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 δ13C values measured in gill and foot tissue further support the hypothesis for a chemoautotrophic-sourced host carbon nutrition.

Introduction

Lucinid clams are infaunal bivalves which 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 named bacteriocytes. Symbiosis is often considered as the major factor shaping the evolution within the family Lucinidae since its emergence in the Jurassic (Little & Vrijenhoek 2003).

Due to ease of access, most information regarding symbiosis in lucinids has been obtained from species collected in shallow, coastal sediments (for 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) indicate 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 class, 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 genuses *Codakia* and *Lingua* collected from similar habitats harbour the same 16S rRNA bacterial phylotype (Durand & Gros 1996, Durand et al. 1996); environmental transmission of bacteria has been demonstrated in *C. orbicularis* in experimental systems (Gros et al. 1996, Gros et al. 2003); and free -living forms of the symbionts were identified in seagrass beds in the clam's habitat (Gros et al. 2003).

Although deep-sea cold seep species of lucinids are known, mainly from empty shells evidence (for review, see Sibuet & Olu 1998), data regarding their symbioses is 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 (1709m 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 to investigate symbiosis in more details. In this study, we describe the bacterial symbionts and their phylogenetic relationship to other lucinid symbionts by comparative 16S rRNA 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 or 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 *Lucinoma kazani* (Salas & Woodside 2002) and herein referred as *Lucinoma* aff. *kazani* were collected using the manned submersible Nautile during the Nautinil cruise to the eastern Mediterranean (2003, 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, 507m 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°31.6N, 30°20.7'E, 1691 m depth, dive NL7) and on ISIS mud volcano (32°21.7'N, 31°23.4' E, 990 m depth, dive NL8) was fixed for transmission electron microscopy (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, CA). The gene encoding 16S rRNA was amplified from each specimen as described elsewhere (Duperron et al. 2005). Fragments of functional genes sequences were amplified from both individuals. Due to limited amounts of amplified DNA, PCR products were pooled prior to cloning. A 395 bp fragment of the gene encoding the alpha 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 and cbbl2c (Elsaied & Naganuma 2001). Denaturation step 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 monooxygenase alpha 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 kit (QIAGEN, Germany) and cloned with a TOPO-TA cloning kit (Invitrogen, California). Inserts were partially sequenced using a vector-specific primer. For 16S rRNA, 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 16S rRNA, and amino acid sequences of APS reductase and RubisCO were compared with sequences available from the EMBL database using BLAST (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 sequences were run with TreeFinder (Jobb 2003) using the maximum likelihood method and a general time reversible model. RELL values from the 1000 best 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. After addition of uranyl acetate, ultrathin sections were observed with an HITACHI H-7500 transmission electron microscope.

Stable isotope analysis

Frozen gill and foot tissue from a specimen were acidified in 0.1N 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.

Results and Discussion

A total of 94 partial 16S rRNA sequences were obtained from the two lucinid clams, representing two distinct bacterial phylotypes. The dominant phylotype, 92 of the 94 sequences, belongs to a Gammaproteobacterium. The occurrence of a unique Gammaproteobacterial 16S rRNA phylotype is 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 shares 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 occured twice in the clone library from one of the specimens and is similar to clone Nubeena322, a spirochete partial 16S rRNA sequence recovered from marine sediments (91% identity, short sequence not included in the phylogenetic reconstruction). The phylogenetic tree (Fig. 1) resembles that presented by Durand and co-workers (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 cluster within this group. The Gammaproteobacterial phylotype appears in a well-supported clade containing symbionts of lucinids, and is 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 clusters 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 *O. ilvae* (Dubilier et al. 1999, Blazejak et al. 2005), nor is it to *Cristispira*, a large spirochete identified in the cristalline style of oysters (Margulis et al. 1991, Paster et al. 1996).

A single APS alpha subunit sequence was retrieved. It shares 90% amino-acid identity to 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 does not cluster with the APS sequence from the Gammaproteobacterium *Allochromatium vinosum*. This relationship might indicate that the sequence derives from a contaminating, non-symbiotic, bacterium from the environment. However, events of lateral transfer of the ApsA gene, which have 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 were obtained from 'blasting' APS amino acid sequences against the 6 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 aminoacid 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. The occurence 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 sequence. No amplification could be obtained for *pmoA*.

