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EXPERIMENTAL ARTICLES

Phylogenetic Characterization of Endosymbionts of the Hydrothermal Vent Mussel *Bathymodiolus azoricus* by Analysis of the 16S rRNA, *cbb*L, and *pmo*A Genes

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Abstract—In order to assess the phylogenetic diversity of the endosymbiotic microbial community of the gills of marine bivalve *Bathymodiolus azoricus*, total DNA was extracted from the gills. The PCR fragments corresponding to the genes encoding 16S rRNA, ribulose-bisphosphate carboxylase (*cbb*L), and particulate methane monooxygenase (*pmo*A) were amplified, cloned, and sequenced. For the 16S rDNA genes, only one phylotype was revealed; it belonged to the cluster of thiotrophic mytilid's symbionts within the *Gammaproteobacteria.* For the RuBisCO genes, two phylotypes were found, both belonging to *Gammaproteobacteria.* One of them was closely related to the previously known mytilid symbiont, the other, to a pogonophore symbiont, presumably a methanotrophic bacterium. One phylotype of particulate methane oxygenase genes was also revealed; this finding indicated the presence of a methanotrophic symbiont. Phylogenetic analysis of the *pmo*A placed this endosymbiont within the *Gammaproteobacteria*, in a cluster including the methanotrophic bacterial genus *Methylobacter* and other methanotrophic *Bathymodiolus* gill symbionts. These results provide evidence for the existence of two types of endosymbionts (thioautotrophic and methanotrophic) in the gills of *B. azoricus* and demonstrate that, apart from the phylogenetic analysis of 16S rRNA genes, parallel analysis of functional genes is essential.

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Deep-sea hydrothermal vents and cold methane seeps are among the most productive habitats on the Earth. The large invertebrate biomass typical of these sites depends on the organic carbon fixed by bacteria that are present as endosymbionts in animal tissue.

Deep-sea mussels of the genus *Bathymodiolus* are the predominant macroorganisms in the communities of deep-sea hydrothermal vents and cold seeps. Endosymbiotic organisms belonging to the *Gammaproteobacteria* have been revealed in all presently knownspecies of *Bathymodiols*.

Information concerning the phylogeny of deep-sea endosymbiotic microorganisms have been derived mostly from the comparison of 16S rDNA sequences. Some species (*B. thermophilus* [1] and *B. septemdierum* [2]) have been found to contain only thioautotrophic endosymbionts. Other species (*B. japonicus* and *B. platifrons* [2]) contain only methanotrophic endosymbionts.

B. puteoserpentis is presently the only mussel species of deep-sea hydrothermal vents for which dual symbiosis has been confirmed, i.e., stable coexistence of thioautotrophic and methanotrophic bacteria in a single cell (bacteriocyte) of the mytilid gills. The presence of two morphologically and phylogenetically different symbiotic populations in *B. puteoserpentis* gills has been confirmed by ultrastructural studies, 16S rDNA sequence analysis, and in situ hybridization [3]. Furthermore, physiological and immunological studies of the expression and activity of the key enzymes of two distinct C_1 assimilation pathways (RuBisCO and methanol dehydrogenase) have confirmed the metabolic activity of both thioautotrophic and methanotrophic endosymbionts [4].

This dual symbiont population enables the host to utilize reduced sulfur compounds and/or methane as sources of metabolic energy, and carbon dioxide and/or methane as the primary sources of biomass carbon. Presumably, the resulting extraordinary metabolic flexibility promotes the wide distribution and predominance of

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Oligonucleotide primers used in this study

Gene	Primer designation	Primer nucleotide sequence[a]	Reference
16S rRNA	27F	5'-AGAGTTTGATCMTGGCTCAG-3'	[8]
	519R	5'-GWATTACCGCGGCKGCTG-3'	
*cbb*L	RubIgF	5'-GAYTTCACCAARGAYGAYGA-3'	[9]
	RubIgR	5'-TCRAACTTGATYTCYTTCCA-3'	
*cbb*M	RuIIF1	5'-GGHAACAACCARGGYATGGGYGA-3'	[9]
	RuIIR2	5'-TGRCCIGCICGRTGRTARTGCA-3'	
*pmo*A	*pmo*A189F	5'-GGNGACTGGGACTTCTGG-3'	[10]
	*pmo*A682R	5'-GAASGCNGAGAAGAASGC-3'	
*mmo*C	*mmo*C542	5'-GGTTCTGCTGTG CCGCACC-3'	[11]
	*mmo*C986	5'-ATCCCGTGCCGCCGGCGACG-3'	
*nif*H	F1	5'-TAYGGNAARGGNGGNATYGGNAARTC-3'	[12]
	R6	5'-TCNGGNGARATGATGGC-3'	

