Microbiology (2002), **148**, 1947–1957 Printed in Great Britain

Molecular characterization and endosymbiotic localization of the gene encoding D-ribulose 1,5-bisphosphate carboxylase–oxygenase (RuBisCO) form II in the deep-sea vestimentiferan trophosome

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To better understand the contribution of micro-organisms to the primary production in the deep-sea gutless tubeworm *Lamellibrachia* sp., the 16S-rDNA-based phylogenetic data would be complemented by knowledge of the genes that encode the enzymes relevant to chemoautotrophic carbon fixation, such as D-ribulose 1,5-bisphosphate carboxylase–oxygenase (RuBisCO; EC 4.1.1.39). To phylogenetically characterize the autotrophic endosymbiosis within the trophosome of the tubeworm *Lamellibrachia* sp., bulk trophosomal DNA was extracted and analysed based on the 16S-rRNA- and RuBisCO-encoding genes. The 16S-rRNA- and RuBisCO-encoding genes were amplified by PCR, cloned and sequenced. For the 16S rDNA, a total of 50 clones were randomly selected and analysed directly by sequencing. Only one operational taxonomic unit resulted from the 16S rDNA sequence analysis. This may indicate the occurrence of one endosymbiotic bacterial species within the trophosome of the *Lamellibrachia* sp. used in this study. Phylogenetic analysis of the 16S rDNA showed that the *Lamellibrachia* sp. endosymbiont was closely related to the genus *Rhodobacter*, a member of the α-*Proteobacteria*. For the RuBisCO genes, only the form II gene (*cbbM*) was amplified by PCR. A total of 50 *cbbM* clones were sequenced, and these were grouped into two operational RuBisCO units (ORUs) based on their deduced amino acid sequences. The *cbbM* ORUs showed high amino acid identities with those recorded from the ambient sediment bacteria. To confirm the results of sequence analysis, the localization of the symbiont-specific 16S rRNA and *cbbM* sequences in the *Lamellibrachia* sp. trophosome was visualized by *in situ* hybridization (ISH), using specific probes. Two types of cells, coccoid and filamentous, were observed at the peripheries of the trophosome lobules. Both the symbiont-specific 16S rDNA and *cbbM* probes hybridized at the same sites coincident with the location of the coccoid cells, whereas the filamentous cells showed no *cbbM*-specific signals. The RuBisCO form I gene (*cbbL*) was neither amplified by PCR nor detected by ISH. This is the first demonstration of chemoautotrophic symbiosis in the deep-sea gutless tubeworm, based on sequence data and *in situ* localization of both the 16S-rRNA- and RuBisCO-encoding genes.

Keywords: *Lamellibrachia* endosymbiont, 16S rDNA, *cbbM*, phylogenetic analysis, *in situ* hybridization

Abbreviations: ISH, *in situ* hybridization; NJ, neighbour-joining; ORU, operational RuBisCO unit; OTU, operational taxonomic unit; RuBisCO, D-ribulose 1,5-bisphosphate carboxylase–oxygenase.

The DDBJ accession numbers for the sequences reported in this paper are AB042416 [ST-Sym(16S)-1], AB032829 [ST-Sym(II)-1] and AB040509 [ST-Sym(II)-2].

INTRODUCTION

The concept of the sustainment of animal life by bacterial chemoautotrophy has been reviewed for the vestimentiferan tubeworms inhabiting hydrothermal vents and cold seeps (Felbeck, 1981; Cavanaugh, 1983; Julian *et al*., 1999). Three lines of evidence currently support this concept. The first piece of evidence for chemoautotrophic symbiosis is based on microscopic observations that demonstrated the intracellular occurrence of prokaryotic cells in the specialized organ of vent tubeworms, the trophosome. The vent tubeworm *Riftia pachyptila* harbours 3×10^9 prokaryotic cells per gram of trophosome (Cavanaugh *et al*., 1981). The trophosome mass often accounts for 40–60 % of the total body mass of the worm, making the worm a 'chemoautotrophic animal' (Felbeck & Childress, 1988).

The trophosome of the vestimentiferan tubeworms consists of lobules (Hand, 1987). Prokaryotic cells are localized in specialized cells – the bacteriocytes – located at the peripheries of these lobules (Cary *et al*., 1993). Morphologically diverse micro-organisms have been observed in the trophosomal tissue of the seep tubeworm *Lamellibrachia* sp. (Naganuma *et al*., 1997a). In the vent tubeworm *Riftia pachyptila*, the trophosome consists of distinct zones that harbour morphologically distinct prokaryotes: rod-like prokaryotes are found in the inner zone of the lobules and coccoid prokaryotes are found in the outer zone of the lobules (Bright *et al*., 2000).

The second piece of evidence for chemoautotrophic symbiosis within the trophosome of vestimentiferan tubeworms is the detection and amplification of bacterial rRNA and rDNA from the contents of the trophosome (Stahl *et al*., 1984; Distel *et al*., 1994; Di Meo *et al*., 2000). Intracellular localization of thiobacillic 16S rRNA sequences in the trophosome of the vent tubeworm *Riftia pachyptila* was demonstrated by *in situ* hybridization (ISH; Cary *et al*., 1993), whereas the 16S rRNA of a member of the *ε-Proteobacteria* was detected and localized in the trophosome of the seep tubeworm *Lamellibrachia* sp. (Naganuma *et al*., 1997b).

The third piece of evidence for chemoautotrophic symbiosis in tubeworms was the detection of enzymes that catalyse the fixation of radioactive bicarbonate via the Calvin–Benson cycle (Felbeck *et al*., 1981). The enzyme that mediates the first step of carbon fixation is D-ribulose 1,5-bisphosphate carboxylase–oxygenase (RuBisCO; EC 4.1.1.39), which serves as an indicator for autotrophy. RuBisCO is localized in intracellular polyhedral bodies (carboxysomes) in the endosymbiont of the vent tubeworm *Ridgeia* spp. (De Burgh *et al*., 1989). The enzyme has three forms, namely form I, form II and archaeal RuBisCO, which are distinct from each other on the basis of their structures, and their physical and chemical properties (Kellogg & Juliano, 1997; Watson *et al*., 1999). The RuBisCO form I large-subunit gene (*cbbL*) was sequenced from the endosymbionts of the vent gastropod *Alvinoconcha hessleri* and the mussel *Bathymodiolus* sp. (Stein *et al*., 1990; Elsaied & Naganuma, 2001), whereas the RuBisCO form II gene (*cbbM*) was sequenced from the endosymbionts of the vent tubeworm *Riftia pachyptila* (Robinson *et al*., 1998) and the cold-seep tubeworm *Lamellibrachia* sp. (Elsaied & Naganuma, 2001).

In this study, we describe the use of both 16S rDNA and RuBisCO sequences to phylogenetically characterize only the autotrophic endosymbiotic bacteria from the natural microbial community within the trophosome of the tubeworm *Lamellibrachia* sp. We also demonstrate the localization of both the endosymbiotic 16S rRNA and RuBisCO form II *cbbM* sequences within the trophosome by using ISH with specific oligo- and polynucleotide probes, respectively.

