Evidence for chemoautotrophic symbiosis in a Mediterranean cold seep clam (Bivalvia: Lucinidae): comparative sequence analysis of bacterial 16S rRNA, APS reductase and RubisCO genes

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Abstract

Symbioses between lucinid clams (Bivalvia: Lucinidae) and autotrophic sulphide-oxidizing bacteria have mainly been studied in shallow coastal species, and information regarding deep-sea species is scarce. Here we study the symbiosis of a clam, resembling *Lucinoma kazani*, which was recently collected in sediment cores from new cold-seep sites in the vicinity of the Nile deep-sea fan, eastern Mediterranean, at depths ranging from 507 to 1691 m. A dominant bacterial phylotype, related to the sulphide-oxidizing symbiont of *Lucinoma aequizonata*, was identified in gill tissue by comparative 16S rRNA gene sequence analysis. A second phylotype, related to spirochete sequences, was identified twice in a library of 94 clones. Comparative analyses of gene sequences encoding the APS reductase α subunit and ribulose-1,5-bisphosphate carboxylase oxygenase support the hypothesis that the dominant symbiont can perform sulphide oxidation and autotrophy. Transmission electron micrographs of gills confirmed the dominance of sulphide-oxidizing bacteria, which display typical vacuoles, and $\delta^{13}C$ values measured in gill and foot tissue further support the hypothesis for a chemoautotrophic-sourced host carbon nutrition.

Introduction

Lucinid clams are infaunal bivalves that live in reduced sediment in both shallow coastal waters as well as at deep-sea cold seeps. Lucinids display typical characteristics of symbiont-associated bivalves, such as reduced gut and palps and large fleshy gills (Fiala-Médioni & Felbeck, 1990; Taylor & Glover, 2000). Hence, all investigated species are associated with symbiotic bacteria found in specialized epithelial cells termed bacteriocytes. Symbiosis is often considered to be the major factor that has shaped evolution within the family Lucinidae since its emergence in the Jurassic (Little & Vrijenhoek, 2003).

Because of ease of access, most information regarding symbiosis in lucinids has been obtained from species collected in shallow, coastal sediments (for a review, see Fiala-Médioni & Felbeck, 1990; Fisher, 1990). Despite attempts (Wood & Kelly, 1989), symbionts have not been successfully grown separately from their host (Cary *et al.*, 1989). However, indirect evidence based on enzyme assays and transmission electron microscopy (TEM) indicates that symbionts are autotrophic sulphide-oxidizing bacteria (Felbeck *et al.*, 1981). In oxygen-depleted environments, symbionts of some species can avoid competition with their host for oxygen resources by growing on nitrate (Duplessis *et al.*, 2004), indicating the existence of environment-specific adaptation or physiological responses. Phylogenetic characterization of the symbionts has shown that they belong to the *Gammaproteobacteria*, and are related to symbionts from thyasirid and solemyid bivalves as well as symbionts from siboglinid tubeworms (Stewart *et al.*, 2005). Host–symbiont specificity is questionable, as several host species of the genera *Codakia* and *Lingua* collected from similar habitats harbour the same 16S rRNA gene bacterial phylotype (Durand & Gros, 1996; Durand *et al.*, 1996); environmental transmission of bacteria has been demonstrated in *Codakia orbicularis* in experimental systems (Gros *et al.*, 1996, 2003a, b); and free-living forms of the symbionts were identified in seagrass beds in the clam's habitat (Gros *et al.*, 2003a, b).

Although deep-sea cold-seep species of lucinids are known, mainly from evidence from empty shell (for a review, see Sibuet & Olu, 1998), data regarding their symbioses are

