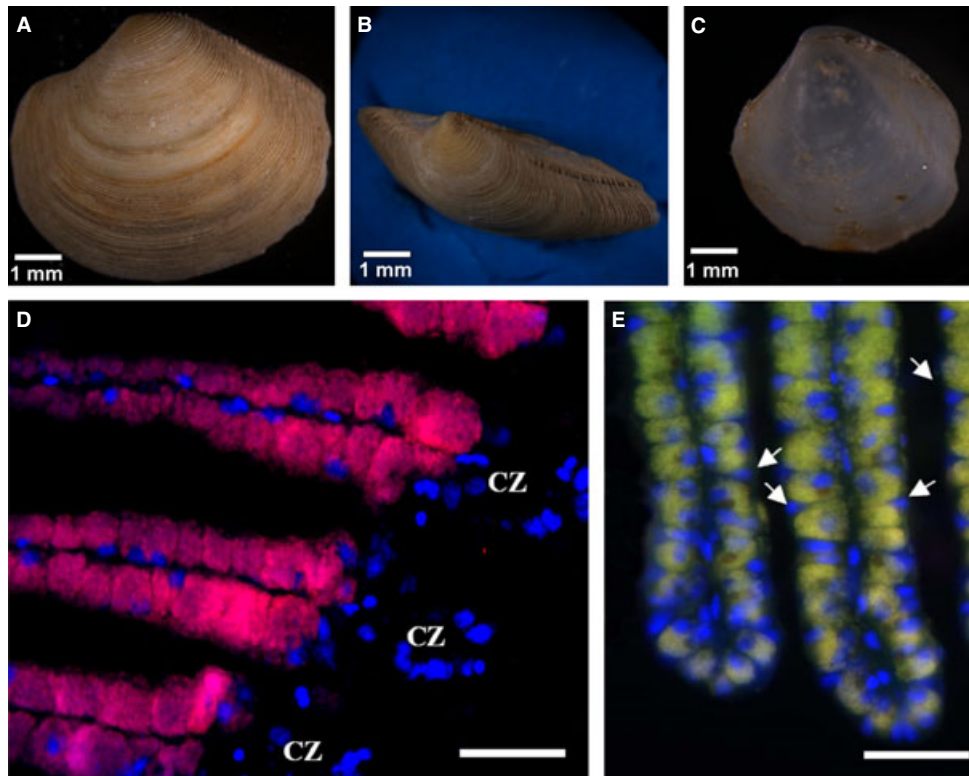


Fig. 1. (A) Lucinid ML (*Myrtea* sp.) collected at 1687 m depth. (B) Detail of the tubercles of a ML specimen (*Myrtea* sp.). The concentric ridges of the shell are obvious and a few spines can be observed on the margin. (C) MT specimen (*Thyasira* sp.) collected at 1693 m depth. (D) FISH hybridization of *Thyasira* sp. gill filaments with the Gam42a probe. No signal was observed from the ciliated zone (CZ), whereas all bacteriocytes from the lateral zone are fully hybridized, indicating a cytoplasmic volume filled with bacteria. Nuclei of thyasirid cells, which were counterstained with DAPI, appear in blue. Scale bar: 25 μ m. (E) FISH micrographs of *Myrtea* sp. gill filaments with the Gam42a probe focusing on the abfrontal zone of the filament. Intercalary cells (arrows) characterized by their nuclei in apical position appear free of bacteria, whereas adjacent bacteriocytes (stained in yellow) are fully hybridized, with the probe confirming the presence of numerous intracellular bacteria in their cytoplasm. Nuclei of lucinid cells, which were counterstained with DAPI, appear blue. Scale bar: 25 μ m.

(Thompson *et al.* 1994). An alignment of bacterial 16S rRNA gene sequences and an alignment of concatenated host 18S and 28S rRNA gene sequences were generated and checked by eye. Gap positions and ambiguously aligned sites were removed using GBLOCKS 0.91b (Castresana 2000). Each alignment was then imported in the TOPALI v2.5 software (Milne *et al.* 2004) to define the substitution model to be used for each dataset (Keane *et al.* 2006). The best model for each dataset was chosen using the Bayesian information criterion (Schwarz 1978). Phylogenetic analyses were then performed, trees being estimated by maximum likelihood (ML) using PHYML (Guindon & Gascuel 2003) and by Bayesian inference (BI) using MRBAYES 3 (Ronquist & Huelsenbeck 2003). Support for individual clades was evaluated using non-parametric bootstrapping obtained from 100 ML replicates (Felsenstein 1985). Posterior probabilities for BI were also evaluated for each node.

Fluorescence *in situ* hybridization (FISH)

Gill tissue was fixed (4% formaldehyde in 0.2- μ m filtered seawater, for 2 h at 4 $^{\circ}$ C), rinsed twice, dehydrated in increasing ethanol series, and stored in ethanol. In the lab, gills were embedded in PEG distearate : 1-hexadecanol (9 : 1) wax, and cut into 7- μ m-thick sections using a Jung microtome. Sections were deposited on Superfrost Plus slides (Roth, Germany) and hybridized as described previously (Duperron *et al.* 2005). Probes Eub338 (GCTGCCTCCCGTAGGAGT) and Non338 were used with 20% formamide as positive and negative controls, respectively (Amann *et al.* 1990). Probes Gam42 (5'-GCCTTCCCACATCGTTT-3', specific for Gammaproteobacteria) and Epsy549 (5'-CAGTGATTCCGAGTAACG-3', specific for Epsilonproteobacteria) were used with 30 and 50% formamide, respectively, to test for the presence of specific bacterial groups (Manz *et al.* 1992; Duperron *et al.* 2007b).

