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Independent Phylogenetic Origins of Methanotrophic and Chemoautotrophic Bacterial Endosymbioses in Marine Bivalves

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The discovery of bacterium-bivalve symbioses capable of utilizing methane as a carbon and energy source indicates that the endosymbionts of hydrothermal vent and cold seep bivalves are not restricted to sulfur-oxidizing chemoautotrophic bacteria but also include methanotrophic bacteria. The phylogenetic origin of methanotrophic endosymbionts and their relationship to known symbiotic and free-living bacteria, however, have remained unexplored. In situ localization and phylogenetic analysis of a symbiont 16S rRNA gene cloned from the gills of a recently described deep-sea mussel species demonstrate that this symbiont represents a new taxon which is closely related to free-living, cultivable Type I methanotrophic bacteria. This symbiont is distinct from known chemoautotrophic symbionts. Thus, despite compelling similarities between the symbioses, chemoautotrophic and methanotrophic symbionts of marine bivalves have independent phylogenetic origins.

The recent discovery of symbioses between methanotrophic bacteria and marine bivalves has demonstrated that bacterium-invertebrate associations of the types that are known to occur in the unique fauna of deep-sea hydrothermal vents and cold seeps are more diverse and more evolutionarily complex than had previously been thought (6). Such symbioses have been observed in a diverse variety of marine invertebrates, including bivalve and gastropod mollusks, vestimentiferans, pogonophorans, annelids, and nematodes. However, until recently, the symbionts known to be associated with these animals have been restricted to a relatively closely related group of sulfur-oxidizing chemoautotrophs which fall within the gamma subdivision of the class *Proteobacteria* (10–13). Although the methanotrophic symbionts have been shown to be physiologically distinct from previously known symbionts of vent and seep organisms, their phylogenetic relationships to known symbionts and free-living bacteria have remained unexplored.

Among bivalves, the newly discovered methanotrophic symbioses bear a striking resemblance to the previously described sulfur-oxidizing chemoautotrophic associations. In both cases, the symbionts perform similar roles in symbiosis by providing the hosts with a substantial fraction of their nutritional carbon and energy needs by utilizing sources otherwise unavailable to metazoans. The hosts, in turn, facilitate the symbionts' simultaneous access to necessary substrates, notably O_2 and H_2S or CH_4, from oxic and anoxic environments, respectively (7). The two types of symbioses also share similarities in habitat, in ultrastructural organization of the symbiont-bearing tissues, and in the symbionts' resistance to growth in pure culture (6, 14, 16). The broad similarities between these symbioses could be the result of common descent, with hosts and symbionts having diverged from a single ancestral symbiosis. Alternatively, methanotrophic and chemoautotrophic symbioses may have arisen independently, drawing their symbionts from phylogenetically distinct groups. The latter alternative would indicate that similarities observed between the symbioses are the result of evolutionary convergence or displacement of a previously existing symbiont population.

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Free-living methanotrophs have been classified into three categories based on biochemical pathways and ultrastructure (2, 20). Type I methanotrophs utilize the ribulose monophosphate (RuMP) pathway of formaldehyde assimilation and have centrally stacked intracytoplasmic membranes, while Type II methanotrophs utilize the serine pathway and have peripheral intracytoplasmic membranes. Type X methanotrophs have centrally stacked membranes but contain enzymes of both serine and RuMP pathways as well as ribulose-1,5-bisphosphate carboxylase-oxygenase (RuBisCO), the key enzyme of the Calvin-Benson cycle of carbon fixation. Analysis of 16S rRNA sequences has demonstrated the phylogenetic diversity of methanotrophic bacteria, showing them to be broadly distributed among the class *Proteobacteria*. Type I and Type X methanotrophs examined to date fall within the gamma subdivision, and Type II methanotrophs are associated with the alpha subdivision (19, 32).