TEM observations on gill sections (Fig. 4) indicate the presence of numerous inclusions, 0.65 to 1.04 µm diameter (n=14), resembling bacterial symbionts reported in other lucinids (see for example Gros et al. 2003). Bacteria occur in vacuoles containing one or two individuals which occupy most of the volume within specialised bacteriocytes. Symbionts lack any internal membrane system such as observed in methanotrophs (Anthony 1982), but display electron lucent vesicles which are interpreted as sulphur granules in other species (Vetter 1985). No morphotype resembling typical spirochetes was seen, suggesting that the retrieved spirochete 16S rRNA phylotype came from an environmental

contaminant. However, the recovered phylotype is related to an unusual coccoid-shaped spriochete *Spirochaeta 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 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 which likely possesses APS reductase- and RubisCO-encoding genes. Larger fragments surrounding these genes should be sequenced to conclude 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 16S rRNA spirochete 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 the sequence herein described, are known to occur in oligochetes (Dubilier et al. 1999, Blazejak et al. 2005). Further work should investigate 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. comm.), 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 due to 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 to coastal species.

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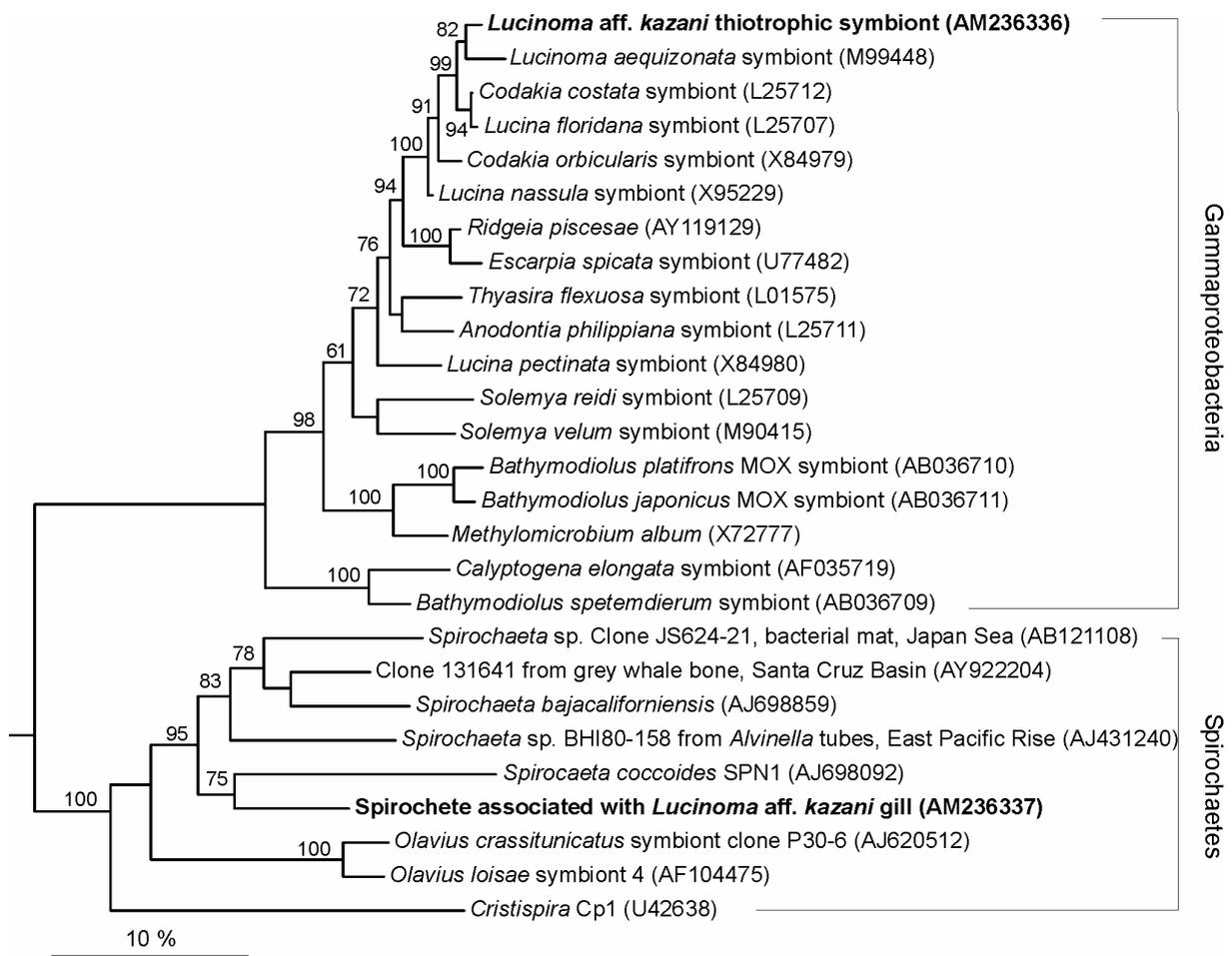
Fig. 1: Maximum likelihood tree displaying the phylogenetic relationships of symbiont from *Lucinoma* aff. *kazani* (in bold) to Gammaproteobacteria and spirochetes, based on 16S rRNA gene sequences (1240 nt positions). Tree was constructed using a GTR model, RELL values were obtained from the 1000 best trees (only >60% 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.
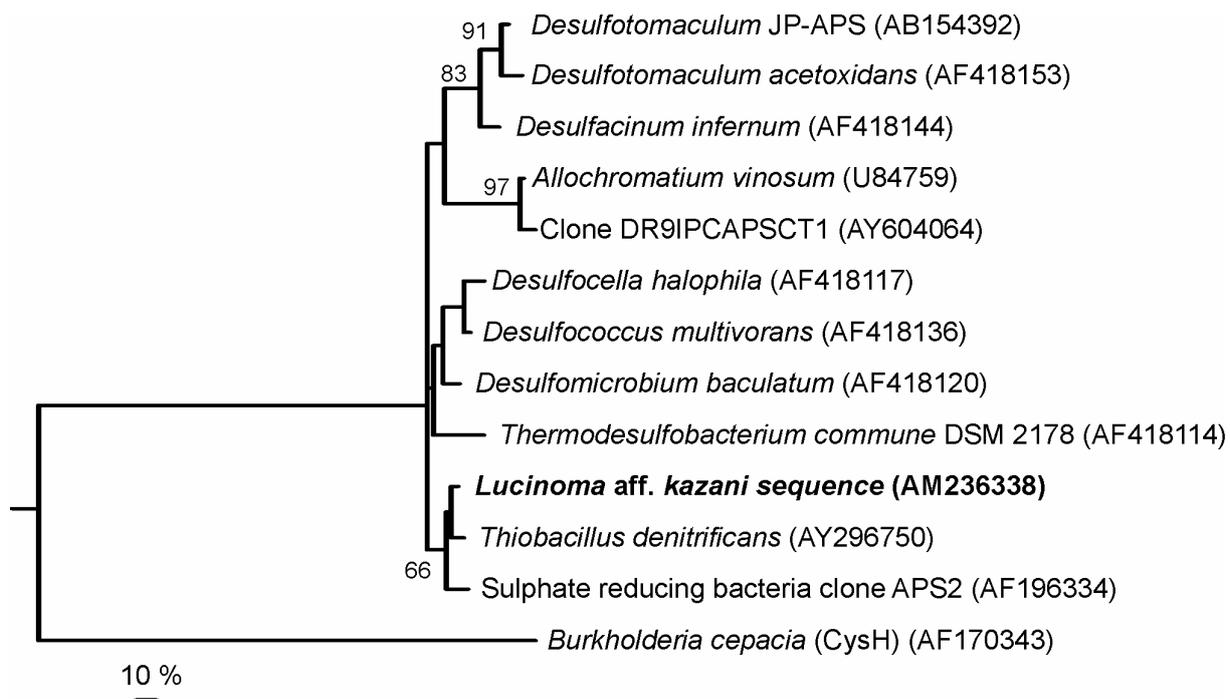
Fig. 2: Maximum likelihood tree displaying the phylogenetic position of the APS reductase alpha subunit sequence retrieved from *Lucinoma* aff. *kazani* (in bold). Tree was contructed