METHODS

Tubeworm collection and pre-treatment of the symbiont-containing trophosome. Individuals of *Lamellibrachia* sp. were collected from a bathyal methane-rich seep [1199 m deep, Sagami Trough (35 ° 00·2′ N, 139 ° 20·5′ E), Japan] during the 1142nd dive of the manned-submersible *Shinkai* 2000 (Japan Marine Science and Technology Centre). Immediately after retrieval, the soft body tissue of the samples was pulled out of the external chitinous tube and rinsed gently in filter-sterilized (0·2 μm filter) autoclaved seawater. Part of the soft body tissue of the specimens was fixed onto a board with 4 % paraformaldehyde in PBS (137 mM NaCl; 2·7 mM KCl; 10·6 mM Na_2HPO_4; 1·5 mM KH_2PO_4; pH 7·5), in preparation for ISH. The other part of the soft body tissue was used for DNA analysis. To remove any contaminating extracellular bacteria and free DNA from the trophosome tissue before DNA extraction, the tissue was treated according to the method of Elsaied & Naganuma (2001). The trophosomal tissue was suspended in TE buffer (10 mM Tris; 1 mM EDTA; pH 8) and incubated with lysozyme (1 mg ml^{-1}) at room temperature for 30 min. The tissue was then treated with DNase (10 μg ml^{-1}) and $MgCl_2$ (0·02 mM ml^{-1}) at 37 °C for 5 min, and washed several times with TE buffer with a higher concentration of EDTA (50 mM Tris; 50 mM EDTA; pH 8) to remove any residual DNase and $MgCl_2$. The cleaned tissue was kept at −80 °C for DNA analysis.

Genomic DNA extraction, PCR, cloning and sequencing of the 16S-rRNA- and RuBisCO-encoding genes. The extraction of trophosomal DNA, amplification of the genes of interest by PCR, and the cloning and sequencing of these genes was performed according to the methods of Distel *et al*. (1994) and Elsaied & Naganuma (2001). The primers used in the amplification of the 16S rDNA were 27F and 1492R, specific for the amplification of eubacterial 16S rDNA (DeLong, 1992). The expected amplified product was equivalent to approximately 1484 bp of *Escherichia coli* 16S rDNA. Approximately 400 bp of *cbbM* were amplified from the bulk trophosomal DNA using specific primers and PCR conditions, as detailed in Elsaied & Naganuma (2001). Fifty clones containing the target inserts were selected randomly from both the 16S rDNA and *cbbM* clone libraries, and these were sequenced. The obtained sequences (1427 bp for the 16S rDNA and 400 bp for *cbbM*) were screened against sequences contained within the databases held at the DNA Database of Japan (DDBJ) (http://www.ddbj.nig.ac.jp) using the program FASTA 3. For the 16S rDNA, the sequences showing 97 % nucleotide identity with each other were grouped into one operational taxonomic unit (OTU) (Godon *et al*., 1997). The resulting nucleotide se-

quences of *cbbM* were translated into amino acids using the program PROTEIN ENGINE (available at the EBI website; http://www.ebi.ac.uk). The current *cbbM* sequences sharing 100 % amino acid identity with each other were grouped into an operational RuBisCO unit (ORU). One OTU resulted from the nucleotide sequence analysis of the 50 16S rDNA clones, and this was named ST-Sym(16S)-1. Two ORUs resulted from the amino acid sequence analysis of *cbbM*, and these were grouped into ST-Sym(II)-1 and ST-Sym(II)-2, representing 38 and 12 clones, respectively, of the 50 *cbbM* clones that were analysed. Each OTU or ORU was represented by the clone that displayed the highest similarity (with respect to nucleotide sequence) with the other clones within the same OTU or ORU. Two phylogenetic trees were constructed based on multiple alignments of the nucleotide and deduced amino acid sequences for the 16S rDNA OTU and the *cbbM* ORUs, respectively, and these included other sequences from the databases. The phylogenetic trees were constructed using the neighbour-joining (NJ) method (Saitou & Nei, 1987), and were visualized using the program TREEVIEW (Page, 1996). The branching patterns of the constructed phylogenetic trees were confirmed by reconstruction of the phylogenies using two other methods of analysis, namely maximum-parsimony and maximum-likelihood, contained within the PHYLIP package (Felsenstein, 1989).

The ST-Sym(16S)-1, ST-Sym(II)-1 and ST-Sym(II)-2 sequences obtained in this study were deposited in the DDBJ under accession numbers AB042416, AB032829 and AB040509, respectively.

Preparation of the oligonucleotide 16S rDNA probes. The oligonucleotide 16S-rRNA-targeted probe specific for the autotrophic *Lamellibrachia* sp. endosymbiont was designed from the sequence ST-Sym(16S)-1, by creating a multiple alignment with this sequence and those sequences retrieved from the database. The alignment was created with the program CLUSTAL W (Thompson *et al*., 1989). The endosymbiont-specific 16S-rRNA-targeted probe Lam-384R (5′-GCCTGCGCCCATTGTCTAAGATCCC-3′) was complementary to nucleotide positions 360–384 of *E. coli* 16S rDNA. The mismatching of the probe target region with 16S rDNA sequences from other species was checked by homology searches of the sequences contained within the databases (DDBJ, EMBL, GenBank and RDP) using the programs BLAST and PROBE MATCH (Altschul *et al*., 1990; Olsen *et al*., 1991).

The universal eubacterial 16S-rRNA-targeted probe EUB338 (5′-GCTGCCTCCCGTAGGAGT-3′), complementary to nucleotide positions 338–355 of *E. coli* 16S rDNA (Amann *et al*., 1990), was used as a positive control, to localize all possible bacterial species within the trophosome. The oligonucleotide probes were chemically synthesized by Roche and were labelled using the Digoxigenin 3′-end Labelling Kit (Boehringer Mannheim), according to the manufacturer's instructions.

Preparation of the RuBisCO form II *cbbM* probe. To obtain a *cbbM* probe, characterized by its efficiency to produce a *cbbM*-specific ISH signal in trophosome tissue, probes with different lengths (short oligonucleotides, typically < 50-mer single-stranded DNA fragments, and long, 400 bp DNA fragments) were designed from the sequence alignments created for ST-Sym(II)-1 and ST-Sym(II)-2 (data not shown). The short probes were prepared and labelled in the same way as the 16S rDNA probes. The 400 bp fragment, representing the full-length ST-Sym(II)-2 sequence, was chosen as the *cbbM* probe, as the nucleotide sequence divergence within the ST-Sym(II)-2 ORU was as low as 1 %. This was contrasted by the higher nucleotide sequence divergence (8·8 %) seen within the ST-Sym(II)-1 ORU, which would have resulted in a degenerative probe that would have given a less specific ISH signal. The *cbbM* ST-Sym(II)-2 fragment was amplified by PCR from the plasmid of a transformed TOP10 *E. coli* clone. The fragment was purified by using the Qiagen QIAquick PCR Purification Kit (Germany) and labelled by random-priming with digoxigenin-11-dUTP, using the DIG-High Prime Kit (Boehringer Mannheim) according to the manufacturer's instructions.