Here we have examined the symbionts found in the gill of a recently described mytilid (unnamed species, family Mytilidae) (32a) collected at a depth of 600 to 700 m on the continental slope off the coast of Louisiana. Several kinds of evidence indicate that the symbionts of this bivalve are methanotrophic bacteria: (i) transmission electron microscopy of gill tissue reveals gram-negative bacterial cells containing complex intracytoplasmic membrane stacks typical of Type I and Type X methanotrophs (8), (ii) methanol dehydrogenase has been detected in gill tissue (17), (iii) carbon isotope values of mytilid tissues show considerable ^{13}C depletion ($\partial^{13}C = -40$ to $-57‰$), reflecting local thermogenic methane sources rather than photosynthetically or chemoautotrophically fixed carbon (4), (iv) utilization of and growth stimulation by methane have been demonstrated in live mytilid specimens in the laboratory (5, 8, 17, 24), and (v) this mytilid species inhabits an environment in which dissolved methane concentrations in excess of 65 μM have been measured (28) and gas has been observed bubbling from the sea floor (3).

Although ultrastructural and biochemical evidence suggests that the symbionts of the Louisiana mytilid are methanotrophs, these data provide little insight into the phylogenetic origin of these bacteria. For example, complex intracytoplasmic mem-

branes observed in the symbionts could indicate affiliation with Type I and Type X methanotrophs (19, 20, 32). Similar structures, however, are also observed in other unrelated bacterial groups, including *Nitrosococcus* species (25). RuBisCO activity reported in the gill tissue of these mussels (17) could indicate that the symbionts are affiliated with chemoautotrophic symbionts and/or with Type X methanotrophs. Alternatively, the methanotrophic symbionts, like the chemoautotrophic symbionts, could represent a lineage as yet unknown among free-living bacteria (10–13). Here we have used comparative phylogenetic analysis of symbiont small-subunit (16S) rRNA sequences and fluorescent in situ oligonucleotide hybridization to examine the phylogenetic relationship of the methanotrophic symbiont of the Louisiana mytilid mussel to other known symbiotic and free-living bacteria.

MATERIALS AND METHODS

Organisms. Specimens of a new species of mytilid mussel (unnamed species, family Mytilidae) (32a) were collected at depths of 600 to 700 m on the continental slope off the coast of Louisiana (27°47′N, 91°32′W) by using deep submersible research vehicle *Sealink*. Mytilids were transported to the laboratory live in chilled seawater (4°C) for dissection.

PCR amplification, cloning, and sequencing. Nucleic acids from symbiont-containing gill and symbiont-free foot tissues (negative controls) were prepared as described elsewhere (10). Universal eubacterial primers 1492r and 27f (26) were used to amplify bacterial 16S rRNA genes contained in the nucleic acids extracted from the bivalve tissues. The PCR products from two specimens were sequenced directly (23) with sequencing primers previously described (26) and Sequenase V.2 t7 polymerase (U.S. Biochemical Corp.). PCR products were also cloned from one of the specimens used for direct sequencing and from a third specimen by using the TA Cloning kit (Invitrogen). A total of 24 clones were screened by restriction analysis with the frequent cutter *Alu*I. Three clones were fully sequenced and five clones were partially sequenced (~600 bp each).

Symbiont-specific probes. In order to confirm that the dominant gene sequence amplified from the gill of the Louisiana mytilid originated from the symbiont rather than from a superficially associated bacterium or chance contaminant, two specific oligonucleotide probes (LA-1 and LA-2) complementary to highly variable regions of the RNA-like strand of the amplified and cloned sequence were designed. Symbiont-specific probe target regions in the 16S rRNA gene encoded by the dominant clone were selected by computer search of sequences published in GenBank by using BLAST (1) and of those published in the rRNA Database Project small-subunit rRNA data base by using CHECKPROBE search algorithms (29). The two symbiont-specific probes (LA-1, CCGCCACTA AACCTGTATATAGG, *Escherichia coli* positions 838 to 859; LA-2, GTAGGGCATATGCGGTATTAGCATGGG, *E. coli* positions 163 to 187) contain at least four mismatches to all published small-subunit rRNA sequences. A negative-control probe was also used in this study (LA-0, GTAGGGCGTAT GCGGTATTAGCCTGGT, *E. coli* positions 163 to 187) which differed at three nucleotide positions from probe LA-2. A eubacterial universal probe (GCTGCCTCCCGTAGGAGT, *E. coli* positions 338 to 356) (22) and a eukaryotic universal probe (GGGCATCACAGACCTG, *E. coli* positions 1209 to 1224) (18) served as positive controls. Probes complementary to these target regions were biotinylated during synthesis by 5′ terminal addition of Biotin-On (Clontech) phosphoramidite and subsequently purified by polyacrylamide gel electrophoresis.