Electron microscopy

For transmission electron microscopy (TEM), gill fragments were pre-fixed for 1 h at 4 °C in 2.5% glutaraldehyde in 0.1 M pH 7.2 cacodylate buffer, and adjusted to 900 mOsM with NaCl and CaCl₂ to improve membrane preservation. Samples were then briefly rinsed in the cacodylate buffer and stored in the same buffer at 4 °C until they were brought to the laboratory. Samples were fixed for 45 min at room temperature in 1% osmium tetroxide in the same buffer before being rinsed in distilled water and post-fixed with 2% aqueous uranyl acetate for another hour. After a rinse in distilled water, each sample was dehydrated through a graded ethanol series and embedded in Epon-Araldite. Thin sections (60 nm thick) were contrasted for 30 min in 2% aqueous uranyl acetate and for 10 min in 0.1% lead citrate before examination in a TEM LEO 912. For scanning electron microscopy (SEM), prefixed samples stored in cacodylate buffer were dehydrated through a graded acetone series before drying with CO₂ in a critical point drier (Polaron; BioRad). The samples were then sputter-coated with gold (Sputter Coater SC500; BioRad) before observation at a SEM Hitachi S-2500 at a 20 kV accelerating tension.

Results

Host characterization

Phylogenetic analysis (using concatenated 18S and 28S rRNA genes) of the specimens confirmed that these species belonged to the genera *Myrtea* and *Thyasira* (Fig. 2). MEDECO Lucinidae (ML) specimens branched into the *Myrtea* clade. All four specimens clustered together, displaying a single shared 28S rRNA gene sequence, and a divergence of around 0.1% for 18S rRNA gene. Sequences clustered with *Myrtea spinifera* and displayed <0.2% and 0.1% difference with *M. spinifera* 28S and 18S rRNA gene sequences, respectively. Sequences from other lucinid species displayed at least 6.8% and 5.3% difference. A similar pattern was observed for MEDECO Thyasiridae (MT) specimens. Indeed, the four MT sequences branched within the Thyasiridae clade, and were almost identical, with a 0.1% difference in 28S rRNA gene and no differences in 18S rRNA gene. Specimens appeared closely related to *Thyasira flexuosa* (0.2% and 0.1% sequence divergence with *T. flexuosa* 28S and 18S rRNA sequences, respectively).

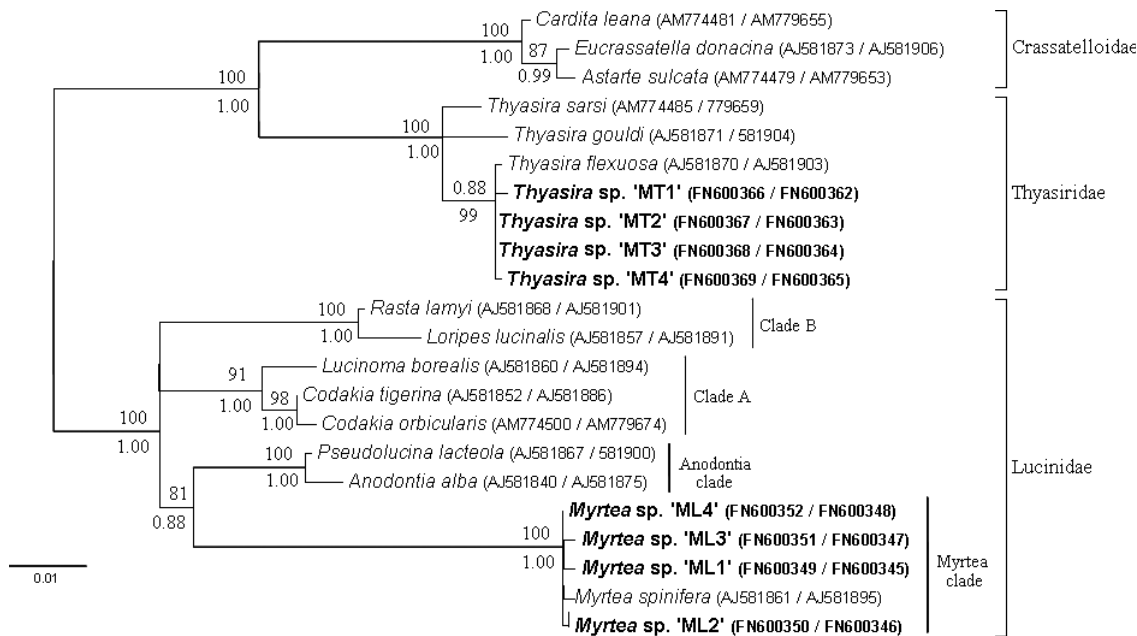


Fig. 2. Phylogenetic tree of bivalve hosts based on concatenated 18S and 28S rRNA gene sequences (1312 positions in the alignment). Tree was reconstructed using maximum likelihood (ML) and Bayesian inference (BI) under K80+I+Γ (BI) or TrNef+Γ (ML) evolutionary models. Robustness of clades was evaluated and only bootstrap values [ML, 100 replicates (above branches)] and posterior probabilities [BI (under branches)] above 80% are displayed. Sequences in bold were obtained in this study and other sequences are from Williams *et al.* (2003) and Taylor *et al.* (2007). Accession numbers: 18SrRNA/28SrRNA. Scale bar represents estimated 1% sequence substitution.

