Proc. Natl. Acad. Sci. USA
Vol. 92, pp. 9598–9602, October 1995
Microbiology

Intracellular coexistence of methano- and thioautotrophic bacteria in a hydrothermal vent mussel

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Communicated by George N. Somero, Oregon State University, Corvallis, OR, July 13, 1995

ABSTRACT **The coexistence of two phylogenetically distinct symbiont species within a single cell, a condition not previously known in any metazoan, is demonstrated in the gills of a Mid-Atlantic Ridge hydrothermal vent mussel (family Mytilidae). Large and small symbiont morphotypes within the gill bacteriocytes are shown to be separate bacterial species by molecular phylogenetic analysis and fluorescent *in situ* hybridization. The two symbiont species are affiliated with thioautotrophic and methanotrophic symbionts previously found in monospecific associations with closely related mytilids from deep-sea hydrothermal vents and hydrocarbon seeps.**

The uptake, maintenance, and regulation of prokaryotic intracellular symbionts is a complex process long recognized as an important source of biological diversity in the evolution of eukaryotic organisms (1). Examples range from the proposed symbiotic origin of mitochondria and chloroplasts to modern symbioses, such as nitrogen-fixing rhizobia in plant-root nodules and chemosynthetic endosymbionts of deep-sea marine invertebrates (for reviews see refs. 2–4). In each case, the symbiont provides the host organism with metabolic pathways not previously associated with the eukaryotic genome. While the intracellular coexistence of multiple endosymbionts is known among single celled eukaryotes (5), this has not been observed in metazoans. In cases where a single metazoan host is known to contain two separate intracellular symbionts—e.g., the S and P symbionts of aphids (6)—the symbionts are found segregated into separate cells or tissues. In fact, with the possible exception of mitochondria and chloroplasts, which are widely thought to have become established as symbionts in ancient single-celled ancestors of modern plants (2), the stable coexistence of more than one bacterial symbiont within a single cell has, to our knowledge, not been demonstrated in a multicellular eukaryote.

Recently morphological and enzymatic data were presented as evidence that two symbiont types coexist in single cells in the gills of several recently described species of mytilid bivalves discovered at deep-sea hydrothermal vents and hydrocarbon seeps (7–9). Previously, mytilid species from these habitats have been shown to obtain the bulk of their nutritional carbon from uncultivable methanotrophic (methane-utilizing) or thioautotrophic (sulfur-oxidizing, CO_2-fixing) bacteria which exist as monocultures within specialized cells (bacteriocytes) of the animals' gill epithelia (8, 10, 11). The recently described mytilid species contain two distinct Gram-negative bacterial morphotypes within individual bacteriocytes: a "large" morphotype (1.5- to 2.0-μm diameter), containing complex intracytoplasmic membranes similar to those observed in type I and type X methanotrophs, and a "small" morphotype (<0.5-μm diameter) lacking internal membranes (7–9) (Fig. 1). Also, enzymes characteristic of both autotrophic and methanotrophic bacteria (ribulose-1,5-bisphosphate carboxylase/oxygenase and methanol dehydrogenase, respectively) are detectable in gill extracts from these species (7–9).

These morphological and enzymatic data, however, are insufficient to demonstrate the presence of two symbiont species in these mytilids. Morphological polymorphism is common within individual species of intracellular symbionts and pathogens (12, 13). In fact, polymorphic symbiont populations have been observed in several thioautotrophic symbioses (10); however, molecular phylogenetic evidence supports the existence of a single symbiont type in all cases examined to date (14–17). Also, both of the enzymes detected in the recently described mytilids are present within individual species of free-living type X methanotrophic bacteria (18), a group shown to be closely related to methanotrophic symbionts of mytilids (19). Thus, these observations could equally likely be explained by the presence of a single polymorphic bacterial species, which, like the type X methanotrophs, contains enzymes common to both methanotrophic and thioautotrophic bacteria. Ultimately, the species composition of these mytilid symbiont populations is a question of symbiont phylogeny rather than physiology or ultrastructure.