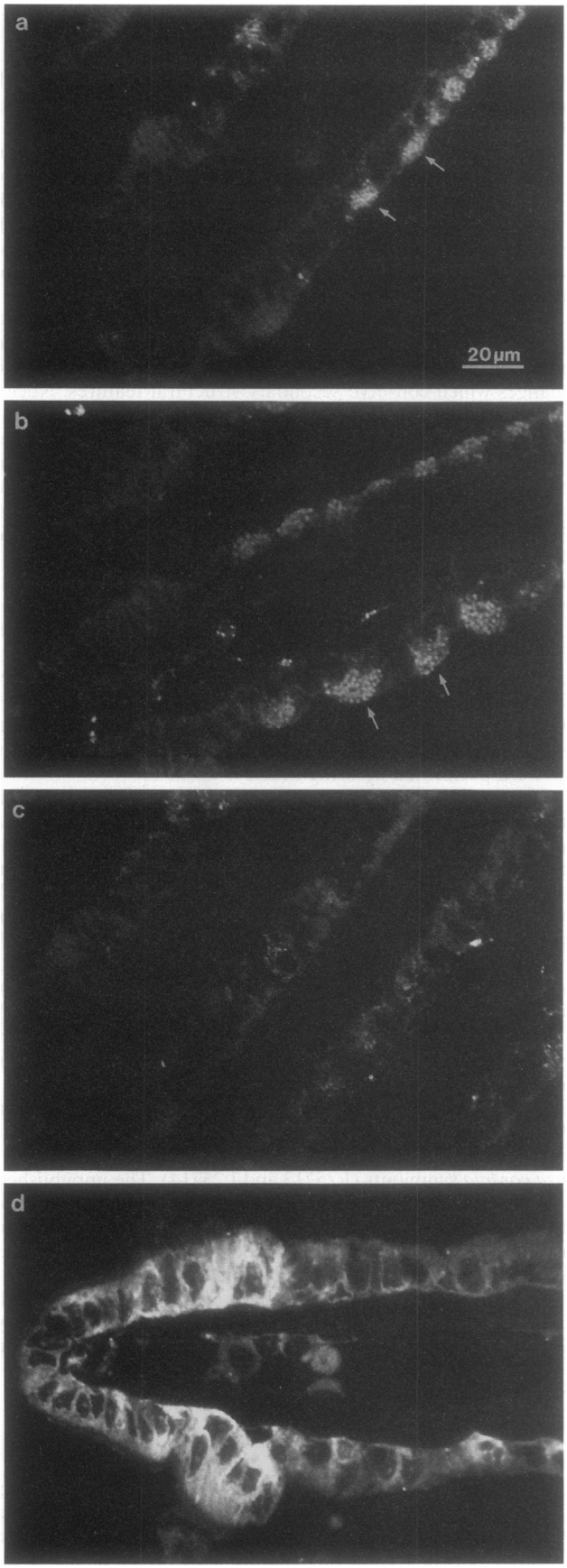
In situ hybridizations. For in situ hybridizations, mytilid gills were dissected, preserved, embedded in paraffin, sectioned, and deparaffinized as described elsewhere (9). Hybridizations were performed at 42°C with subsequent washes (three washes for 15 min each) at 46°C in 0.2× SET (9). Sections were then incubated in avidin-conjugated fluorescein isothiocyanate (Vector Laboratories) for 10 min on ice and washed (three washes for 10 min each at 4°C) in 1× carbonate buffer (27). Sections were observed and photographed with a Zeiss Axioskop microscope and with a Bio-Rad MRC600 confocal microscope.