Characterization of symbiont sequences

Of 17 examined clones, all four ML specimens yielded a single 16S rRNA phylotype. Like all lucinid symbionts analyzed to date, the ML-associated phylotype was related to sulphur-oxidizing Gammaproteobacteria. According to the phylogenetic tree (Fig. 3) this phylotype clustered, and displayed above 98% sequence identity, with various symbionts of lucinids, including *Lucinoma* aff. *kazani* and *Lucinoma* sp., collected in the Mediterranean Sea and gulf of Cadiz, respectively (Duperron *et al.* 2007a; Rodrigues *et al.* 2008).

Twenty-three 16S rRNA gene clones were analyzed from the four MT specimens. All but one corresponded to a single phylotype related to the sulphur-oxidizing symbiont of *Thyasira flexuosa* (98.7% sequence identity) (Distel & Wood 1992). The second phylotype, identified only once, displayed 92% and 90% identical positions, respectively, with sequences from *Helicobacter* sp. and another *Epsilonproteobacterium*, possibly wrongly labelled as '*Vibrio* sp.', recovered from the sea fan (Gorgonacea) *Muricea elongata* (L.K. Ranzer, unpublished observations).

FISH on gill tissue

Gill tissue of both ML and MT specimens hybridized successfully with the general Eubacteria probe Eub338, and with the Gammaproteobacteria-specific probe Gam42. In ML specimens, positive signals were localized within a single cell type occurring in the lateral zone of gill filaments, which corresponds to documented bacteriocytes (Fig. 1E) (Fisher 1990). Most of the bacteriocyte volume was composed of bacteria. No signal was recovered from intercalary cells. In MT specimens, bacteria also comprised most of the bacteriocyte volume (Fig. 1D). Attempts to hybridize Epsilonproteobacteria using probe Epsy549 yielded very few positive bacterial cells in MT specimens, localized only around the ciliated zone (not shown).

Electron microscopy on gill tissue

Gills of ML specimens were similar in morphology to those of other Lucinidae, as described by Frenkiel & Moužza (1995), especially concerning the ciliated zone

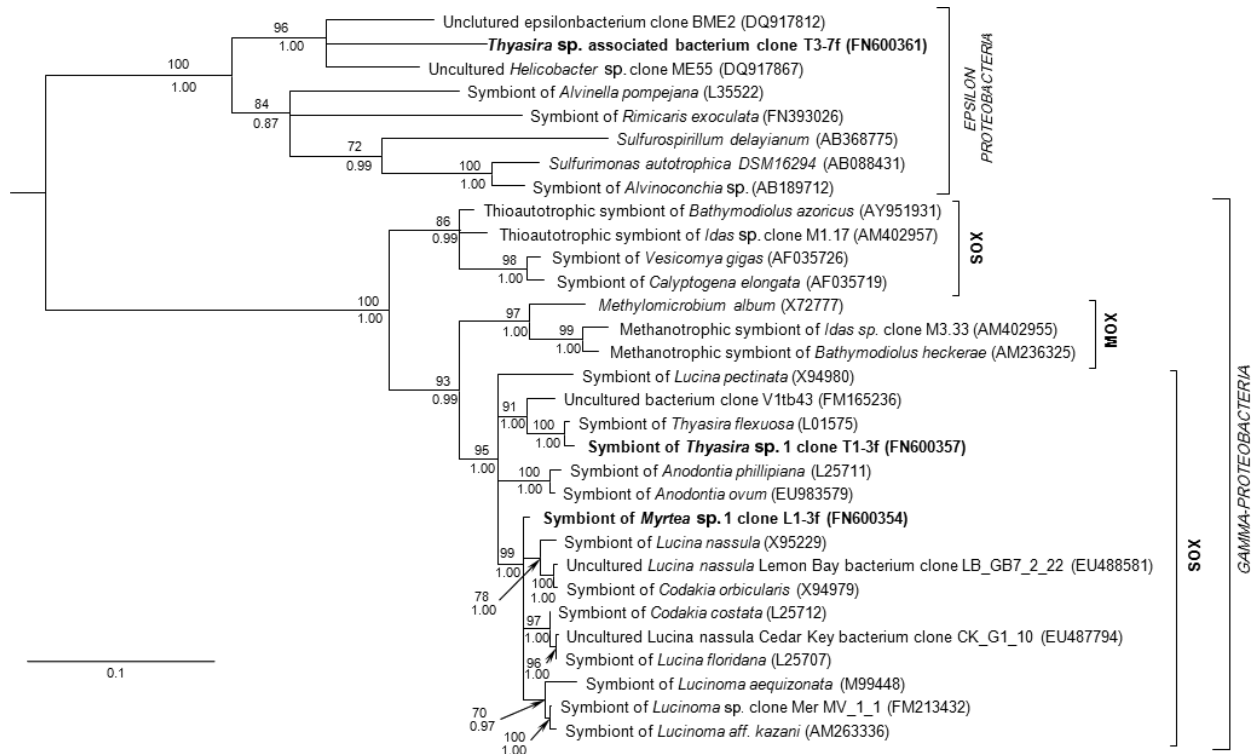


Fig. 3. Phylogenetic tree of bivalve-associated bacteria based on 16S rRNA gene sequences (1146 positions in the alignment). Tree was reconstructed using maximum likelihood (ML) and Bayesian inference (BI) under GTR+I+ Γ (BI) or K81+ Γ +I (ML) evolutionary models. Robustness of clades was evaluated and only bootstrap values [ML, 100 replicates (above branches)] and posterior probabilities [BI (under branches)] above 70% are displayed. Sequences in bold were obtained in this study. Groups of sulphur- (SOX) and methane-oxidizers (MOX) are highlighted. Scale bar represents estimated 10% sequence substitution.