The specificities of the 16S rDNA and the RuBisCO *cbbM* probes for their target sequences were checked by Southern dot-blot hybridization using a positive high-bond nylon membrane (Amersham Pharmacia Biotech), according to the method of Cary *et al*. (1993). The efficiency of these probes to produce ISH signals was also evaluated, using sections of the trophosomal tissue.

Detection of the RuBisCO form I *cbbL* gene by PCR and ISH. The primers and PCR conditions used to test the amplification of the *cbbL*-encoding gene from bulk trophosomal DNA were as described in Elsaied & Naganuma (2001). *cbbL* was not amplified from the *Lamellibrachia* sp. trophosomal DNA. To confirm the absence of this gene in the *Lamellibrachia* sp. trophosome, ISH was performed using a *cbbL*-specific probe. Approximately 800 bp were amplified by PCR from the *cbbL* gene of the endosymbiont of the deep-sea mussel *Bathymodiolus* sp. (Elsaied & Naganuma, 2001). The 800 bp *cbbL* probe, named Bathy(*cbbL*)-1, was labelled by random-priming with digoxigenin-11-dUTP, using the DIG-High Prime Kit. The efficiency of Bathy(*cbbL*)-1 to produce a *cbbL*-specific ISH signal was tested on a section of gill tissue from *Bathymodiolus* sp. The probe was also used to test for the possible presence of RuBisCO form I *cbbL* within the trophosome of the tubeworm, *Lamellibrachia* sp., used in this study.

ISH procedure. To visualize the localization of the endosymbiotic 16S-rRNA and RuBisCO sequences within the tubeworm trophosome, ISH was performed on consecutive paraffin cross sections of the trophosome, using a modified method of Naganuma *et al*. (1997b). Before ISH, the trophosomal-tissue sections were de-paraffinized in xylene and were rehydrated by immersing them in a decreasing series of ethanol (100, 95, 90, 80 and 70 %) for 5 min at each level, followed by a final wash for 5 min in H_2O. The sections were then treated with 0·2 % Triton X-100 in PBS for 1·5 min, followed by digestion with 100 μg proteinase K ml^{-1} at 37 °C for 15 min. The digested sections were treated with 0·1 % glycine and were post-fixed in 4 % paraformaldehyde for 5 min. They were then washed in PBS. The sections were treated with 0·25 % (v/v) acetic anhydrite in 0·1 M triethanolamine (pH 8) for 10 min, and equilibrated in 2 × SSPE [diluted from a 20 × SSPE stock solution (3 M NaCl; 0·2 M $NaH_2PO_4.H_2O$; 20 mM EDTA; pH 7·4)]. Sections for ISH were dehydrated in an ascending series of ethanol (70, 95 and 100 %; 5 min each dehydration step) and then air-dried. Slides can be stored at room temperature until the hybridization step.

Slides were prepared for hybridization by immersing them in hybridization buffer that contained 0·5 mg salmon-sperm DNA ml^{-1}, 0·25 mg yeast tRNA ml^{-1} and 5 × Denhardt's reagent in 2 × SSPE. They were then incubated for 40 min at 37 °C. One hundred nanograms of the digoxigenin-labelled probe was mixed with 100 μl of the fresh hybridization buffer, and this mixture was added to each slide section. The slides

were then sealed with rubber cement. The probe and target sequences were denatured by placing the slides on a heating plate at 100 °C for 10 min. The slides were then incubated in a sealed humid chamber overnight at 43 °C for hybridization. Thereafter, the sections were washed consecutively in 2 ×, 1 × and 0·2 × SSPE for 30 min at room temperature, followed by a final high-stringency wash in 0·2 × SSPE at 45 °C for 1 h to remove any unbound probe. The sections were then equilibrated in pre-blocking buffer (100 mM Tris; 150 mM NaCl; pH 7·5) for 5 min and incubated in pre-blocking buffer supplemented with 1% blocking agent (Boehringer Mannheim) for 30 min at room temperature. An alkaline-phosphatase-conjugated digoxigenin-specific IgG (anti-dig) (Boehringer Mannheim) was used to detect the hybridization signals with enhanced sensitivity. Approximately 100 µl of diluted anti-dig (1 : 400 blocking buffer) was applied to each section under a coverslip, and these were incubated in a sealed humidified chamber overnight at room temperature. To remove any unbound anti-dig, the sections were washed twice in pre-blocking buffer for 15 min, with shaking. They were then equilibrated in colouring buffer (100 mM Tris base; 100 mM NaCl; 50 M $MgCl_2$; pH 9·5). The sections were incubated with a colour solution containing 0·4 mg nitrotetrazolium blue chloride ml^{-1}, 0·19 mg 5-bromo-4-chloro-3-indolyl-phosphate *p*-toluidine salt ml^{-1}, 50 mM $MgSO_4$ and 100 mM Tris buffer (pH 9·5) in the dark until the colour developed. Once the colour had started to precipitate, the reaction was stopped by washing the sections with 1 × TE buffer (10 mM Tris; 1 mM EDTA; pH 8·0). The tissue sections were then examined using a light microscope.

To visualize the lobular structure of the trophosomal tissue, non-hybridized trophosome sections were stained by the standard double-staining method, using a haematoxylin/eosin dye mixture (Dealtry & Rickwood, 1992).

RESULTS AND DISCUSSION

Occurrence of an autotrophic endosymbiotic species within the trophosome of *Lamellibrachia* sp., based on 16S rDNA sequence analysis

Only one OTU was recorded from the sequence analysis of a total of 50 16S rDNA clones. This may indicate the existence of only one endosymbiotic species within the trophosome of the *Lamellibrachia* sp. studied here. The phylogenetic analyses performed using NJ, maximum-parsimony and maximum-likelihood methods (only the tree based on NJ is shown in Fig. 1) placed the *Lamellibrachia* sp. endosymbiont within the cluster of *α-Proteobacteria*, which includes autotrophic bacteria of the genera *Rhodobacter* and *Rhodospirillum*. This is in contrast with other tubeworm endosymbionts, which commonly belong to the *γ-Proteobacteria* (Feldman *et al*., 1997; Di Meo, 2000). The bootstrap value of 87% obtained from NJ analysis demonstrates that the association of our endosymbiont with *Rhodobacter sulfidophilus* (Imhoff, 1989) is statistically significant, suggesting that the *Lamellibrachia* sp. endosymbiont is a member of the genus *Rhodobacter*. The occurrence of an endosymbiont belonging to the *α-Proteobacteria* in the tubeworm *Lamellibrachia* sp. suggests that this endosymbiont may form a unique phylogenetic lineage among the endosymbionts of other tubeworms (Feldman *et al*., 1997).

Occurrence of a single RuBisCO gene in the trophosome of the *Lamellibrachia* sp.