Phylogenetic analysis. Sequence alignments were performed manually by using the Genetic Data Environment sequence editor (31) and were verified by analysis of compensatory base changes which conserve secondary structural features. Nucleotide positions which were undetermined or of ambiguous identity, insertions and deletions, alignment gaps, and sequence regions which could not be aligned with certainty in one or more taxa were eliminated from consideration. A total of 1,018 nucleotide positions were utilized in this analysis. Evolutionary-distance analyses were performed by using the following programs contained in the Phylip 3.4 package (15) and were implemented through the Genetic Data Environment sequence editor on a Sun SPARC station IPC. Evolutionary distances were estimated by using DNADIST with Jukes and Cantor correction. The SEQBOOT program was used to generate 100 bootstrapped datum sets. Tree construction and bootstrap analyses were performed by using FITCH with random sequence addition and global rearrangement. Maximum-parsimony analysis and parsimony bootstrap analysis were performed by using PAUP.

Sequences used in this investigation include (with GenBank accession numbers in parentheses) those of a *Bathymodiolus thermophilus* symbiont (M99445), a *Calyptogena magnifica* symbiont (M99446), a Louisiana mytilid symbiont (U05595), a *Lucinoma aequizonata* symbiont (M99448), *Methylomonas albus** (M95659), *Methylobacterium extorquens** (M95656), *Methylobacillus flagellatum* (M95651), *Methylococcus luteus** (M95657), *Methylomonas methanica* (M29022), *Methylophilus methylotrophus* (M29021), *Methylomonas rubra* (M95662), *Methylosinus sporium* (M95665), *Methylomonas pelagica** (U05570), *Methylomonas* sp. strain A4* (M95658), *Nitrosococcus oceanus* (M96398), a *Riftia pachyptila* symbiont (M99451), a *Solemya reidi* symbiont (L25709), a *Solemya velum* symbiont (M90415), *Thiomicrospira thyasirae* (L01478 and L01479), a *Thyasira flexuosa* symbiont (L01575), *Thiobacillus hydrothermalis* (M90662), *Thiomicrospira* strain L-12 (L01576), and *Vesicomya cordata* symbionts (L25713 and L25714). The unpublished sequence of *Methylococcus capsulatus* and unpublished updated versions of sequences from organisms marked with an asterisk were provided by J. P. Bowman, L. I. Sly, and E. Stackebrandt, Center for Bacterial Diversity and Identification, Department of Microbiology, University of Queensland, Brisbane, Australia. The *M. pelagica* sequence was provided by S. J. Giovannoni (Oregon State University). Sequences for the remaining species were obtained from the Ribosomal Database Project.

RESULTS

PCR amplification (30) was used to obtain bacterial 16S rRNA gene sequences from the symbiont-containing gill tissue of three mytilid specimens collected by deep submersible research vehicle *Sealink* on separate expeditions to the Loui-



siana site. The results of direct sequencing of PCR products show evidence of amplification of a single plausible bacterial 16S rRNA sequence from the gill tissue samples, as confirmed by conservation of primary sequence and secondary structural features. Screening by restriction analysis and sequencing of cloned PCR products confirm these products to be dominated by a single 16S rRNA sequence. Of three restriction patterns observed, two represented the two possible orientations of a single insert in the vector. These accounted for 23 of 24 clones (95.8%) examined. The sequence of the dominant clone was identical to the direct PCR sequence.