(Fig. 4A). Gills displayed very abundant, mostly intracellular bacteria within vacuoles located within bacteriocytes, *i.e.* modified epithelial cells from the lateral zone (Fig. 4B–D) (Fisher 1990). Bacteria corresponded to a single $\sim 1\text{-}\mu\text{m}$ -diameter morphotype displaying a typical gram-negative envelope and no unique internal structures apart from the nucleoid (Fig. 4E,F). No sulphur granules were seen within bacterial cells. Besides bacteriocytes, intercalary cells and granule cells were seen, both of which were devoid of bacteria (Fig. 4B–D). No mucocyte was observed in the samples analyzed.

The lateral zone of the gill of MT specimens consisted of host cells completely filled with bacteria (Fig. 5A–C). Although the presence of nuclei, located close to blood lacuna, supports the hypothesis of host cells, these cells rather resembled bags filled with bacteria. Indeed, bacteria were not embedded within vacuoles (as observed in lucinids), and no host cytoplasm could be seen between bacteria (Fig. 5B–E). Except for the presence of a typical gram-negative double membrane, the morphology of bacteria differed from that described above from ML specimens, as bacterial symbionts displayed sulphur granules and glycogenic storage granules (Fig. 5E,F). Another distinctive feature was the presence of virus-like inclusions within some bacteria in both specimens investigated (Fig. 5F). These inclusions were sometimes abundant within a section of a single bacterium. Bacteriocytes containing infected symbionts presented large lysosomal structures probably synthesized to destroy infected bacteria before the lysis and liberation of new free viruses inside the bacteriocyte (Fig. 5C,D). A second bacterial morphotype with a helicoid morphology was sometimes observed on the outside of symbiont-containing cells, between two adjacent gill-filaments (Fig. 6A,B). Such spirochete-like bacteria were infrequently observed attached to the apical pole of the bacteriocytes in SEM (Fig. 6C,D). Their low frequency indicates that they are probably environmental bacteria rather than true symbionts.

Discussion

Host identification

Unexpectedly, the specimens studied and recovered here from the pockmarks of the Central Zone, north of the Nile deep-sea fan, are different species from the ones documented by Olu-Le Roy *et al.* (2004) from mud volcanoes and fault ridges in the Olimpi and Anaximander areas of the Eastern Mediterranean. During their study, Olu-Le Roy *et al.* reported the presence of *Myrtea amorphosa* and *Thyasira striata*. In contrast, our molecular results rather indicate that ML specimens are actually closely related to *Myrtea spinifera*, and MT specimens to

Thyasira flexuosa. Indeed, sequence divergences of 28S and 18S rRNA gene sequences from documented species is very low, ranging from 0.0 to 0.2%, and it is documented in gastropod and bivalve molluscs that members of a same species often share identical 18S rRNA and 28S rRNA gene sequences (Distel 2000; Lorion *et al.* 2009). However, it must be noted that these conserved gene sequences are sometimes not sufficient to discriminate sister species, so sequencing of additional genes such as the cytochrome oxidase I (COI) is recommended (Lorion *et al.* 2009). Unfortunately, despite attempts and the use of several published primers and programs, COI amplification could not be obtained here.

Myrtea spinifera and *T. flexuosa* have been found previously co-occurring with a rich population of Siboglinida *Siboglinum fjordicum* in the reducing environment of a shallow Norwegian fjord (Southward *et al.* 1979), but not yet been found in deep sites. *Myrtea spinifera* is reported from the Western Mediterranean (Williams *et al.* 2003) and Norway (Dando *et al.* 1985) at depths between 33 and 627 m. *Myrtea spinifera* is similar in appearance to *M. amorphosa*, and therefore the identification of *M. amorphosa* at deepwater cold seeps in various publications (Olu-Le Roy *et al.* 2004; Bayon *et al.* 2009) should be treated with caution, due to the possibility of misidentification. The ML specimens presented here display all the main morphological shell characteristics of *M. spinifera*, namely the concentric ridges which continue to the anterior and posterior dorsal margin as raised tubercles (Fig. 1A,B). Nevertheless, the sculpture on these specimens is almost twice as fine as that on NE Atlantic *M. spinifera* shells (P.G. Oliver, personal communication). According to the original description, *M. amorphosa* presents a sulcus, and the number of concentric ridges is higher (around 66 as opposed to 40 in *M. spinifera*) (Sturany 1896). Overall, ML specimens bear a greater resemblance to *M. spinifera*.