comparatively scarce. This is due to the infaunal habitat of lucinids, preventing observation and specific collection of living specimens on the sea floor. *Lucinoma kazani* was recently described based on few living specimens recovered from gas-saturated sediments at Kazan mud volcano (1709 m depth, eastern Mediterranean) (Salas & Woodside, 2002; Olu-LeRoy *et al.*, 2004). This species was suggested to harbour sulphide-oxidizing symbionts based on scanning electron micrographs, but this hypothesis was not tested (Salas & Woodside, 2002). During the *Nautinil* cruise in 2003, several new cold-seep sites were discovered and explored in the vicinity of the Nile deep-sea fan. Specimens of a lucinid resembling *L. kazani* (based on shell morphology) were sampled from sediment cores on the North Alex and Isis mud volcanoes, and from pockmarks in the central area, allowing investigation of symbiosis in greater detail. Here we describe the bacterial symbionts and their phylogenetic relationship to other lucinid symbionts based on comparative 16S rRNA gene sequence analysis, and we assess their metabolic potential by sequencing target functional genes encoding proteins involved in the sulphide oxidation pathway (APS reductase) and in carbon fixation using the Calvin Benson cycle [Ribulose 1,5 bisphosphate carboxylase oxygenase (RubisCO)]. The structure of the association and its nutritional role are investigated using TEM and stable isotope analysis.

Materials and methods

Samples

Specimens of lucinid clams resembling *L. kazani* (Salas & Woodside, 2002) and herein referred to as *Lucinoma* aff. *kazani* were collected in 2003 using the manned submersible *Nautile* during the *Nautinil* cruise to the eastern Mediterranean (chief scientist: J.P. Foucher). These bivalves were buried in sediments below the oxic/anoxic interface. Two specimens from a sediment core collected on the North Alex mud volcano (31°58.2′N 30°08.2′E, 507-m depth) were dissected and stored in liquid nitrogen for DNA studies and stable isotope measurements. Gill tissue of two specimens from pockmarks of the Central area (32°31.6′N, 30°20.7′E, 1691 m depth, dive NL7) and from the Isis mud volcano (32°21.7′N, 31°23.4′E, 990-m depth, dive NL8) was fixed for TEM (see below).

Methane measurements

To confirm the presence of methane in the sediment, two 750-mL water samples were collected a few centimetres above the seafloor, using titanium syringes coupled to a funnel. Methane concentrations were measured onboard by gas chromatography according to the protocol described in Sarradin & Caprais (1996).

Gene amplification, cloning and sequencing

DNA was extracted from the gill tissue of the two North Alex specimens using a FastDNA® SPIN KitPrep (Qbiogene). The gene encoding 16S rRNA gene was amplified from each specimen as described by Duperron *et al.* (2005). Fragments of functional gene sequences were amplified from both individuals. Owing to limited amounts of amplified DNA, PCR products were pooled prior to cloning. A 395-bp fragment of the gene encoding the α subunit of the APS reductase was amplified using primers APS1-FW and APS4-RV (Deplancke *et al.*, 2000). An initial denaturation step (92 °C, 4 min) was followed by 33 cycles at 92 °C (1 min), 58 °C (1 min) and 72 °C (1 min). The reaction ended with a final elongation step at 72 °C (10 min). A 691-bp fragment of the gene encoding RubisCO form I was amplified using primers cbbl1b (5′ CACC TGGACCACVGTBTGG 3′) and cbbl2c (5′ CGGTGYATGTGC AGCAGCATGCCG 3′) (Elsaied & Naganuma, 2001). Denaturation was followed by 33 cycles at 92 °C (1 min), 48 °C (1.5 min) and 72 °C (1 min). A final elongation step was added. The presence of a particulate methane mono-oxygenase α subunit gene (*pmoA*) was tested by amplifying a gene fragment using the PCR protocol described in Holmes *et al.* (1995). PCR products were purified using a Qiagen kit and cloned with a TOPO-TA cloning kit (Invitrogen). Inserts were partially sequenced using a vector-specific primer. For the 16S rRNA gene, 94 clones were partially sequenced (47 from each individual) and 25 fully sequenced. A total of 19 APS and 12 RubisCO clones were fully sequenced.