Two oligonucleotide probes (LA-1 and LA-2), complementary to highly variable regions of the RNA-like strand of the putative symbiont 16S rRNA sequence, were synthesized and tested in situ on tissue sections from the gills of the Louisiana mussels. These probes, which contain at least four mismatches to all published small-subunit rRNA sequences, are expected to hybridize specifically with targets containing the putative symbiont sequence. Epifluorescence (data not shown) and confocal microscopy (Fig. 1b and 2b) demonstrate that the symbiont-specific probes bind to the apical region of the bacteriocytes in the gills of the Louisiana mytilid. Transmission electron micrographs (Fig. 2a) show that most of the bacteriocyte volume in the apical region is composed of symbiont cells while the bacteriocyte's cytoplasm is largely confined to the basal region. Identical hybridization patterns are observed for the symbiont-specific probes and a eubacterial universal probe (22) (Fig. 1a) applied as a positive control. A second symbiont-specific probe (LA-2) gave results identical to those given by LA-1 (data not shown). A negative-control probe (LA-0) which differed at three nucleotide positions from probe LA-2 did not bind to gill sections (Fig. 1c), while a eukaryotic universal probe (18) bound to symbiont-free ciliated cells at the distal end of the gill filament and to the basal region of the bacteriocytes (Fig. 1d). High-magnification confocal images demonstrate that the symbiont-specific probe binding is localized to structures within the gills which are identical in size, shape, location, and distribution to the symbionts as observed by electron microscopy (Fig. 2).

Phylogenetic analysis based on 16S rRNA sequences, using an evolutionary-distance method, indicates that the symbiont of the Louisiana mussel represents a new taxon closely related, but not identical, to known free-living and cultivable Type I methanotrophic bacteria (Fig. 3). A similar result was observed by using maximum-parsimony methods. Six shortest trees of 1,793 steps were found, each tree identical to the distance tree shown here with respect to the branching order of Type I methanotrophs and placement of the Louisiana mytilid symbiont. Bootstrap analyses using both evolutionary-distance and maximum-parsimony methods strongly support (97 of 100 and 93 of 100 trees, respectively) the placement of the Louisiana mytilid symbiont within a monophyletic group entirely com-

FIG. 1. Confocal micrographs showing in situ hybridization of symbiont-specific and control oligonucleotide probes in gill tissue sections of the Louisiana mytilid. Hybridizations were performed with the following fluorescence-labeled oligonucleotides: the eubacterial universal probe (a), the symbiont-specific probe (LA-1) (6), the negative-control probe (LA-0, differing from LA-2 by three nucleotides) (c), and the eukaryotic universal probe (d). The symbiont-specific probes and the universal eubacterial probe bind to spherical structures (arrows) within the gill epithelial cells (bacteriocytes). The eukaryotic universal probe binds to the symbiont-free cells associated with the ciliated regions at the distal end of the filament and to the basal region of the bacteriocytes, where most of the host cytoplasm is located.