Originally described by Montagu (1803) from the south coast of England, *T. flexuosa* has been recorded as widely distributed in the intertidal and shelf to over 3000 m (Dando *et al.* 1985; López-Jamar & Parra 1997; Oliver & Killeen 2002; Williams *et al.* 2003; Dufour 2005; Dufour & Felbeck 2006). Its occurrence in California has been suggested, but molecular confirmation is needed (Distel *et al.* 1994). As for *Myrtea*, *Thyasira* identification is also complex and, in the case of *T. flexuosa*, this species can be easily confused with *Thyasira polygona* and *Thyasira gouldi*. Compared to *T. polygona*, *T. flexuosa* is bisinuate, and has a longer and less pronounced lunule margin, an auricle which is always strongly elevated, and sharper posterior folds (Fig. 1C). The bisinuate form is characteristic of both *T. flexuosa* and *T. gouldi* but most *T. gouldi* shells have

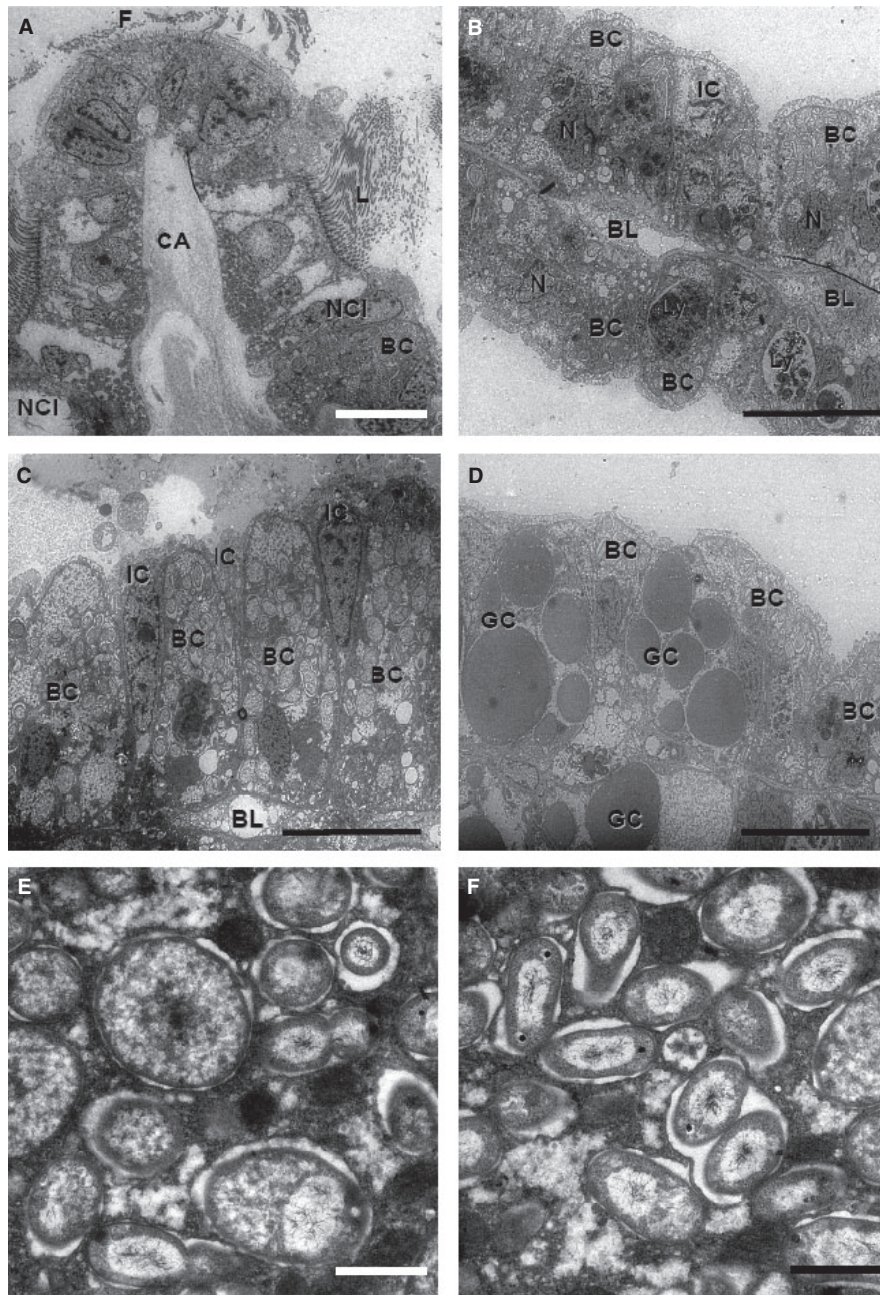


Fig. 4. TEM observations from freshly collected ML individuals (*Myrtea* sp.). (A) The ciliated zone of the gill filament from adult individuals is composed of frontal (F) and lateral (L) ciliated cells organized along a collagen axis (CA). The intermediary zone is limited to one non-ciliated cell (NCI) in contact with the last eulateral cell. The lateral zone contains mostly bacteriocytes (BC), filled with chemoautotrophic bacteria. Scale bar: 10 μm . (B,C) The pseudostratified epithelium of the lateral zone is composed of three cell types. The most prevalent cells are the bacteriocytes (BC), which are characterized by a basal nucleus (N), a rounded apical pole with microvilli, and a cytoplasmic volume filled with intracellular bacteria. Intercalary cells (IC), characterized by a trumpet shape and an apical nucleus, are regularly interspersed among bacteriocytes (BC). BL, blood lacuna; Ly, lysosomes. Scale bar: 10 μm . (D) Few granule cells (GC) are present in the abfrontal zone of the gill filament. Such cells, which are interspersed with bacteriocytes (BC), are characterized by large electron-dense granules. Scale bar: 10 μm . (E,F) High magnifications focusing on the gram-negative bacterial symbionts individually enclosed in vacuoles inside bacteriocytes. Electron-dense granules located in the cytoplasm of the bacteria probably correspond to glycogenic storage. The size difference of adjacent gill endosymbionts inside a single bacteriocyte is probably due to the section orientation. Notice that, exceptionally, bacterial division can occur inside a vacuole. Sulphur granules are not obvious in gill endosymbionts, in contrast to most sulphur-oxidizing symbionts described in the literature, colonizing the few individuals collected from a cold seep in the Nile Deep-sea fan. Scale bars: 1 μm .