The RuBisCO form II gene (*cbbM*) was amplified from the bulk trophosomal DNA, whereas the RuBisCO form I gene (*cbbL*) was not amplified from the same bulk trophosomal DNA. The existence of *cbbM* and the absence of *cbbL* in the tubeworm endosymbiont could be explained in two ways. The first possible explanation is based on the observed $\delta^{13}C$ value of −11 ‰ for the vestimentiferan organic carbon assimilated by RuBisCO form II, whereas the molluscan organic carbon assimilated by RuBisCO form I has a $\delta^{13}C$ value of −30 ‰ (Robinson & Cavanaugh, 1995). The form II enzyme has a smaller kinetic isotope effect with respect to CO_2 than does form I (Tabita, 1988). Thus, the ^{13}C-enriched stable carbon isotope signature observed in the intact −11 ‰ vestimentiferan symbioses appears to be at least partially due to this diminished enzymic discrimination (Robinson & Cavanaugh, 1995). However, the $\delta^{13}C$ value for the current vestimentiferan *Lamellibrachia* sp. tissue used in this study was not determined and future measurement is needed to support this explanation. The second explanation is based on the chemical and kinetic properties of RuBisCO form II. The RuBisCO form II enzyme potentially functions better in micro-aerobic conditions with a high CO_2 concentration (Haygood, 1996). The vestimentiferan tubeworms bear endosymbionts in the trophosome, which is buried deep within the body of the animal. In this site, the low O_2 and high CO_2 concentrations are tightly controlled by the blood flow of the animal. The micro-aerobic condition within the trophosome allows the chance for the expression of *cbbM* (Stein & Felbeck, 1993). Thus, the physiological constraints within the host animal may allow preferential selection of the *cbbM*-bearing endosymbionts.

The occurrence of *cbbM* within the trophosome of *Lamellibrachia* sp. supports the results of the 16S rDNA analyses, which showed the presence of an *α-Proteobacteria*-like endosymbiont. This is because the autotrophic *α-Proteobacteria* have the ability to fix CO_2 with the RuBisCO form II enzyme, using sulfide and thiosulfate as electron donors (Imhoff, 1989). These conditions are optimum within the trophosome (Nargang *et al*., 1984; Tabita, 1995; Haygood, 1996). Moreover, the endosymbiont of *Riftia pachyptila* bears *cbbM* that is closely related to that of an autotrophic member of the *α-Proteobacteria*, *Rhodospirillum rubrum* (Robinson *et al*., 1998). This implies that *Proteobacteria*-like *cbbM* may be distributed widely among the vestimentiferan tubeworms. Studies on *cbbM* genes from other species of vestimentiferan endosymbionts are needed to support these observations.

Analysis of the *cbbM* sequences of ORU ST-Sym(II)-1 and ST-Sym(II)-2

To show the catalytic site amino acids within the ORUs, ST-Sym(II)-1 and ST-Sym(II)-2, their sequences were aligned with the sequences retrieved from the databases. The alignment of these partial amino acid sequences

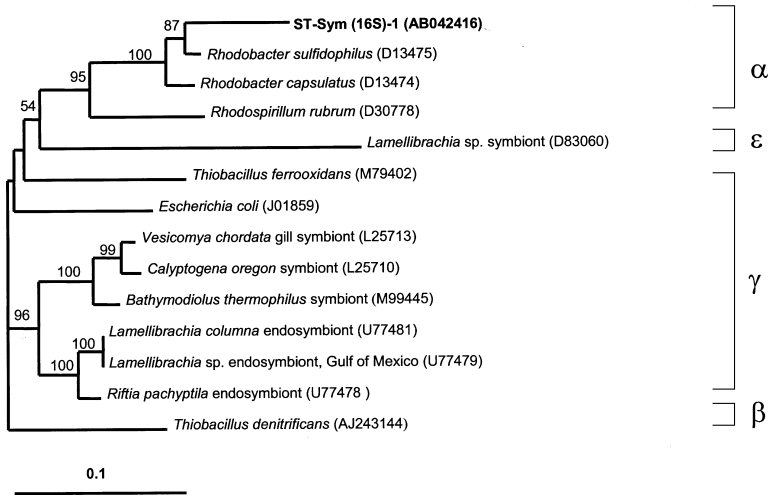

Fig. 1. Phylogenetic tree based on the complete nucleotide sequences of the ST-Sym (16S)-1 OTU and other species retrieved from the DDBJ database. The tree was constructed using the NJ method with the Kimura-2 correction parameter. Bootstrap values, calculated from 1000 replicates, are expressed as percentages; only those values greater than 50% are shown at the nodes of the tree. α, β, ε, and γ represent the different classes of the *Proteobacteria*. Bar, 0·1 substitutions per site.

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ST-Sym(II)-1                    IIKPKLGLRPKPFADAAYQFWLGG-DFIKNDEPQGNQVFCPTKKVMPLVA 49
ST-Sym(II)-2                    IIKPKLGLRPEPFANAAYQFWLGG-DFIKNDEPQGNQVFCPLKKVLPLVH
ST-Sed(II)-1                    IIKPKLGLRPEPFAEAAYQFWLGG-DFIKNDEPQGNQVFCPMKKVIPLVA
ST-Sed(II)-2                    IIKPKLGLRPEPFAEAAYQFWLGG-DFIKNDEPQGNQVFCPMKKVIPLVY
Riftia pachyptila endosymbiont  IIKPKLGLRPEPFAEAAYQFWLGG-DFIKNDEPQGNQPFSPMKKTIPLVA 214
Rhodospirillum rubrum           IIKPKLGLRPKPFAEACHAFWLGG-DFIKNDEPQGNQPFAPLRDTIALVA 212
Thiobacillus denitrificans      IIKPKLGLRPEPFAKAAYQFWLGG-DFIKNDEPQGNQVFCPLKKVLPLVY 212
Synechococcus sp. PCC6301       TIKPKLGLSAKNYGRAVYECLRGGLDFTKDDENINSQPFQRWRDRFLFVA 219
Methanococcus jannaschii        IVKPKVGLKTEEHAKVAYEAWVGGVDLVKDDENLTSQEFNKFEDRIYKTL 201
                                 :***:** .: .. . :    ** *: *:**   .* *   .. :  .

ST-Sym(II)-1                    DALARAQDETGEAKLFSANITADDHHEMCARADYILETFGENASHVAFLV 97
ST-Sym(II)-2                    DAMKRAQDETGEPKIFSMNITADDHHEMCARADFGLELFGEDAPRLAFLV
ST-Sed(II)-1                    DAMKRAQDETGEAKLFSANITADDHYEMLARADYVLETFGENASHVAFLV
ST-Sed(II)-2                    DAMKRAMDETGRAKLFSANITADDHYEMIARGEYVLETFGPDADKVAFLV
Riftia pachyptila endosymbiont  DAMRRAQDETGEAKLFSANITADDPAEMIARGEFVLETFGFEASQVAFLV 262
Rhodospirillum rubrum           DAMRRAQDETGEAKLFSANITADDPFEIIARGEYVLETFGENASHVALLV 260
Thiobacillus denitrificans      DAMKRAQDDTGQAKLFSMNITADDHYEMCARADYALEVFGPDADKLAFLV 260
Synechococcus sp. PCC6301       DAIHKSQAETGEIKGHYLNVTAPTCEEMMKRAE-----FAKELGMPIIMH 266
Methanococcus jannaschii        EMRDKAEEETGERKAYMPNITAP-YREMIRRAE-----IAEDAGSEYVMI 244
                                :   ::  :**. * .  *:**    *:  *.:     :. :     .:

ST-Sym(II)-1                    DGYVGGPGMVTTARR--NYPSQYLHYHRAGHGAVTS              133
ST-Sym(II)-2                    DGYVGGPGMVTTARR--NYPSHYLHYHRAGHGAVTS
ST-Sed(II)-1                    DGYVGGPGMITTARR--QYPNQYLHYHRAGHGAVTS
ST-Sed(II)-2                    DGYVGGPGMITTARR--NFPSQYLHYHRAGHGAVTS
Riftia pachyptila endosymbiont  DGYVAGPTAVATARR--NFPNQFLHFHRAGHGAVTS              298
Rhodospirillum rubrum           DGYVAGAAAITTARR--RFPDNFLHYHRAGHGAVTS              296
Thiobacillus denitrificans      DGYVGGPGMVTTARR--QYPGQYLHYHRAGHGAVTS              296
Synechococcus sp. PCC6301       DFLTAGFTANTTLAKWCRDNGVLLHIHRAMHAVIDR              300
Methanococcus jannaschii        DVVVCGFSAVQSFRE--EDFKFIIHAHRAMHAAMTR              278
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Fig. 2. Alignment of the deduced partial RuBisCO *cbbM* amino acid sequences of ST-Sym(II)-1 and ST-Sym(II)-2 with other *cbbM* sequences and representatives of the form I and archaeal RuBisCOs from *Synechococcus* sp. PCC6301 and *Methanococcus jannaschii*, respectively. Multiple sequence alignments were created by using CLUSTAL W. The accession numbers for the amino acid sequences that were used to create the multiple alignment are as follows: ST-Sym(II)-1, AB032829; ST-Sym(II)-2, AB040509; ST-Sed(II)-1, AB040504; ST-Sed(II)-2, AB040505; *Riftia pachyptila* endosymbiont, AF047688; *Rhodospirillum rubrum*, X00286; *Thiobacillus denitrificans*, L37437; *Synechococcus* sp. PCC6301, X03220; *Methanococcus jannaschii*, U67564. The amino acids that were identical in all of the aligned sequences are marked with asterisks, conserved substitutions are marked with colons, and semi-conserved substitutions are marked with periods. The shaded regions represent the known catalytic-site amino acids (Schneider *et al.*, 1990). The characteristic RuBisCO motif sequence, GGDFIKNDE, is indicated by a thick, solid line. The numbers of the aligned *cbbM* amino acid positions are shown at the right side.

corresponded to amino acid positions 164–296 of the *cbbM* of *Rhodospirillum rubrum* (Schneider *et al.*, 1990) (Fig. 2). The two *cbbM* ORUs shared 86% amino acid sequence identity and had the characteristic RuBisCO motif sequence (GGDFIKNDE) that has been found in other RuBisCOs (Schneider *et al.*, 1990; Newman & Gutteridge, 1993). The ST-Sym(II)-1 and ST-Sym(II)-2 *cbbM* amino acid sequences also showed conservation of the known consensus catalytic site amino acids. The conserved region is flanked by a lysine at position 191, corresponding to the sequence of *Rhodospirillum rubrum*. This conserved region has been identified in other RuBisCOs as the binding site for CO_2, and has been shown to be involved in carbamate formation during enzyme activation (Miziorko & Lorimer, 1983; Schneider *et al.*, 1990). The other known active

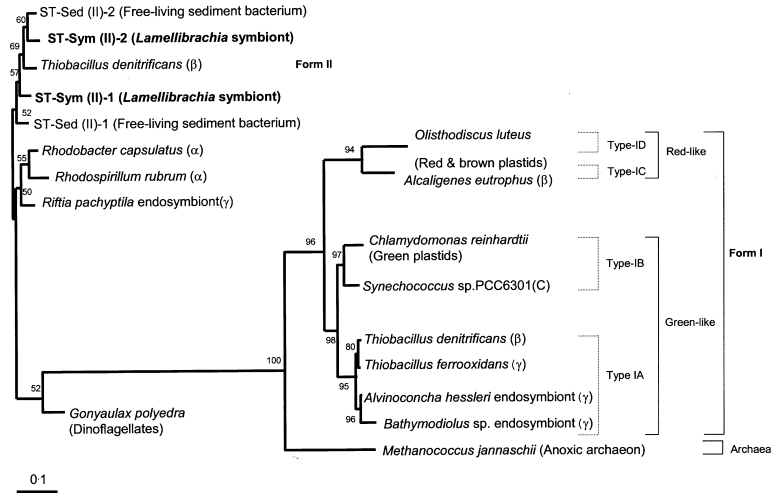

Fig. 3. Phylogenetic tree based on the deduced partial RuBisCO *cbbM* amino acid sequences of ST-Sym(II)-1 and ST-Sym(II)-2 and those retrieved from the databases. The tree was constructed using the NJ method with the Kimura-2 correction parameter. Bootstrap values, calculated from 1000 replications, are expressed as percentages and only those values greater than 50 % are shown at the nodes of the tree. The Greek letters in parentheses (*α, β, γ*) represent the classification of the species within the *Proteobacteria* made on the basis of 16S rDNA data or on the results from other studies. C in parentheses indicates a member of the *Cyanobacteria*. Bar, 0·1 substitutions per site.

ST-Sym(16S)-1(α)	**GGGATCTTAGACAATGGGCGCAGGC 346**
Lamellibrachia sp. symbiont(ε)	GGAATATTGCACAATGGAGGAAACT 379
Lamellibrachia columna symbiont(γ)	GGAATATTGGACAATGGGCGCAAGC 359
Riftia pachyptila symbiont(γ)	GGAATATTGGACAATGGGCGAAAGC 362
Rhodobacter sulfidophilus (α)	GGAATCTTGGACAATGGGGGAAACC 383
Rhodospirillum rubrum (α)	GGAATATTGCGCAATGGGGGCAACC 326
Bathymodiolus endosymbiont(γ)	GGAATATTGGACAATGGGCGAAAGC 376
Escherichia coli(γ)	GGAATATTGCACAATGGGCGCAAGC 384

Fig. 4. Alignments of the 16S rDNA sequences in the target region of the *Lamellibrachia* sp. symbiont-specific probe Lam-384R [ST-Sym(16S)-1 sequence]. The shaded regions represent the mismatches with the corresponding nucleotides in the ST-Sym(16S)-1 sequence. The accession numbers for the species shown in the alignment are: ST-Sym(16S)-1, AB042416; *Lamellibrachia* sp. endosymbiont, D83060; *Lamellibrachia columna* endosymbiont, U77481; *Riftia pachyptila* endosymbiont, U77478; *Rhodobacter sulfidophilus*, D13475; *Rhodospirillum rubrum*, D30778; *Bathymodiolus* endosymbiont, AB056868; *E. coli*, J01859. The letters in parentheses (*α, ε, γ*) represent the classification of the species within the *Proteobacteria*. The numbers of the aligned 16S rDNA positions are shown at the right side.

binding sites, such as those around the lysine at position 166 and the histidine at position 287, are conserved in the current ORUs (Schneider *et al*., 1990).