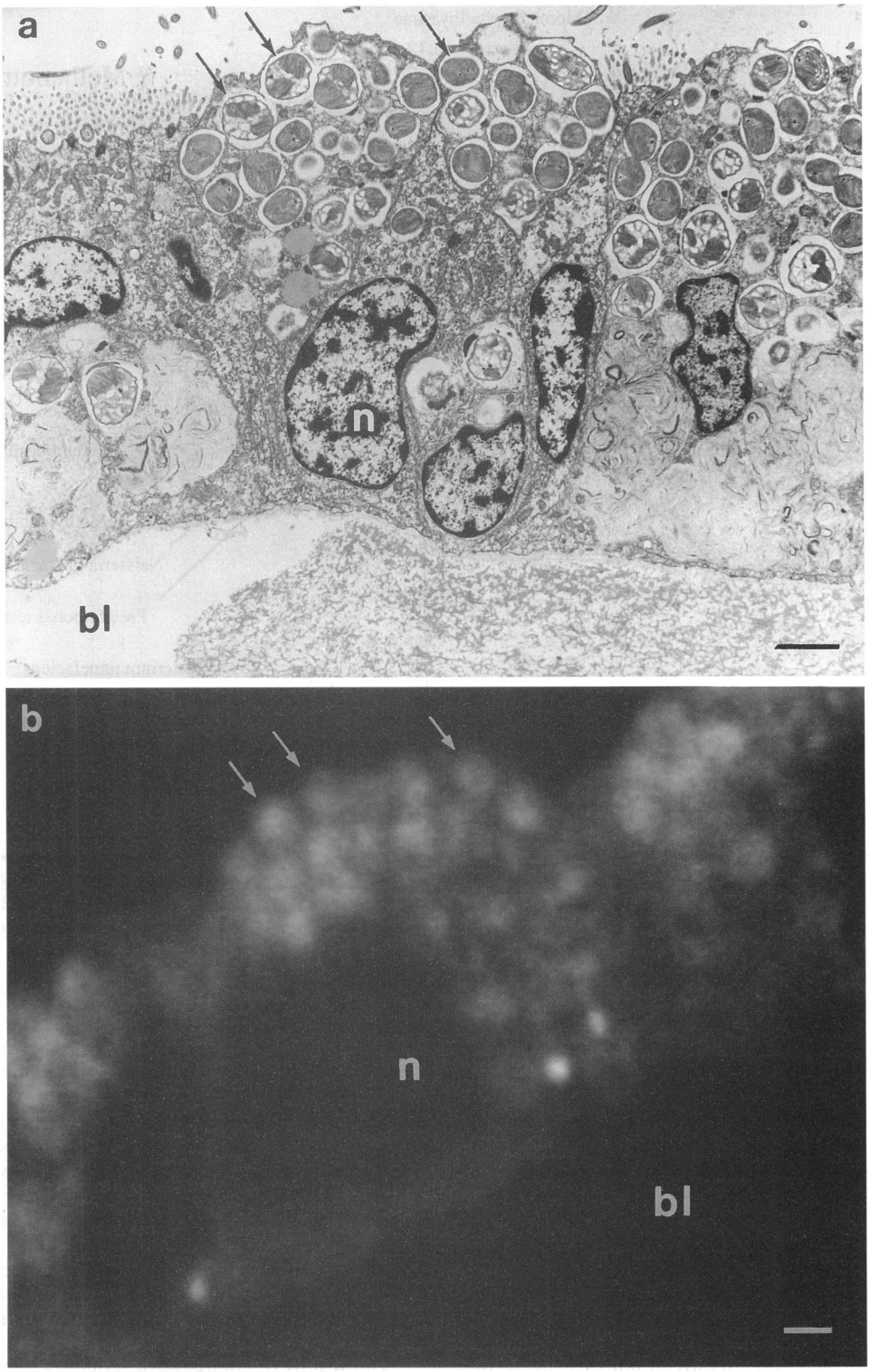


FIG. 2. High-magnification micrographs of symbiont-containing gill tissue of a Louisiana mytilid. (a) Transmission electron micrograph showing methanotrophic bacterial symbionts (arrows) within gill epithelial cells (bacteriocytes). Symbionts are concentrated in the apical region of the cell, closest to the external environment. Gill tissues were prepared for transmission electron microscopy according to a method described previously (17). (b) Confocal micrograph showing binding of symbiont-specific probe LA-1 to structures (arrows) within the bacteriocyte which are identical to symbiont cells in size, shape, and distribution. n, nucleus of bacteriocyte; bl, blood space. Scale bars = 2 μm. (Fig. 2a is courtesy of C. R. Fisher, Pennsylvania State University.)