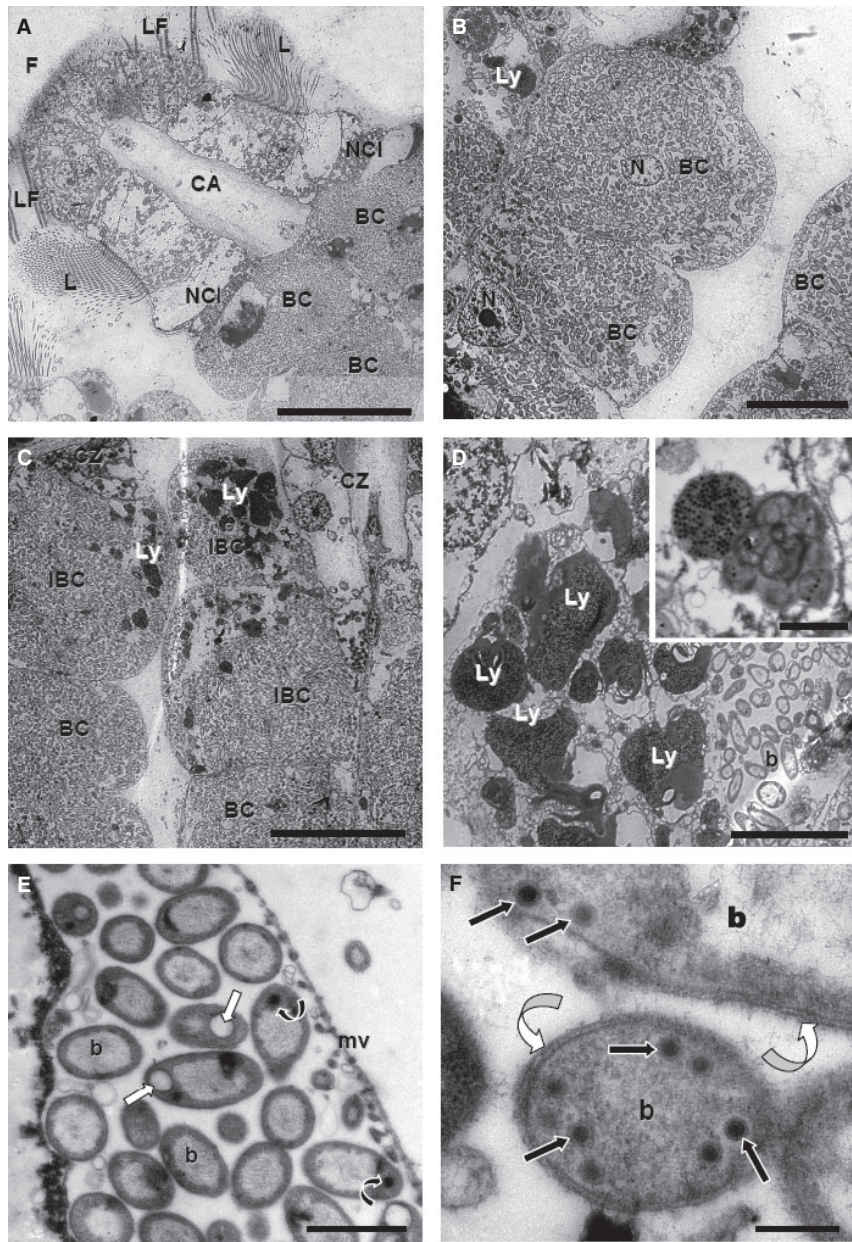


Fig. 5. TEM observations from freshly collected MT individuals (*Thyasira* sp.). (A) The ciliated zone of the gill filament from adult individuals is composed of frontal (F), laterofrontal (LF) and lateral (L) ciliated cells organized along a collagen axis (CA). The intermediary zone is not obvious in this species and is probably represented by a single non-ciliated intermediary cell (NCI). Bacteriocytes (BC) filled with chemoautotrophic symbionts are the most prevalent cells of the lateral zone. Scale bar: 20 μm . (B) Bacteriocytes (BC), which are the most prevalent cells in the gill filament, have a basal nucleus (N) and a rounded apical pole in contact with the pallial seawater. The cytoplasm is crowded by non-encapsulated bacteria. Scale bar: 10 μm . (C) In these two gill filaments, the first bacteriocytes of the lateral zone just below the ciliated zone (CZ) are characterized by numerous electron-dense lysosomal structures mostly located at the apical pole of the bacteriocytes. The bacteriocytes containing such structures are considered infected bacteriocytes (IBC) compared to normal bacteriocytes (BC). Scale bar: 20 μm . (D) Higher magnification showing the concentration of secondary lysosomes (Ly) in infected bacteriocytes. Each lysosome contains numerous dark inclusions that could be virus-like inclusions. Scale bar: 5 μm . Insert: a lysosome with both degraded bacteria and virus inclusions. Scale bar: 1 μm . (E) Intracellular gill endosymbionts of *Thyasira* sp. Such Gammaproteobacteria are characterized by a double membrane typical of gram-negative bacteria. The bacterial cytoplasm contains numerous non-membrane-bound inclusions, probably glycogenic storage (curved arrows). The large electron-lucent vacuole (straight arrows) is a periplasmic sulphur granule frequently present in most sulphur-oxidizing bacteria. Mv, microvilli from the host cell. Scale bar: 1 μm . (F) Details of two infected gill endosymbionts. The cytoplasm is crowded with intracellular virus-like inclusions (straight arrows). Notice the two membranes (curved arrows) typical of gram-negative bacteria. Scale bar: 200 nm.