Species belonging to different phylogenetic groups, such as the *α*-, *β*-, and *γ*-*Proteobacteria*, are usually displayed in individual clades in 16S-rDNA-based phylogenetic trees, where the species that belong to one phylogenetic group are always related to each other, in some way, within the clade (Fig. 1). In contrast to the 16S-rDNA-based phylogeny, the phylogram based on the RuBisCO genes localizes the species from the different 16S-rDNA-based phylogenetic groups into one clade. This can be seen in Fig. 3, where ST-Sym(II)-1 and ST-Sym(II)-2 (*α*-*Proteobacteria*) and *Thiobacillus denitrificans* (a member of the *β*-*Proteobacteria*) group together, and the *cbbM* sequence of the *Riftia pachyptila* endosymbiont (a member of the *γ*-*Proteobacteria*) clusters with members of the *α*-*Proteobacteria*, *Rhodobacter capsulatus* and *Rhodospirillum rubrum* (Hernandez *et al*., 1996; Robinson *et al*., 1998). The inconsistency between the 16S-rDNA- and the RuBisCO-based phylograms is probably due to conservation of the RuBisCO genes among different phylogenetic groups of the *Proteobacteria*, *Cyanobacteria* and green eukarya (Delwiche & Palmer, 1996). The conservation of the RuBisCO sequences has been suggested to be due to multiple horizontal gene transfers, involving the transfer of RuBisCO genes between the different phylogenetic lineages (Delwiche & Palmer, 1996; Watson & Tabita, 1997).

Three phylogenetic trees based on the partial *cbbM* amino acid sequences of ST-Sym(II)-1, ST-Sym(II)-2 and sequences from the databases were constructed using the NJ, maximum-likelihood (ML) and maximum-parsimony (MP) methods. These trees yielded similar, but not identical, topologies; hence, only the NJ-based tree is shown here (Fig. 3). Both NJ and MP analyses grouped the two endosymbiont ORUs into one cluster with *cbbM* sequences from ST-Sed(II)-1 and ST-Sed(II)-2, recovered from sediment in the close vicinity of the studied tubeworm colony (Elsaied & Naganuma, 2001). However, ML analysis did not group the current symbiont ORUs and those from ambient sediment bacteria into one cluster. Both ST-Sym(II)-1 and ST-Sym(II)-2 shared 95 and 90 % aa identity with the ORUs ST-Sed(II)-1 and ST-Sed(II)-2, respectively (Fig. 2).

The phylogenetic similarity between the tubeworm endosymbiont and ambient free-living bacteria based on the RuBisCO genes has also been demonstrated on the basis of 16S rDNA analysis (Di Meo *et al*., 2000). This symbiont–ambient relationship implies that gutless tubeworms may acquire their endosymbionts through random ingestion of ambient bacteria during only a limited period when the tubeworm larva has a mouth opening (Di Meo *et al*., 2000). After acquisition of these ambient bacteria, adaptive species that bear a suitable RuBisCO form (I or II) may be selected as endosymbionts. Although the mechanism of acquisition and selection of the endosymbionts has not been examined so far, observations indicate that the tubeworms living in different habitats, i.e. seep-dwelling *Lamellibrachia* sp. and vent-dwelling *Riftia pachyptila*, prefer to har-

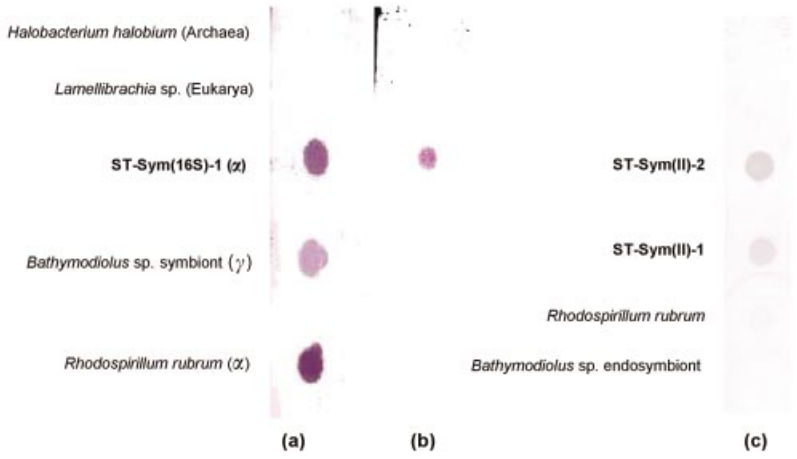

Fig. 5. Southern dot-blot hybridization analyses demonstrating the specificity of the 16S rDNA and *cbbM* probes. In (a) and (b), amplified rDNAs from a variety of species, including members of the archaea, the eukarya, bacterial symbionts (the current *Lamellibrachia* sp. endosymbiont, and the *Bathymodiolus* endosymbiont from which the RuBisCO form I probe was amplified) and a cultured member of the α-*Proteobacteria*, *Rhodospirillum rubrum*, were used as templates. (a) Hybridization of the universal 16S rDNA probe EUB338 to the rDNAs. (b) Hybridization of the *Lamellibrachia* sp. endosymbiont-specific 16S rDNA probe Lam-384R to the rDNAs. (c) Hybridization of the RuBisCO *cbbM* probe ST-Sym(II)-2 to the amplified *cbbM* products from the current *Lamellibrachia* sp. endosymbiont and from *Rhodospirillum rubrum*, and to the RuBisCO form I *cbbL* amplified from the *Bathymodiolus* endosymbiont. DNA (50 ng) was blotted for each dot on the membrane. The probes were labelled with digoxigenin and the dot colours were developed by the reaction of an alkaline phosphatase on the 5-bromo-4-chloro-3-indolyl-phosphate *p*-toluidine salt.

bour *cbbM*-bearing endosymbionts (Robinson *et al*., 1998). Establishment of the host–symbiont relationship is an intriguing process in terms of the evolution and biogeography of vestimentifera. More tubeworm species should be collected and analysed to explain the idea of the selection of *cbbM*-bearing endosymbionts. In terms of a genetic comparison between the *cbbM* genes of tubeworm endosymbionts, ST-Sym(II)-1 and ST-Sym(II)-2 shared 79 and 75% aa identity, respectively, with the *cbbM* amino acid sequence of the *Riftia pachyptila* endosymbiont from a hydrothermal vent in the East Pacific Rise (Robinson *et al*., 1998) (Fig. 2). This genetic variation among the RuBisCOs of vestimentiferan endosymbionts may be influenced by a variety of factors, including host genus, geographic variations in the location of the tubeworm and the sea-bed composition.