Sequence analysis and phylogenetic reconstruction

Nucleotide sequences of the 16S rRNA gene, and amino acid sequences of APS reductase and RubisCO genes were compared with sequences available from the EMBL database using a BLAST search (Altschul *et al.*, 1990). Sequences displaying highest similarities as well as representative sequences for known groups were included in further analyses. Sequences were aligned using CLUSTALX. Phylogenetic analyses of the 16S rRNA gene sequences were run with TREEFINDER (Jobb, 2003) using the maximum-likelihood method and a general time reversible model. RELL values representing the percentage of trees in which a given node appears in the 1000 most probable trees were used as support values for the nodes. Phylogenies of the APS and RubisCO genes were reconstructed with the PHYLIP package (Felsenstein, 2002) using a maximum-likelihood method with a JTT model. Bootstrap values were obtained using the same method.

Transmission electron microscopy

Sample fixation, inclusion and sectioning were performed according to the procedure described in Duperron *et al.* (2005).

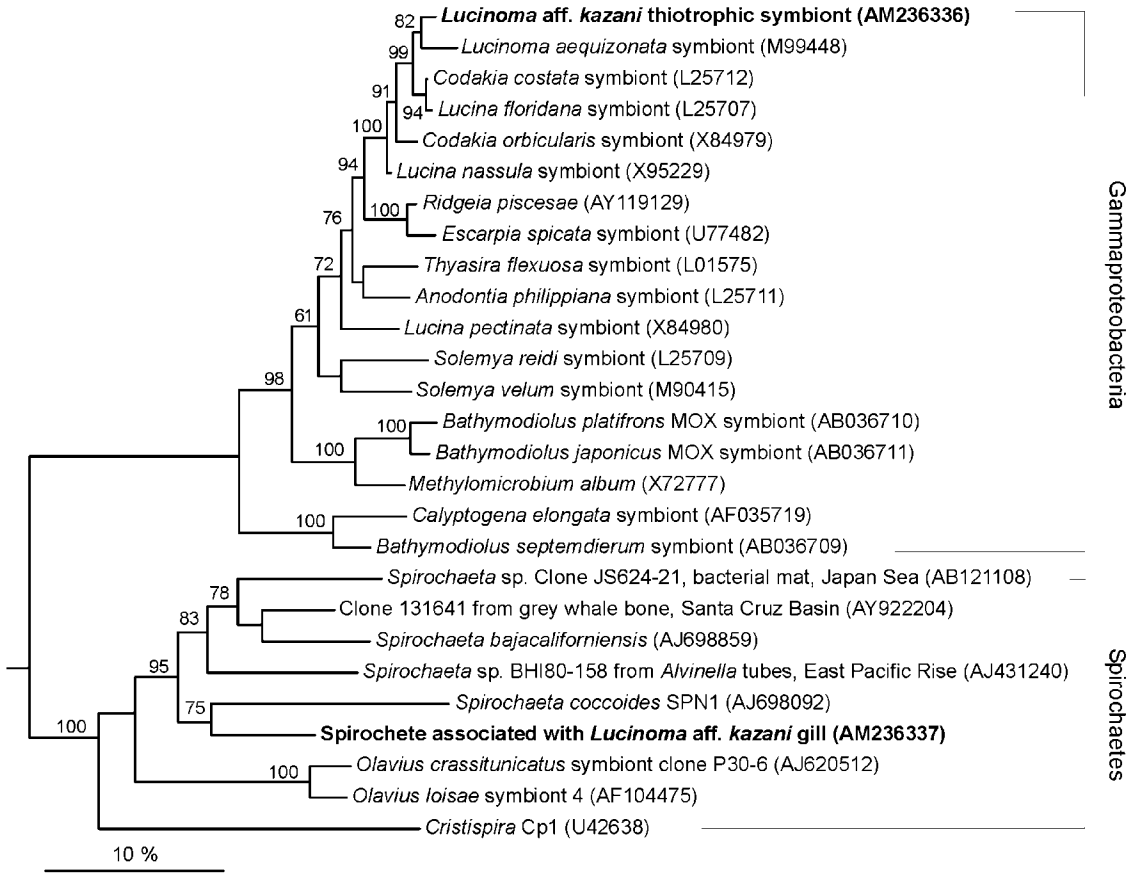

Fig. 1. Maximum-likelihood tree displaying the phylogenetic relationships of symbionts from *Lucinoma* aff. *kazani* (in bold) to *Gammaproteobacteria* and spirochetes, based on 16S rRNA gene sequences (1240 nt positions). The tree was constructed using a GTR model, and RELL values were obtained from the 1000 best trees (only values > 60% are shown), Likelihood: −12774. Within *Gammaproteobacteria*, all symbionts are from putative sulphide oxidizers, except those labelled MOX (methane-oxidizers). Scale bar represents 10% estimated base substitution.