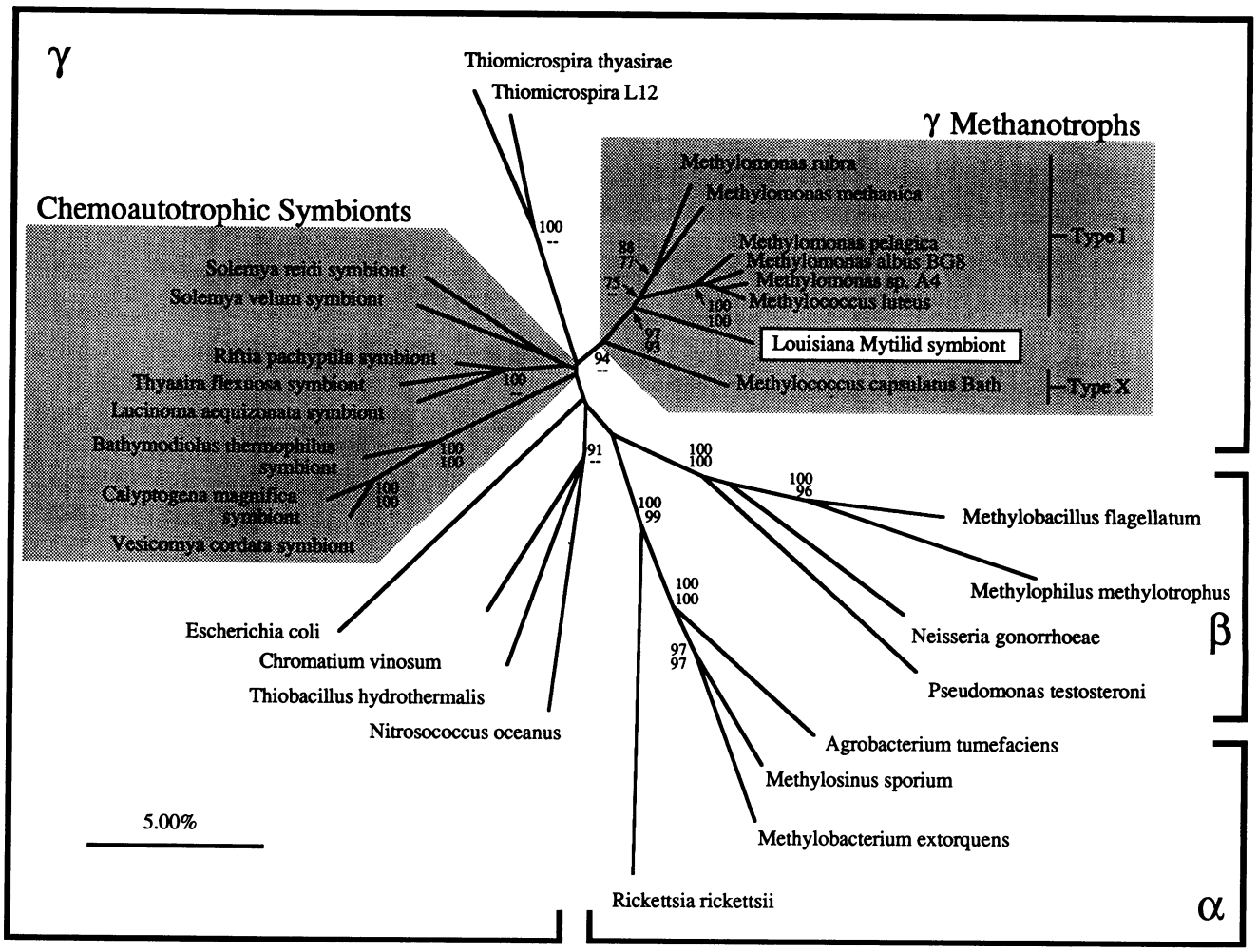


FIG. 3. Evolutionary-distance tree based on 16S rRNA sequences from the Louisiana mytilid symbiont and selected free-living and symbiotic representatives of the class *Proteobacteria*. Greek letters on the borders indicate alpha, beta, and gamma *Proteobacteria* subdivisions. Bootstrap values for selected nodes which are supported in more than 75 of 100 trees by distance analysis (upper numbers) and parsimony analysis (lower numbers) are shown. The scale bar indicates five nucleotide substitutions per sequence position.

posed of methanotrophic bacteria, including all Type I methanotrophs examined to date (Fig. 3). Type X methanotrophs are also included in this group with a high level of confidence by distance analysis (94 of 100 trees) (Fig. 3). Analyses including unpublished sequences from additional Type I and Type X methanotrophs (2a) and additional methanotrophic and chemoautotrophic symbionts (unpublished data) do not significantly alter tree topologies or bootstrap values.