[a] Designations of degenerated positions are as follows: Y = T/C; R = A/G; S = G/C; M = C/A; K = G/T; W = A/T; N = G/A/T/C.

such animal–bacterial communities in many hydrothermal fields.

Ultrastructural, biochemical, and immunological data, as well as the results of gene-specific PCR, suggest the presence of a dual symbiosis in *B. azoricus* [5, 6]. However, no phylogenetic analysis of the prokaryotic symbiotic community of this organism has yet been performed, either based on the sequence analysis of 16S rRNA genes or based on the analysis of functional genes. However, morphological and enzymatic data alone are insufficient to prove the presence of two symbiont species in this mytilid mussel. These data can also be explained by the presence of a single polymorphic bacterial species similar to type X methanotrophs, which contain the key enzymes for both methanotrophic growth and autotrophic CO_2 fixation.

The majority of autotrophic organisms assimilate CO_2 via the Calvin cycle, with RuBisCO (ribulose-1,5-bisphosphate carboxylase/oxygenase) as the key enzyme. Eubacterial RuBisCO occurs in two main forms. Form I is the most widespread; it is present in most autotrophic bacteria, in algae, and in terrestrial plants. Form I RuBisCO consists of eight large (L) and eight small (S) subunits, which are encoded by the *cbb*L and *cbb*S genes, respectively. Only the large subunits perform the catalytic function. Form II of RuBisCO (the *cbb*M gene) is less frequent and occurs only in bacteria. It consists of large subunits only (L_n); the number of these subunits varies from two to eight, depending on the organism. Some autotrophic bacteria, including thioautotrophs, possess both form I and form II RuBisCO. The endosymbionts of deep-sea mollusks usually have form I RuBisCO, whereas endosymbionts of vestimentifers possess form II RuBisCO. The different kinetic characteristics of these two forms have been hypothesized to be the reason for this distribution of RuBisCO forms, together with the physiological limitations imposed by the host organism, which may enable preferential selection of the endosymbionts with the required RuBisCO form [7].

Methane monooxygenase, the key enzyme of methanotrophic metabolism, also occurs in two main forms. The membrane-bound methane monooxygenase is present in the cells of all methanotrophic bacteria with the exception of representatives of the genus *Methylocella*; the α subunit of this enzyme is encoded by the *pmo*A gene. Methanotrophs of types II and X, as well as some type I methanotrophs, additionally contain soluble methane monooxygenase. This enzymatic complex includes protein C, encoded by the *mmo*C gene.

The goal of the present work was the study of the phylogenetic diversity of *Bathymodiolus azoricus* endosymbionts by 16S rRNA gene sequence analysis supplemented by phylogenetic analysis of the functional genes encoding the key autotrophic and methanotrophic enzymes.

MATERIALS AND METHODS

Specimen collection. *Bathymodiolus azoricus* specimens were collected at the Mid-Atlantic Ridge Lucky Strike site (37° N; 1608–1670 m) in 2002 using the *Mir-1* and *Mir-2* deep submergence research vessels (DSRVs). The gills of the mytilids collected in the moire zone of the hydrothermal vents were immediately dissected and repeatedly washed with filtered (0.2 μm) and autoclaved seawater to eliminate surface contamination. Gill samples were stored in 40% ethanol at 4°C prior to analysis.

DNA extraction, PCR amplification, cloning, and sequencing. DNA extraction from the tissue samples was performed as described earlier [6]. Partial amplification of the genes encoding 16S rRNA, the large subunit of form I RubisCO (*cbb*L), the large subunit of

form II RuBisCO (*cbb*M), particulate methane monooxygenase (*pmo*A), soluble methane monooxygenase (*mmo*C), and nitrogenase (*nif*H) was performed.

The oligonucleotide primers used in the present work are listed in the table.