After addition of uranyl acetate, ultrathin sections were observed with a Hitachi H-7500 transmission electron microscope.

Stable isotope analysis

Frozen gill and foot tissue from a specimen were acidified in 0.1 N HCl, rinsed and dessiccated in a 46 °C chamber. After grinding, stable isotope values of nitrogen and carbon were measured in a Finnigan Delta S isotope ratio mass spectrometer.

Sequence accession numbers

Nucleotide sequences were deposited in the EMBL database under accession numbers AM236336 and AM236337 (16S rRNA gene sequences), AM236338 (APS gene sequence), and AM236339 and AM236340 (RubisCO sequences).

Results and discussion

A total of 94 partial 16S rRNA gene sequences were obtained from the two lucinid clams, representing two distinct bacterial phylotypes. The dominant phylotype, 92 of the 94 sequences, belonged to a gammaproteobacterium. The occurrence of a unique gammaproteobacterial 16S rRNA gene phylotype was supported by the very low level of variation between sequences (<0.2%), and by the absence of shared nucleotide substitutions (Duperron *et al.* in press). This phylotype shared 97% sequence identity with the thiotrophic symbiont of *Codakia costata*, a coastal lucinid from sediments in shallow tropical areas (Distel *et al.*, 1994). The second phylotype occurred twice in the clone library from one of the specimens and was similar to clone Nubeena322, a spirochete partial 16S rRNA gene sequence recovered from marine sediments (91% identity, short sequence not included in the phylogenetic reconstruction). The phylogenetic tree (Fig. 1) resembled that presented by Durand *et al.* (1996), with symbionts of *Thyasira flexuosa*, *Lucina pectinata* and *Anodontia phillipiana* branching at the base of the clade containing sequences from symbionts of thyasirids and lucinids. Symbionts from siboglinid tubeworms also clustered within this group. The gammaproteobacterial

phylotype appeared in a well-supported clade containing symbionts of lucinids, and was closely related to the symbiont of *Lucinoma aequizonata*, a clam collected in reduced sediment at a depth of 500 m off the coast of California (Distel *et al.*, 1988). Therefore, it is likely that it belongs to a bacterial symbiont. The spirochete-related phylotype clustered with *Spirochaeta coccoides*, a coccoid-shaped spirochete recently discovered in the hindgut of a termite (Dröge *et al.*, 2006). It is not closely related to spirochete symbionts previously identified in the oligochetes *Olavius algarvensis* and *Olavius ilvae* (Dubilier *et al.*, 1999; Blazejak *et al.*, 2005), nor to *Cristispira*, a large spirochete identified in the crystalline style of oysters (Margulis *et al.*, 1991; Paster *et al.*, 1996).

A single APS α subunit sequence was retrieved. It shared 90% amino-acid identity with that of the betaproteobacterium *Thiobacillus denitrificans*, its closest relative in the tree, together with a clone from the intestinal tract of a mouse (Fig. 2). The sequence did not cluster with the APS sequence from the gammaproteobacterium *Allochromatium vinosum*. This relationship might indicate that the sequence derives from a contaminating, nonsymbiotic, bacterium from the environment. However, lateral transfer of the ApsA gene, which has been reported in sulphate-reducing bacteria (Friedrich, 2002), could explain the discrepancy in the tree if the APS sequence actually belongs to the gammaproteobacterial symbiont. This hypothesis is further supported by the fact that APS reductase activities have been measured in many lucinid clam gills (Felbeck *et al.*, 1981; Dando *et al.*, 1985; Spiro *et al.*, 1986). Spirochetes are not known to possess APS reductase, and no significant result was obtained from 'blasting' APS amino acid sequences against the six available spirochete complete genomes. It is therefore unlikely that the APS sequence belongs to the putative spirochete symbiont.