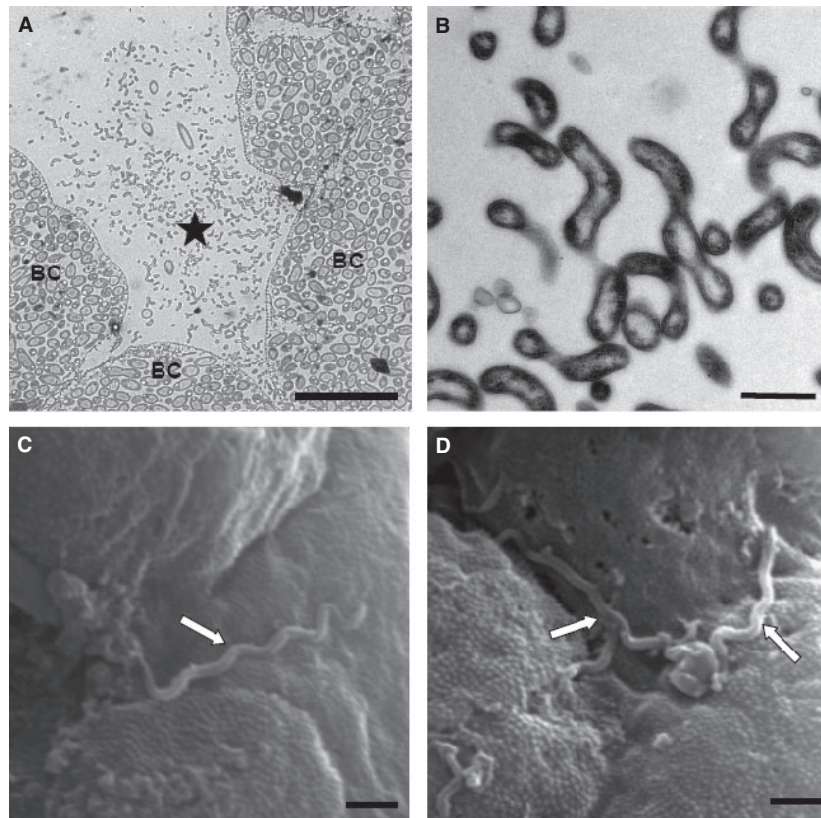


Fig. 6. Ultrastructural observation of helicoid-shaped bacteria on gill filaments of MT specimens (*Thyasira* sp.). (A) TEM. In a few sections, bacteria (star) are located outside the bacteriocytes (BC) between adjacent filaments. Compared to the intracellular gill endosymbionts, they appear thinner and 'curved'. Scale bar: 5 μm . (B) TEM. Higher magnification focusing on the extracellular bacteria, which are gram-negative bacteria according to their envelope structure. Scale bar: 500 nm. (C,D) SEM. Spirochete-morphotype bacteria (arrows) located on the apical pole of bacteriocytes. Scale bar: 2 μm .

a longer auricle, rounded posterior folds and weak sub-marginal and posterior sulci (Oliver & Killeen 2002). In this case, direct comparison of shells of MT specimens from this study with shells of *T. striata* kindly provided by Dr K. Olu allowed us to observe that both species possess coarse micro-spines. For this study, only small specimens were available; these specimens are very similar to *T. flexuosa*, but the presence of dense and coarse micro-spines differentiates them from typical *T. flexuosa*, where the micro-spines are not abundant (P.G. Oliver, personal communication). Without larger specimens, the designation of MT remains *Thyasira* sp.

The presence of two species closely related to shallow species in the deep pockmarks of the Eastern Mediterranean calls into question the species distribution and the evolutionary processes allowing closely related species to occupy such distinct habitats. In spite of their molecular similarity, shallow and deeper species present morphological differences that prevent us from reaching definitive conclusions about the species name in both

Myrtea and *Thyasira*. Minor shell differences and small molecular distances could result from recent evolution at seeps. Additional genes, as well as population genetic studies on many more specimens than available here, will be needed to test whether there is reproductive isolation from shallower populations, and to evaluate the specific status of these two bivalves.