The PCR products obtained by amplification of the 16S rRNA genes and the *cbb*L and *pmo*A genes were cloned in the pGEM-T vector (Promega, United States). The clones containing the inserts of the expected size were subdivided into subgroups by using single nucleotide track sequencing [13] with appropriate forward primers.

Complete sequencing by the Sanger method [14] was performed with a Silver Sequencing kit (Promega, United States) for the representatives of the clone groups revealed. For the *pmo*A gene, the PCR products were also sequenced directly.

Phylogenetic analysis of the sequences. Preliminary analysis of the new sequences was performed with the BLAST software package (http://www.ncbi.nlm.nih.gov/ BLAST). The BioEdit program (http://jwbrown.mbio.ncsu.edu/BioEdit/bioedit.html) was used for editing, aligning, and translation of the sequences. The phylogenetic trees were constructed using the methods implemented in the TREECONW software package (http://bioc-www.uia.ac.be/u/yvdp/treeconw.html).

Nucleotide sequence accession numbers. The GenBank accession numbers for the nucleotide sequences of the symbiotic bacteria are AY945758 to AY945761; that of the type strain of the methylotrophic bacterium *Methylobacter psychrophilus* is AY945762.

RESULTS

Occurrence of a thioautotrophic endosymbiotic component in the gills of *B. azoricus*, revealed by 16S rDNA sequence analysis. A total of 55 PCR clones of partial 16S rDNAs (approximately 500 bp of the most variable 5' portion of the gene) from the gill tissue were analyzed to reveal homogeneity or heterogeneity of the endosymbiont population. The single nucleotide track sequencing demonstrated that all 55 clones were identical. This conclusion was supported by the observation that the 16S rDNA fragments from three randomly selected clones were identical (100% of sequence similarity). Therefore, the existence of only one 16S phylotype, named BA-Sym(16S), can be suggested.

Phylogenetic analysis of the 16S rDNA placed this 16S phylotype in a cluster of *Gammaproteobacteria* that comprised all the previously studied *Bathymodiolus* spp. thioautotrophic symbionts, the majority of other thioautotrophic symbionts associated with marine invertebrate hosts, and some uncultured free-living bacteria from the same environments (Fig. 1a). The 16S rDNA sequence of the studied thioautotrophic symbiont of *B. azoricus* was identical (100% of sequence similarity) to the analogous sequences of *B. azoricus* and *B. puteoserpentis* thioautotrophic gill symbionts from the Mid-Atlantic Ridge Rainbow hydrothermal vent (although these sequences are available in GenBank, they have not been previously analyzed). The similarity to the thioautotrophic symbiont revealed by phylogenetic analysis of the *B. puteoserpentis* bacterial community from the same environment [3] was 99.6%. The sequence similarities to other analyzed gill symbionts and environmental bacterial clones varied from 96.2 to 97.8%. The free-living autotrophic bacteria of the genera *Thiomicrospira, Thioalkalimicrobium*, and *Hydrogenovibrio* were only distantly related to the thioautotrophic symbiont cluster.

Occurrence of a methanotrophic endosymbiotic component in the gills of *Bathymodiolus azoricus*, revealed by *pmo*A sequence analysis. PCR amplification of the total DNA isolated from gill samples revealed a fragment of the *pmo*A gene, encoding particulate methane monooxygenase (ca. 500 bp); the soluble methane monooxygenase gene (*mmo*C) was not amplified by PCR from the same DNA. The sequences of the 44 clones thus obtained were identical to the sequence obtained by direct sequencing of the *pmo*A PCR product. This finding confirms the presence of a single bacterial phylotype, named BA-Sym(M), and thus indicates the existence of one methanotrophic symbiont. Phylogenetic analysis of the deduced *Pmo*A amino acid sequence placed the *B. azoricus* methanotrophic endosymbiont within the *Gammaproteobacteria* in a clade adjacent to the cluster containing the pmoA sequences of type I methanotrophs (Fig. 1b). This sequence, with a high level of bootstrap support (92%), formed a monophyletic cluster with the *Bathymodiolus* sp. symbiont and an uncultured bacterium from a deep-sea hydrothermal vent (94.4% of amino acid sequence similarity). This cluster was most closely related to *Methylobacter psychrophilus* (89.4% of amino acid sequence similarity) and some unidentified *Methylobacter* strains from different environments (89.4–93.1% of amino acid sequence similarity).