Two closely related RubisCO form IA sequences were recovered (>98.5% and > 97.0% base and amino acid identity, respectively), both displaying 96% amino acid identity to the sequence of the *Solemya velum* thiotrophic endosymbiont, which appeared as their closest relative in the tree (Fig. 3; Schwedock *et al.*, 2004). These sequences cluster within a clade that includes RubisCO form IA sequences from chemoautotrophic symbionts associated with molluscs. This supports the hypothesis that they derive from the thiotroph-related symbionts and not from a contaminating bacterium. Alternatively, they could originate from the spirochete putative symbiont, and although autotrophy has been reported in spirochetes (Madigan *et al.*, 2002), no spirochete sequence resembling RubisCO could be retrieved using BLAST analysis. The occurrence of two related APS sequences might indicate the presence of two distinct symbiont strains even though they were not distinguished based on their 16S rRNA gene sequence. No amplification could be obtained for *pmoA*.

TEM observations on gill sections (Fig. 4) indicated the presence of numerous inclusions, 0.65–1.04-μm diameter ($n = 14$), resembling bacterial symbionts reported in other

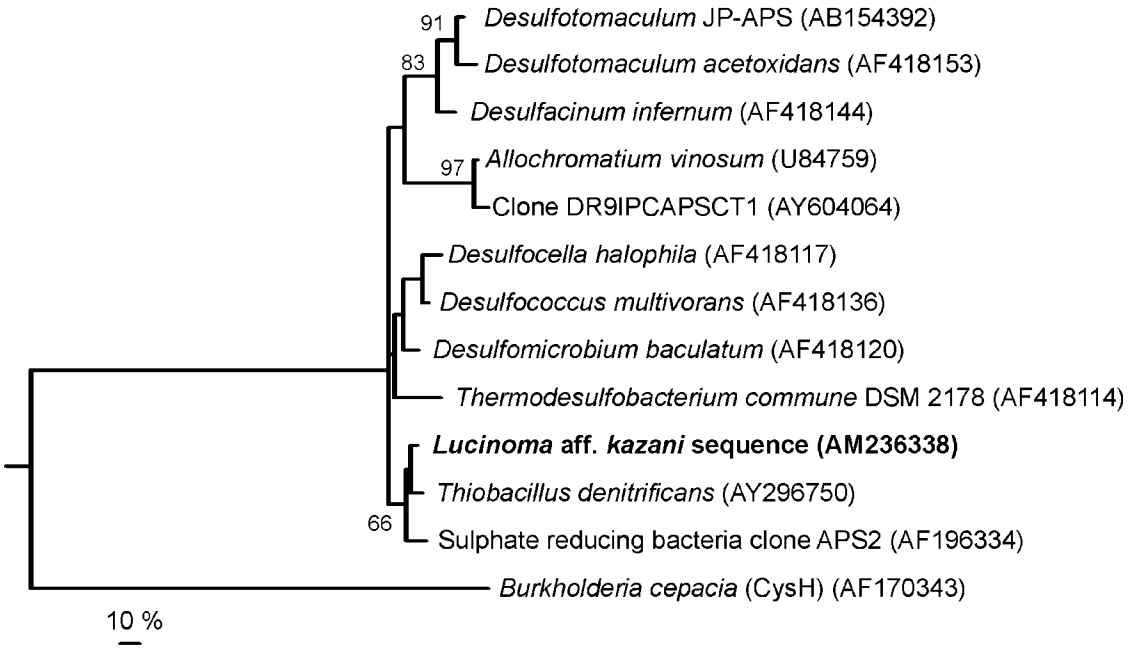

Fig. 2. Maximum-likelihood tree displaying the phylogenetic position of the APS reductase α subunit sequence retrieved from *Lucinoma* aff. *kazani* (in bold). The tree was contructed based on 125 amino acid positions using a JTT model, sequence input order was randomized, and bootstrap values were obtained from 500 replicates (only values > 60% are shown). Scale bar represents 10% estimated amino acid substitution.