Specificity and efficiency of the probes used for ISH

The portion of the16S rDNA sequence used to construct oligonucleotide probe Lam-384R showed minimal nucleotide mismatching (four mismatches) with the 16S rDNA sequence of the *Lamellibrachia columna* symbiont (Feldman *et al*., 1997), and had three mismatches with all of the aligned symbiont rDNA sequences (Fig. 4). Lam-384R was specific for the *Lamellibrachia* sp. endosymbiont, as shown by Southern dot-blot hybridization (Fig. 5b). The use of short oligonucleotide probes to localize *cbbM* sequences within the trophosome by ISH was not successful in yielding a visible signal. This was due to the existence of a single copy of the RuBisCO gene in the genome: a single copy of *cbbM* is not sufficient to give a signal when ISH is performed with a short oligonucleotide probe (Tabita, 1988). This is in good contrast with the 16S rRNA, which has a high copy number within bacterial cells and can be detected using short, specific oligonucleotide probes such as Lam-384R (Cary *et al*., 1993). The polynucleotide *cbbM* probe ST-Sym(II)-2 (400 bp) was efficient in yielding a sufficient *cbbM* ISH signal, but the use of this probe resulted in the loss of the ability to distinguish between the subtle differences in the sequences of ST-Sym(II)-1 and ST-Sym(II)-2, as indicated by dot-blot hybridization on the nylon membrane (Fig. 5c). This was the result of a trade-off between signal intensity and specificity. Hence, the ST-Sym(II)-2 probe was used to visualize all of the possible RuBisCO form II sequences within the trophosome, including the ST-Sym(II)-1 sequence. However, ISH with the symbiont-specific 16S rDNA probe Lam-384R was used as a control for the localization of the signal produced by the *cbbM* probe, ST-Sym(II)-2. The ISH signals of the symbiont-specific 16S rDNA probe Lam-384R and the *cbbM* probe ST-Sym(II)-2 overlapped almost completely in two consecutive trophosome sections (Fig. 6b, c). This reflects the exact localization of the endosymbiont *cbbM* sequences within the trophosome.

ISH using the 16S rDNA and RuBisCO probes

Our initial approach to studying chemoautrotrophic endosymbiosis within the trophosome was to co-localize the symbiotic 16S rRNA and *cbbM* sequences within this tissue. However, the application of the fluorescence *in situ* hybridization (FISH) technique to the trophosomal tissue of the *Lamellibrachia* sp. was unsuccessful. This was due to high background fluorescence, which resulted from the accumulation of sulfur particles in the trophosome that made the tissue prone to auto-fluor-

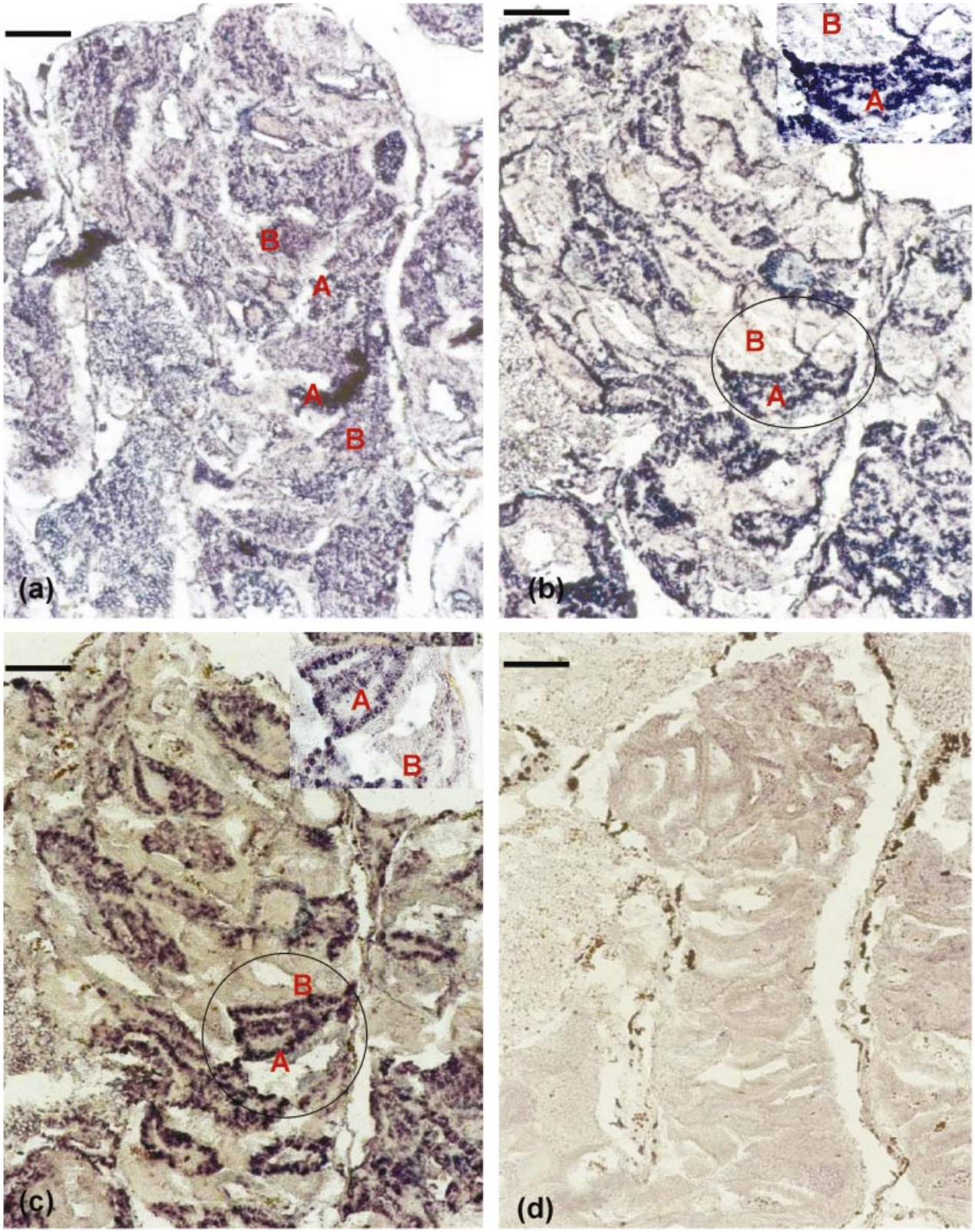

Fig. 6. For legend see facing page.

escence (Fisher, 1990; Naganuma *et al*., 1997a). This is in contrast with the use of FISH to detect symbiotic bacteria in the hydrothermal vent mussel gill that has direct contact with ambient water. The mussel gill may

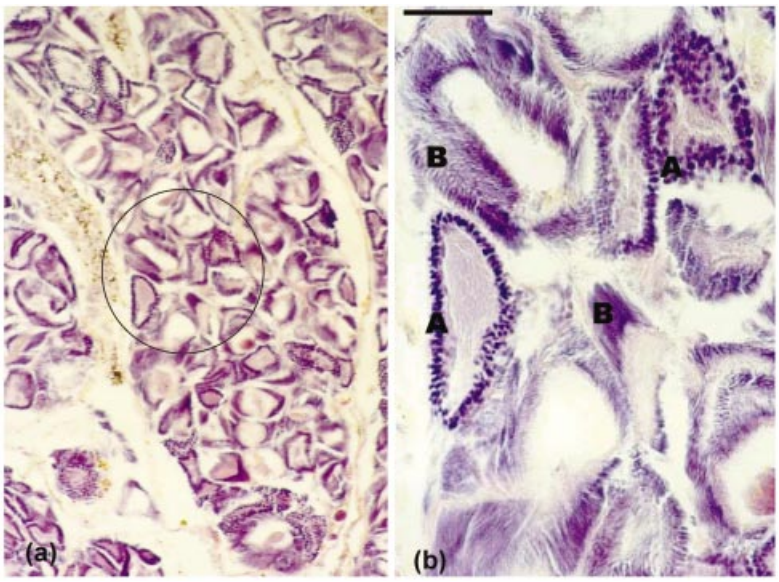

Fig. 7. (a) Cross-section of trophosomal tissue stained with haematoxylin/eosin. The staining shows the histological structures of the symbiont-containing region. (b) Magnified part of the trophosome section showing the trophosome lobules whose peripheries contain two types of cells, i.e. the coccoid (A) and the filamentous (B) cells. Bar, 50 μm.