Since methanotrophic bacteria, including *Methylobacter* species, are know to be capable of dinitrogen fixation and to possess the genes determining this capability [12], experiments were performed in order to reveal the nitrogenase gene (*nif*H) in the total DNA of gill samples. However, no combination of the specific primers used resulted in amplification of PCR fragments analogous to the those obtained for *M. psychrophilus*, the closest relative of the methanotrophic symbiont.

Occurrence of an autotrophic endosymbiotic component in the gills of *Bathymodiolus azoricus*, revealed by *cbb*L gene sequence analysis. PCR amplification of the total DNA isolated from gill samples revealed a fragment of the *cbb*L gene (ca. 800 bp), encoding the form I RuBisCO large subunit; however, the RuBisCO form II gene (*cbb*M) was not amplified.

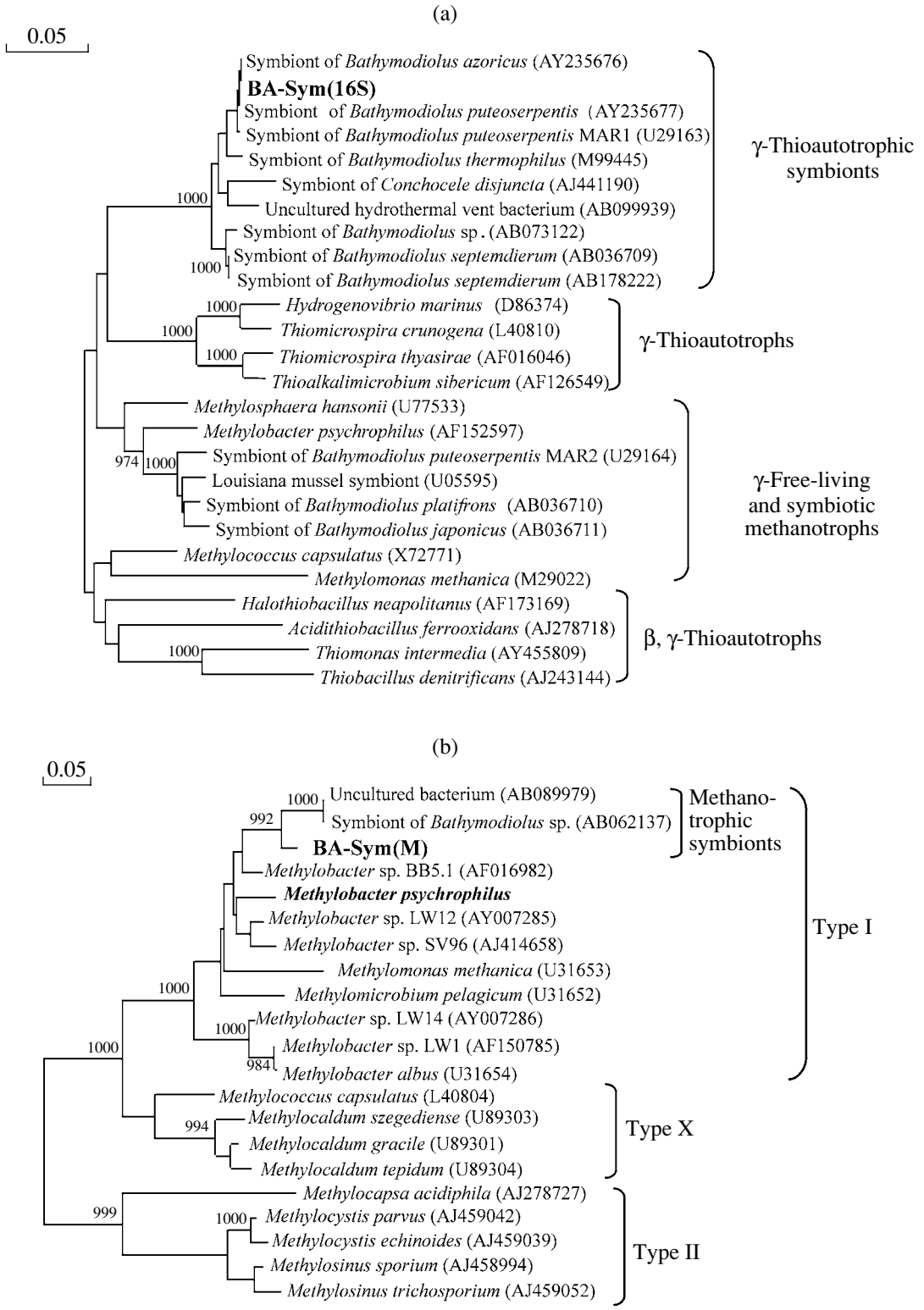

Fig. 1. Position of *B. azoricus* endosymbionts in phylogenetic trees constructed based on the analysis of (a) nucleotide sequences of 16S rRNA genes and deduced amino acid sequences of (b) *pmo*A genes, encoding membrane-bound methane monooxygenase (pMMO), and (c) *cbb*L genes, encoding the large subunit of form I RuBisCo. The sequences determined in this work are set in boldface. The topology of the trees and evolutionary distances were determined by the neighbor-joining method. Numerals show the significance of the branching order as determined by bootstrap analysis (values above 950 were considered significant).

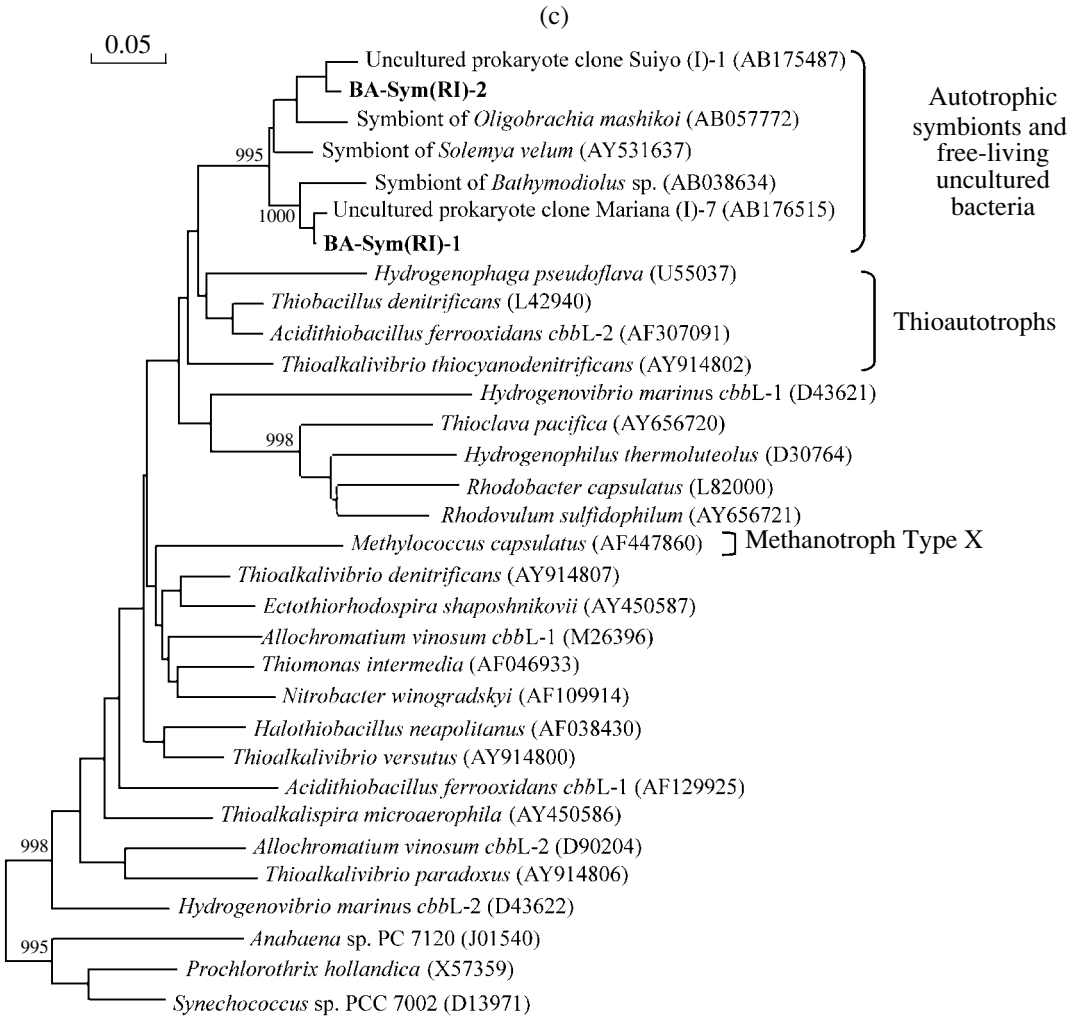

Fig. 1. (Contd.)