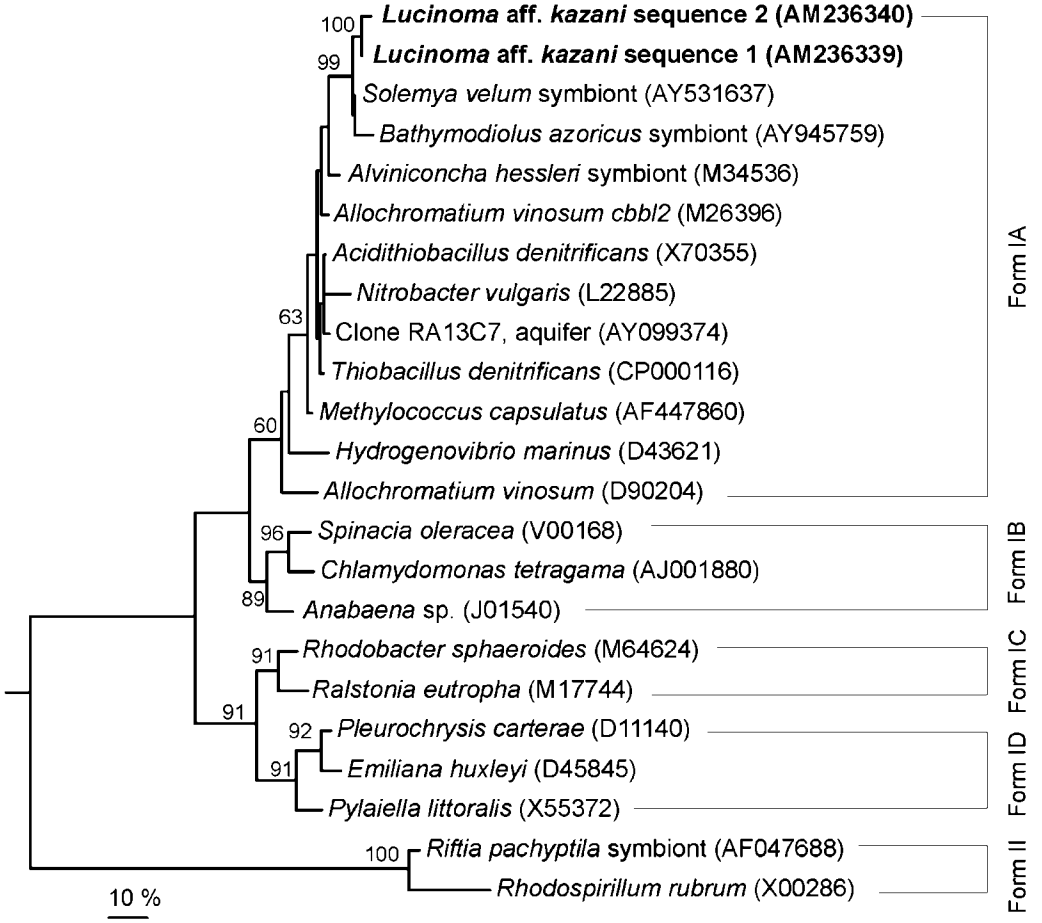

Fig. 3. Maximum-likelihood tree displaying the phylogenetic position of the two RubisCO sequences retrieved from *Lucinoma* aff. *kazani* (in bold). Both sequences from *L.* aff *kazani* cluster together within the Form IA cluster, and are related to sequences from *Gammaproteobacteria*. The tree was constructed based on 230 amino acid positions using a JTT model, sequence input order was randomized, and bootstrap values were obtained from 200 replicates (only values > 60% are shown). Scale bar represents 10% estimated amino acid substitution.

lucinids (see, for example, Gros *et al.*, 2003a, b). Bacteria occur in vacuoles containing one or two individuals that occupy most of the volume within specialized bacteriocytes. Symbionts lack any internal membrane system such as observed in methanotrophs (Anthony, 1982), but display electron-lucent vesicles that are interpreted as sulphur granules in other species (Vetter, 1985). No morphotype resembling typical spirochetes was observed, suggesting that the retrieved spirochete 16S rRNA gene phylotype came from an environmental contaminant. However, the recovered phylotype was related to an unusual coccoid-shaped spriochete, *S. coccoides* (Dröge *et al.*, 2006), so perhaps this bacterium simply does not display the typical spirochete morphology.