DISCUSSION

As is the case for all chemoautotrophic symbionts examined to date by molecular phylogenetic methods (10–13), the symbiont populations within the gills of the Louisiana mytilid appear to be composed predominantly, if not exclusively, of a single symbiont type. Direct sequencing of PCR products and screening of cloned PCR products from several mussel specimens support this conclusion. Although the predominance of a single 16S rRNA gene sequence in amplification products could be the result of bias in amplification efficiencies, the results of in situ hybridizations suggest that this is not the case. In situ hybridizations (Fig. 1 and 2) demonstrate that the gene amplified and cloned from the gill of the Louisiana mytilid contains the same target sequences as those found in the ribosomes of the symbiont cells. This confirms that the amplified and cloned sequence originated from the symbiont genome rather than from a superficially associated bacterium or chance contaminant. Essentially identical staining intensities and patterns were observed with both the universal eubacterial probe, which binds with 16S rRNAs of all eubacteria, and the symbiont-specific probes tested. This indicates that the cloned symbiont sequence is sufficiently abundant and appropriately distributed to account for all bacterial probe binding in the gill sections. These results are consistent with electron micrographic evidence showing a single symbiont morphotype in this host species (17).

The results of comparative sequence analyses indicate that the symbiont of the Louisiana mytilid arose from a lineage that is distinct from that (or those) which gave rise to the known chemoautotrophic symbionts of bivalves and tubeworms (Fig. 3). This clearly distinguishes the methanotrophic symbiont from all previously examined symbionts of hydrothermal vent and hydrocarbon seep organisms (10, 11, 13). Instead, these data suggest that the Louisiana mytilid symbiont arose from a

bacterial lineage that also gave rise to Type I and Type X methanotrophs. This indicates that bacterial endosymbiosis has arisen at least twice in the course of the evolution of vent and seep bivalves by the acquisition of symbionts from phylogenetically divergent bacterial lineages. These results do not support the alternative hypothesis that methanotrophic and chemoautotrophic endosymbionts arose from a common ancestral symbiont which may, like the Type X methanotrophs, have contained genes for both methane oxidation and Calvin-Benson cycle carbon fixation. Similarities observed between the two types of symbioses could be the result of evolutionary convergence, with each type of symbiosis having arisen de novo in a separate host species. Alternatively, similarities may be due to common ancestry of the hosts, with one symbiosis having arisen from the other by displacement of an existing symbiont population. Phylogenetic examination of additional symbionts and comparable phylogenetic examination of hosts (work in progress in this laboratory) should help to distinguish between these alternatives.

Although its relationship with Type I methanotrophs is strongly supported, the Louisiana mytilid symbiont is a deeply divergent member of this lineage and may differ considerably from cultivable Type I methanotrophs with respect to its metabolic capabilities and physiological characteristics. In this respect, the methanotrophic symbiont may provide valuable insights into the physiological diversity of Type I methanotrophs. RuBisCO activity in the gill tissue of the Louisiana mussel has been reported (17). This could indicate either the presence of a RuBisCO gene in the mytilid symbiont, as is observed in Type X methanotrophs, or the presence of a second bacterial species in or on the mytilid gills. In light of the data presented here, confirmation of the former possibility would suggest that the presence of RuBisCO genes is the ancestral condition among gamma methanotrophs but that these genes have been lost in modern free-living Type I methanotrophs.

The affiliation of the uncultivable symbiont of the Louisiana mytilid with free-living and cultivable methanotrophs parallels the relationship of the uncultivable luminous symbionts of flashlight fish and anglerfish with cultivable luminescent vibrios and photobacteria (21). It has been proposed that the physiological characteristics which make the fish symbionts difficult to grow in pure culture may be evolutionary adaptations to an obligate symbiotic existence. A similar argument can be made for the mytilid symbiont. Alternatively, the mytilid symbionts may be capable of free-living existence but may enter a reproductively incompetent developmental stage upon infection of the host, as has been suggested for *Rhizobium* root nodule symbionts of leguminous plants (33). Examination of free-living bacteria from the host environment by using symbiont-specific oligonucleotide probes will help to evaluate these alternatives and will provide important insights regarding the ontogeny and evolution of eukaryote-prokaryote symbioses.

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