serve as the drainage site for deposited sulfur and, consequently, yields less background fluorescence than the vestimentiferan internal organ, the trophosome (Fisher, 1990; Distel & Cavanaugh, 1994; Distel *et al*., 1995). However, the detection signal has been shown to be enhanced by using a digoxigenin-labelled probe via the formation of an insoluble coloured precipitate. This probing method is not subject to background interference caused by the tissue, and was found to be suitable in the detection of dual symbiosis in the deep-sea cold-seep thyasirid clam *Maorithyas hadalis* (Fujiwara *et al*., 2001). Thus, ISH using digoxigenin-labelled probes is most helpful when a strong background fluorescence, or cellular auto-fluorescence, prevents or disturbs the detection of probe-conferred fluorescence, e.g. the detection of endosymbiotic bacteria in the trophosome of *Lamellibrachia* sp. (current study) and the detection of symbiotic *Cyanobacteria* in plant sections (Zarda *et al*., 1991; Amann *et al*., 1995; Naganuma *et al*., 1997a).

The vestimentiferan trophosome consists of lobules. The symbiont-bearing cells, the bacteriocytes, are concentrated at the peripheries of the lobules (Hand, 1987). Two types of cells, coccoid and filamentous, were apparently observed at the peripheries of the lobules stained with haematoxylin/eosin (Fig. 7a, b). Both of the cell types were *in situ* hybridized with the universal eubacterial 16S-rRNA-targeted probe EUB338 (Fig. 6a). The occurrence of more than one bacterial morphotype in the vestimentifera trophosome has been reported in a relatively small number of studies (Cavanaugh *et al*., 1981; Cavanaugh, 1985; Fisher, 1990; Naganuma *et al*., 1997a, b; Bright *et al*., 2000). The occurrence of multiple bacterial morphotypes within the trophosome may reflect the complexity of the geochemical processes seen in tubeworm habitats. Some seep habitats, such as the area of sample collection for this study, have abundant methane in the water and sediment, but hydrogen sulfide is only present in the sediment (Sakai *et al*., 1987). The seep-dwelling tubeworms are known to incorporate hydrogen sulfide from the sediment via their posterior end, which is buried in the sediment (Julian *et al*., 1999). Hence, the existence of a diverse microflora in the trophosome of the tubeworm *Lamellibrachia* sp. has been hypothesized to be correlated with the need to utilize sulfur compounds available from the surrounding environment, using methane as a reducing agent (Naganuma *et al*., 1997a; Naganuma, 1998).

The polynucleotide *cbbM* probe ST-Sym(II)-2, which hybridized with all possible *cbbM* sequences within the trophosome, and the 16S rDNA probe Lam-384R produced signals in the same coccoid cells (Fig. 6b, c). This implies that the coccoid cells may carry ST-Sym(II)-1 and ST-Sym(II)-2, the two *cbbM* ORUs recorded by amino acid analysis. It is not clear whether these two *cbbM* sequences occur in only one endosymbiotic species or in two endosymbiotic species. However, the 16S rDNA analysis showed only one sequence for one endosymbiotic species, which was localized in the coccoid cells. Moreover, this result indicates that the coccoid cells at the peripheries of the lobules are the sites of CO_2 fixation within the trophosome. These coccoid cells have been observed to accumulate sulfur, as shown by X-ray microanalysis of the trophosome content

Fig. 6. Micrographs of four consecutive cross sections of the trophosomal tissue showing ISH with the digoxigenin-labelled probes. (a) Hybridization with the universal eubacterial probe EUB338. Higher colour intensity in some coccoid cells (A) compared to that in the filamentous cells (B) indicates a gradient of available 16S rRNA and, possibly, a gradient in metabolic activity. (b) Hybridization with the endosymbiont-specific 16S rDNA probe Lam-384R. (c) Hybridization with the RuBisCO form II *cbbM* probe ST-Sym(II)-2. (d) Hybridization with the RuBisCO form I *cbbL* probe Bathy(*cbbL*)-1 as a negative control. Bar, 50 μm.

(Naganuma *et al*., 1997a). This sulfur accumulation seems to be related to autotrophy, in which sulfide is oxidized to elemental sulfur.

The filamentous cells showed a hybridization signal with the universal 16S rDNA probe EUB338, but not with the 16S-rDNA-specific probe Lam-384R nor with the RuBisCO form II probe ST-Sym(II)-2 (Fig. 6a–c). This may indicate that these filamentous bacterial cells have a different function, and that they are not involved in carbon fixation. Alternatively, these filamentous cells may have been extracellular bacteria which could not be detected by 16S rDNA sequence analysis, as only the autotrophic endosymbiotic bacteria contained within the trophosome were targeted, and the trophosomal tissue used for DNA extraction was pre-treated to remove any extracellular bacteria. The same filamentous cells have been observed previously in the trophosome of the same *Lamellibrachia* sp. used in this study and are related to the *ε-Proteobacteria* (Naganuma *et al*., 1997a). Certain species belonging to the *ε-Proteobacteria* are known to inhabit animal cavities as extracellular microaerophilic bacteria and are capable of sulfur oxidation (Simbert, 1984; Penner, 1988; Naganuma *et al*., 1997a, b). This seems to be an adaptive trait for the environmental conditions within the trophosome. The use of the term 'symbiotic' to describe these filamentous cells should be avoided until their commensal functions have been demonstrated.

No ISH signal was observed with the RuBisCO form I *cbbL* probe, Bathy(*cbbL*)-1, when it was applied to the trophosome section (Fig. 6d). This result was in agreement with the inability to amplify the RuBisCO form I gene (*cbbL*) from the bulk trophosomal DNA.

The use of ISH for the localization of the RuBisCO form II sequences will facilitate our understanding of the autotrophic microbial processes within the trophosome. Further investigations should be done to show the metabolic relationships between autotrophic and non-autotrophic microflora within the trophosome of this gutless, methane-seep tubeworm, namely *Lamellibrachia* sp.

ACKNOWLEDGEMENTS

We thank all of the teams of the DSV *Shinkai* 2000 for their help in the collection of samples. Our grateful thanks also go to Drs Y. Yoshimura and Zheng Weiming, Hiroshima University, for their help in the preparation of histological sections for ISH. This work was partly supported by the Special Co-ordination Fund 'Archaean Park Project' from the Ministry of Education, Culture, Sports, Science and Technology (MEXT) of Japan, and by the Collaborative Research Fund 'Strategy for Life under Extreme Conditions' of the Graduate University for Advanced Studies, Japan.

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Received 23 February 2001; revised 3 January 2002; accepted 1 February 2002.