Carbon isotopic signatures of −30.5‰ and −28.2‰ were measured, respectively, in the gill and foot tissue of a specimen. These values are in the range of values reported elsewhere for lucinids harbouring thiotrophic symbionts (reviewed in Fisher, 1990), and further support the hypothesis for a carbon nutrition source based on bacterial chemoautotrophy.

Our results suggest that the dominant gill endosymbiont of *Lucinoma* aff. *kazani* is a gammaproteobacterium that probably possesses APS reductase- and RubisCO-encoding genes. Larger fragments surrounding these genes should be sequenced to verify this definitely. To date, all lucinids investigated from both coastal and deep-sea environments, at least 30 species from 18 different genera, were shown to harbour this type of symbiosis, although we provide the first APS reductase and RubisCO gene sequences (Taylor & Glover, 2000). In this study, we report for the first time a spirochete 16S rRNA gene sequence from the gill tissue of a lucinid. We provide no evidence that spirochetes actually occur as symbionts in lucinids, but spirochete symbionts, not closely related to that providing the sequence described herein, are known to occur in oligochetes (Dubilier *et al.*, 1999; Blazejak *et al.*, 2005). Further work should investigate

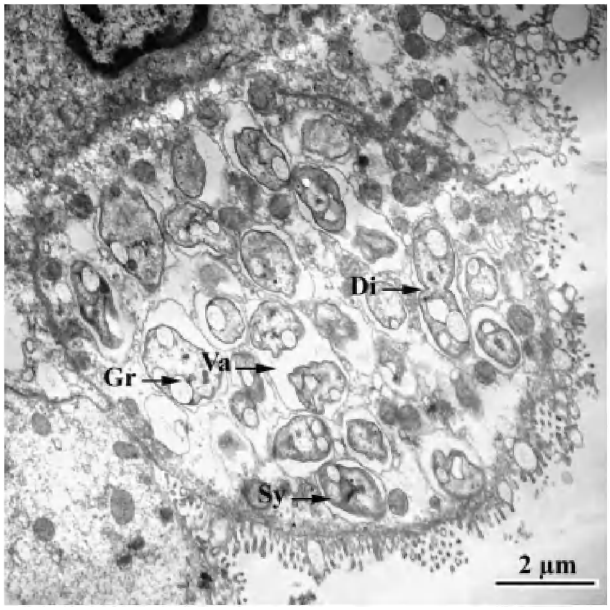

Fig. 4. TEM micrograph from a bacteriocyte within a gill of *Lucinoma* aff. *kazani* displaying symbionts (Sy) possessing empty vesicles previously described as remnants of sulphur granules (Gr). Symbionts are located in vacuoles (Va), and dividing stages occur (Di). Scale bar represents 2 µm.

the true status of spirochetes in lucinids, for example using fluorescence *in situ* hybridization techniques.

Sediments at the North Alex mud volcano are rich in methane. Gas bubbles were observed (A. Prinzhofer, pers. commun.), and methane concentrations of 34 and 640 µM were measured from two water samples collected just above the seafloor. This indicates the presence of methane in the underlying sediment. The occurrence of *Lucinoma* aff. *kazani* might be related to local enrichments in sulphide resulting from the activity of microbial consortia mediating the anaerobic oxidation of methane coupled to sulphate reduction (Boetius *et al.*, 2000). The *L. kazani* shell morphotype also occurs at several cold-seep sites in the eastern Mediterranean (Salas & Woodside, 2002; Olu-LeRoy *et al.*, 2004). Investigating the distribution of this lucinid and the degree of phylogenetic relatedness between populations of hosts and symbionts would greatly improve our knowledge of the biogeography and evolution of symbiosis in deep-sea lucinids, which are currently poorly documented compared with those for coastal species.

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