based on 125 aa positions using a JTT model, sequence input order was randomized, bootstrap values were obtained from 500 replicates (only >60% shown). Scale bar represents 10% estimated amino acid substitution.
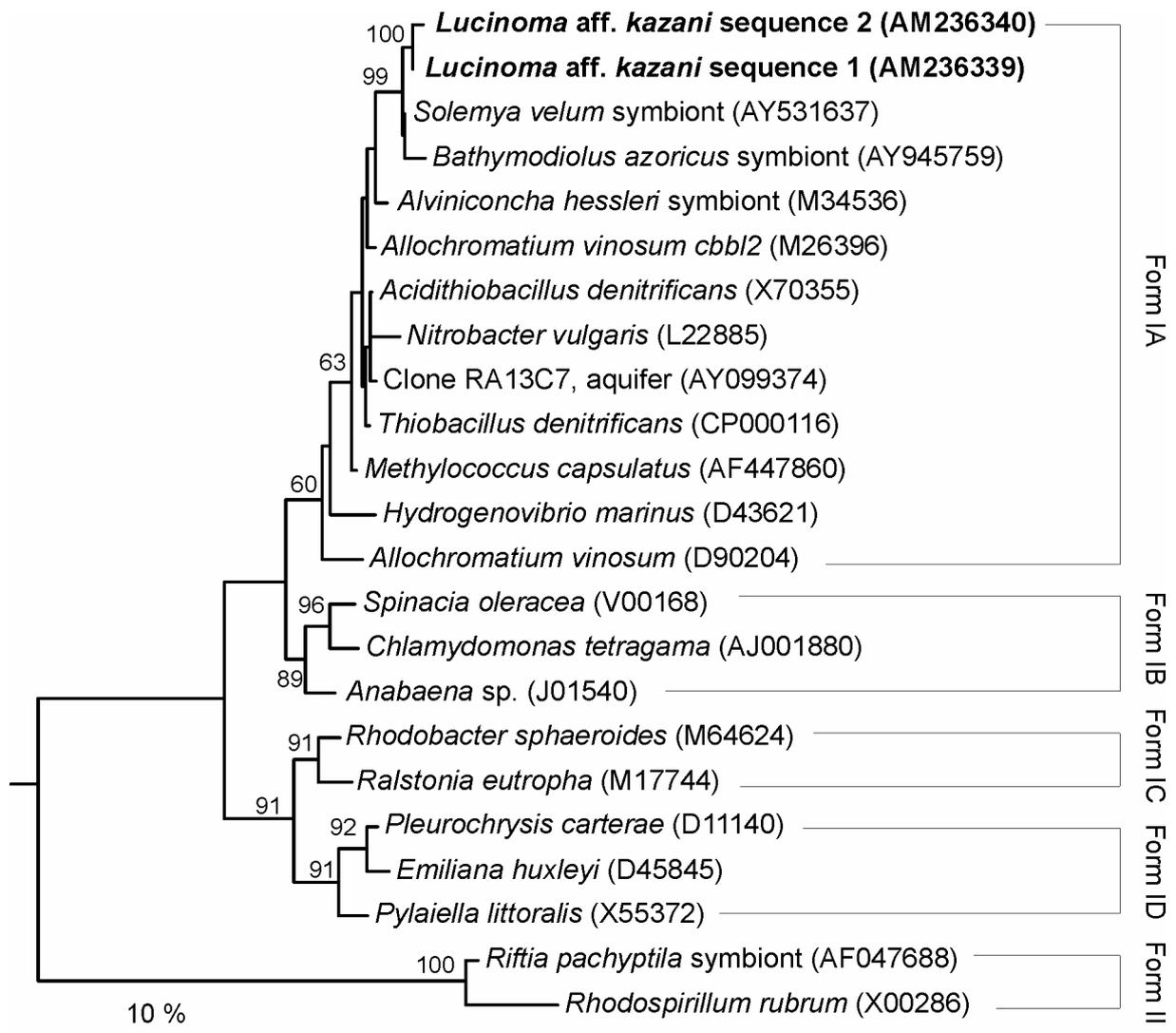
Fig. 3: Maximum likelihood tree displaying the phylogenetic position of the two RubisCO sequences retrieved from *Lucinoma* aff. *kazani* (in bold). Both sequences from *L. aff kazani* cluster together within the Form IA cluster, and are related to sequences from Gammaproteobacteria. Tree was contructed based on 230 aa positions using a JTT model, sequence input order was randomized, bootstrap values were obtained from 200 replicates (only >60% shown). Scale bar represents 10% estimated amino acid substitution.
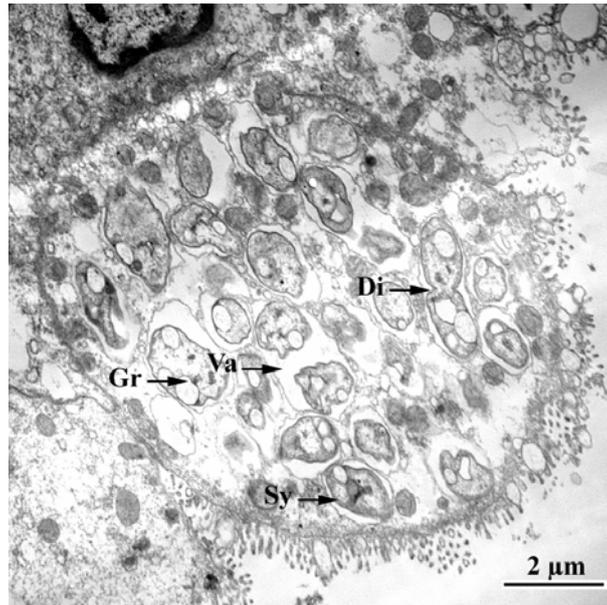
Fig. 4: TEM micrograph from a bacteriocyte within a gill of *Lucinoma* aff. *kazani* displaying symbionts (Sy) possessing empty vesicles previously described as remnants of sulfur granules (Gr). Symbionts are located in vacuoles (Va), and dividing stages occur (Di). Scale bar represents 2 µm.