The resulting nucleotide sequences of *cbb*L were conceptually translated into amino acid sequences, and the sequences exhibiting 100% amino acid identity with each other were grouped into *cbb*L phylotypes. Two *cbb*L phylotypes, named BA-Sym(RI)-1 and BA-Sym(RI)-2 were obtained, which represented 46 and 4 clones, respectively, of the 50 analyzed *cbb*L clones. The two *cbb*L phylotypes shared 93% amino acid sequence similarity. The clones formed a single cluster (with a 95% level of "bootstrap" support) with the analogous genes from symbiotic and free-living uncultured bacteria (Fig. 1c).

The closest relatives of the predominant clone BA-Sym(RI)-1 were an uncultured bacterium Mariana(I)-7 (98.9% of amino acid sequence similarity) detected in the hydrothermal vent plumes of the Mariana Trench, and the *Bathymodiolus* sp. endosymbiont (95.0% of amino acid sequence similarity). The closest relatives of the minor clone BA-Sym(RI)-2 were an uncultured bacterium Suiyo (I)-1 (97.1% of amino acid sequence similarity) from the hydrothermal vent of Suiyo Seamount, and the endosymbiont of *Oligobrachia mashikoi* (*Pogonophora*) (95.0% of amino acid sequence similarity).

The phylogenetic cluster containing the conceptually translated sequences of *cbb*L genes of symbiotic (including the symbiont of *B. azoricus*) and free-living uncultured bacteria was most closely related to the cluster of the corresponding sequences belonging to the chemotrophic gamma- and betaproteobacteria of the genera *Acidithiobacillus*, *Thiobacillus* and *Hydrogenophaga* (84.5–91.2%).

DISCUSSION

Analysis of 16S rRNA gene sequences is widely used in molecular ecological investigations of prokaryotic diversity in natural communities. It concerns also the studies of endosymbionts of various marine invertebrates, including *Bathymodiolus*. Works using this

approach have revealed in the gills of this mollusk a single thioautotrophic symbiont [1, 2], a single methanotrophic symbiont [2, 15], or both symbionts together [3].

Two groups of endosymbionts—thioautotrophs and methanotrophs—have previously been revealed in the gills of *B. azoricus* by the radioisotopic, cytological, and enzymatic analyses, as well as by PCR amplification with gene-specific primers [5, 6].

However, our analysis of 16S rRNA genes indicated the presence of only one symbiont, which was identical to the thioautotrophic symbionts found previously in the gill tissue of *B. azoricus* and *B. puteoserpentis* [3, 16]. The host species *B. azoricus* and *B. puteoserpentis* are very closely related and differentiated mostly by their habitats; their thioautotrophic symbionts were also closely related or identical according to the results of 16S rRNA gene sequencing. They, however, exhibited pronounced differences from the symbionts of other, less closely related mussel species, *B. thermophilus* and *B. septemdierum*. This finding confirms the previously revealed species specificity of symbiotic communities [1].

Our results of 16S rRNA gene analysis indicated the presence of only one (thioautotrophic) symbiont in *B. azoricus* gill tissue and thus contradicted the earlier evidence of the existence of a dual bacterial symbiosis in this mollusk. Therefore, PCR detection of methane monooxygenase genes was performed in order to identify the possible methanotrophic symbiont. The only sequence type of the *pmo*A gene detected was closely related to the analogous gene of the methanotrophic symbiont of another *Bathymodiolus* species. Methanotrophic *Bathymodiolus* symbionts are undoubtedly type I methanotrophs. Among the methanotrophs described, *M. psychrophilus* is phylogenetically their closest relative (the *pmo*A gene sequence of this species was determined in the present work).

Since *nif*H gene fragments were not revealed in the methanotrophic symbiont, nothing definite can be said concerning its ability to fix dinitrogen. The absence of the PCR product may indicate the inefficiency of the primer system applied. This is, however, hardly probable, since this system has proved efficient for all the methanotrophs studied [12]. The loss of the ability to fix dinitrogen can therefore be hypothesized for the methanotrophic symbiont; the tissues of the mussel host possibly satisfy its nitrogen requirements.