Symbiotic relationships

Clone libraries from representatives of both species revealed a single dominant gammaproteobacterial 16S rRNA gene phylotype. *Myrtea* sp. yielded a single sequence related to other lucinid symbionts. The dominance of a single bacterial type inside bacteriocytes, as described in other lucinids analyzed to date (Durand *et al.* 1996; Duperron *et al.* 2007a), is further supported by FISH signals obtained using probes Gam42 and Eub338, which fully overlap. The closest relatives of the recovered phylotype include sulphur-oxidizing symbionts

from three deep-sea *Lucinoma* species from the Nile deep-sea fan (507–1691 m), the gulf of Cadiz (358 m) and the Santa Barbara Basin (500 m) (Distel *et al.* 1988; Duperron *et al.* 2007a; Rodrigues 2009). Other relatives are associated with shallow-water *Lucina* and *Codakia* species from reduced sediments and seagrass beds (Distel *et al.* 1994; Durand *et al.* 1996; A.M. Green-Garcia, unpublished observations). Apart from symbionts of *Lucina pectinata* (Durand *et al.* 1996) and *Anodontia* sp. (Ball *et al.* 2009), lucinid symbionts form a monophyletic group displaying only up to 3.7% sequence variation among members, despite their wide geographical and depth distribution. Such a low level of divergence is surprising because symbiosis in the Lucinidae has been suggested to date back to at least the Silurian (*Ilioma prisca*), with evidence for association with seep sites since the Jurassic, indicating that lucinids and their symbionts may have shared a long evolutionary history (Newell 1969; Boss 1970 cited in Distel *et al.* (1994)). Low variation could result from the wide distribution of connected populations of free-living symbionts establishing occasional associations with lucinids (Gros *et al.* 1996). Alternatively, if a symbiont lineage has recently evolved to become more efficient in establishing symbiosis with lucinids, and if this gives this lineage an advantage, this lineage could have replaced 'older' symbionts in several lucinid species. The morphology of the association in *Myrtea* sp., with a single bacterium per vacuole within host gill bacteriocytes, is highly similar to the structure observed in other species such as *Codakia orbicularis* (Frenkiel & Moužka 1995), with no evidence for the presence of additional bacterial types, as recently reported in *Anodontia ovum* and *Lucinoma* aff. *kazani* (Duperron *et al.* 2007a; Ball *et al.* 2009).

A dominant 16S rRNA gene phylotype was recovered from *Thyasira* sp., closely related to the sequence from a *Thyasira flexuosa* sulphur-oxidizing symbiont from Plymouth Sound, at 15 m depth (Distel & Wood 1992). Unfortunately, this is the only full-length sequence available from a thyasirid symbiont, apart from *Maorithyas hadalis*, which harbours an unusual type of dual symbiosis among thyasirids (Fujiwara *et al.* 2001; Dufour 2005). In this study, we present the first FISH pictures obtained from a thyasirid bivalve gill. Hybridizations confirm the dominance of Gammaproteobacteria inside bacteriocytes, based on the observation of fully overlapping signals between the two probes tested. This and the absence of FISH signals for the Epsilonproteobacteria-specific probe confirm that the epsilonproteobacterial sequence is very unlikely to be a second symbiont. More data would be needed to test whether, as in lucinids, several thyasirid species share very similar symbionts, and whether a single given symbiont associates with all

representatives of a given host species, whatever their geographical localization and depth. Gill morphology of *Thyasira flexuosa* has already been described by Dufour (2005) as 'Type 3 gills' with bacteria separated from the outside environment by a membrane. Bacteria are located inside host cells, although not individually enclosed in vacuoles as described in lucinids such as *Myrtea* (Southward 1986; Herry & Le Pennec 1987). Bacteriocytes appear as 'bags' filled with bacteria with no visible vacuoles surrounding either a single bacterium or a group of bacteria.

Possibility of parasitism in *Thyasira* sp.

Interestingly, virus-like inclusions were present in many bacteria within the gill of *Thyasira* sp. These inclusions can be abundant within a section of a single bacterium, suggesting a rather high level of infection. The presence of such inclusions was previously reported from symbionts of *Thyasira flexuosa* from Plymouth Sound and thus seems to be a recurrent feature of this particular association (Dando & Southward 1986; Southward & Southward 1991). This could indicate large-scale infection by viruses, as reported for *Bathymodiolus* mussels (Ward *et al.* 2004). Few bacteriocytes display obvious signs of viral infection of the symbionts, suggesting that infection is limited to individual bacteriocytes, or that release of viral particles is synchronized in many bacteria from a single bacteriocyte, perhaps through a lytic pathway. Infected bacteriocytes are indeed characterized by numerous bacterial symbionts filled with viruses and large lysosomal structures synthesized by the host cell, probably to remove infected bacteria before the lysis and liberation of new free viruses inside the bacteriocyte. Such infected bacteria may be considered permissive cells as they seemingly allow virus replication. The infected bacteria probably break open, allowing the viruses to access nearby bacterial cells, contaminating the whole bacteriocyte. The bacteriocyte could be then destroyed by the host.

In this study we report *Myrtea* sp. and *Thyasira* sp., two bivalves closely related to species from shallow sediments, from a deep-sea cold seep site in the Eastern Mediterranean. Both species display symbioses involving thiotroph-related Gammaproteobacteria very similar to symbionts reported from their shallow-water relatives. Representatives of the lucinid and thyasirid bivalve families occur both in coastal sediment and at deep-sea chemosynthesis-based sites, suggesting a wide ecological plasticity. Whether hosts or symbionts are responsible for this ability to colonize diverse sites remains to be determined, and the evolutionary and functional aspects of the associations deserve further study.

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