The inability of the standard 16S rRNA gene analysis to reveal the methanotrophic symbiont may be due to the different amplification efficiency on different DNA templates. However, since the samples were collected relatively far from the zone of high methane concentration, a relatively low quantity of methanotrophic symbionts is a more probable explanation. The rates of CO_2 fixation and methane oxidation, as well as the ratio of the two types of symbionts, were previously demonstrated to vary depending on the environmental conditions [6]. The low concentration of the DNA of the methanotrophic component in the total DNA is therefore quite probable. The PCR conditions (concentration of the DNA template and the number of cycles) can sometimes result in random fluctuations of the course of the reaction and, therefore, in the amplification mostly of the 16S rDNAs of the predominant components of a community [17, 18].

A comparison of our data with the results of the earlier investigation of the endosymbiont diversity in the *Oligobrachia mashikoi* pogonophore [19] confirm this possibility. The only 16S phylotype revealed in that work was found to be remotely related to the methanotrophic bacteria of the genus *Methylobacter* and to methanotrophic symbionts of *Bathymodiolus* sp. Analysis of the *cbb*L genes also revealed a single phylotype related to *Gammaproteobacteria.* These results have been attributed to the existence of a single methanotrophic symbiont capable of methane oxidation and CO_2 fixation, similarly to bacteria of the genus *Methylococcus*. It is quite probable, however, that in the study of *O. mashikoi*, as in the present study, the 16S rDNA analysis just failed to reveal one of the components of a dual symbiosis, namely, the thioautotroph responsible for the *cbb*L phylotype.

In order to obtain more detailed information concerning the *B. azoricus* autotrophic symbiont, we performed an additional phylogenetic analysis of the translated *cbb*L gene sequences. Although the 16S rDNA analysis revealed only one type of 16S rRNA genes, we found in the gill samples two sequence types of *cbb*L genes determining form I RuBisCO. Similar results were obtained in an earlier study of the thioautotrophic symbionts of the *Lamellibrachia* sp. trophosome, although no explanation has been provided. In that study, only one sequence type of alphaproteobacterial 16S rRNA genes was detected, together with two closely related sequence types of *cbb*M genes determining form II RuBisCO [20].

Our results can be explained either (1) by the existence of a minor thioautotrophic symbiont that cannot be differentiated cytologically with 16S rDNA analysis; (2) by the presence of two copies of the *cbb*L gene in the genome of a single thioautotrophic symbiont, similarly to such free-living thioautotrophs as *Acidithiobacillus ferrooxidans* [21], *Allochromatium vinosum* [22], *Hydrogenovibrio marinus* [23], and *Thiomicrospira* sp. (unpublished data); or (3) by the existence of two *cbb*L genes belonging to two symbionts, one thioautotrophic and one methanotrophic, the latter able to employ both pathways of C_1 assimilation. The simultaneous presence of *cbb*L and *pmo*A genes in the genome has so far been demonstrated only for methanotrophs of the genus *Methylococcus*; however, the phylotypes specific to this genus have not been revealed in our work. It has, however, been previously stated that the presence of the RuBisCO genes in the genomes of type I methanotrophs is not impossible [15, 19]. The

phylogenetic relatedness of the minor *cbb*L phylotype discovered in the present work and with the analogous sequence of the hypothetical type I methanotrophic symbiont of *O. mashikoi* may support this supposition. Further experiments employing in situ hybridization are required to confirm the presence of two *cbb*L phylotypes and to determine to which organisms they belong.

Thus, the simultaneous phylogenetic analysis of the 16S rRNA genes and of the genes encoding the key enzymes of auto- and methanotrophy in the gill tissue of *B. azoricus* mussels confirmed the coexistence therein of a thioautotrophic and a methanotrophic (type I) endosymbiont.

The present work has demonstrated that the analysis of the diversity of functional genes can significantly supplement the results of conventional phylogenetic analysis of 16S rRNA genes and provide better understanding of microbial communities. Moreover, such works provide the basis for the development of specific probes for in situ hybridization and for further research into various communities, including endosymbionts of invertebrates.

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