Here, phylogenetic analysis and *in situ* hybridization based on 16S rRNA sequences were used to examine the phylogenetic identity of the two symbiont morphotypes populating the bacteriocytes (Fig. 1) of an as yet unnamed mussel species of the family Mytilidae (R. D. Turner, personal communication) collected from active hydrothermal vent sites on the MAR. These results demonstrate the intracellular coexistence of two bacterial species within a single metazoan cell.†

MATERIALS AND METHODS

Organisms. Mytilids (referred to here as MAR mytilids) were collected in 1986 and 1993 by DSRV (deep submergence research vessel) *ALVIN* from an active deep-sea hydrothermal vent (Snakepit site; 23° N, depth, 3476 m) and transferred to the surface in an insulated container. Mytilids dissected aboard the ship were either frozen in liquid nitrogen or fixed for microscopy. Other mytilids were frozen whole at −70°C. Specimens were transferred to the laboratory on dry ice and stored at −80°C.

PCR and Cloning of 16S rRNA Genes. DNA was extracted from the symbiont-containing gill tissue by using guanidinium isothiocyanate lysis medium as described (16). Bacterial 16S rRNA genes were amplified from the symbiont-containing gill tissue of these mytilids by PCR with primers (1492r and 27f) targeted to sequences universally conserved among eubacteria, as described in ref. 19. PCR products from two mytilid specimens were inserted into a plasmid vector (pCR II; Invitrogen) and examined individually. Clones were screened by digestion with the frequently cutting restriction enzyme *Alu* I, grouped by fragment-size pattern, then examined by single

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Abbreviation: MAR, Mid-Atlantic Ridge.
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†The sequences reported in this paper have been deposited in the GenBank data base (accession nos. U29163 and U29164).

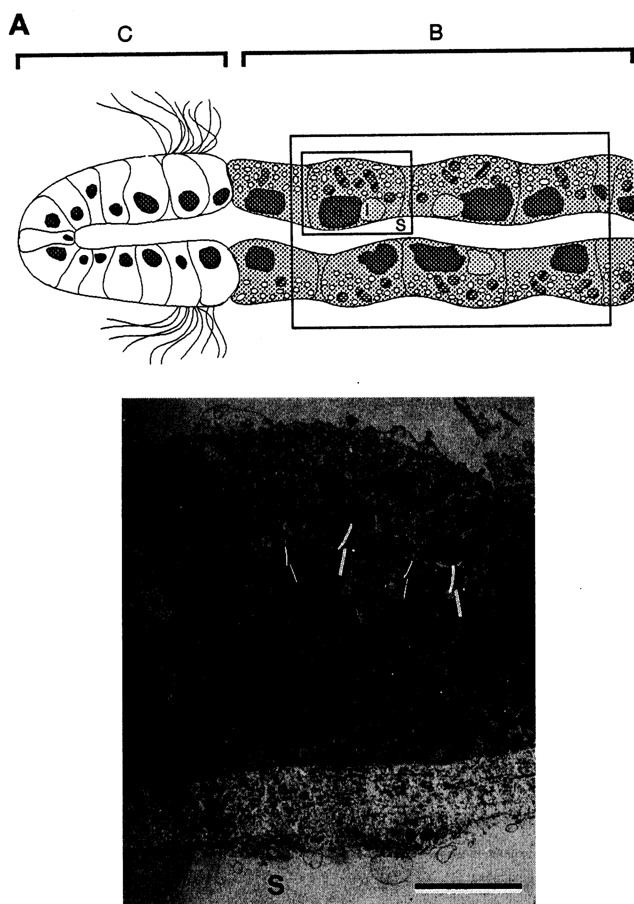

FIG. 1. Transverse section of Mid-Atlantic Ridge (MAR) mytilid gill filament, showing symbiont-containing gill epithelial cells (bacteriocytes). (*A*) Diagram of gill filament. Bacteriocytes are confined to the region proximal to the ciliated border of the gill. Small and large boxes show positions of Figs. 1*B* and 3, respectively. B, symbiont-containing bacteriocyte region; C, symbiont-free ciliated region. (*B*) Transmission electron micrograph. Large and small symbionts (large and small arrows, respectively) are located in the apical region of the cells, while nuclei and lysosomal residual bodies occupy the region closest to the blood sinus. Note centrally stacked intracytoplasmic membranes in large symbionts. (Bar = 5 μm.) l, Lysosomal residual body; n, bacteriocyte nucleus; s, blood sinus.

nucleotide track sequencing (20) by using a universally conserved primer (519r). Sequencing was performed manually as in ref. 19. Fifteen clones were partially sequenced and five were fully sequenced.

Phylogenetic Analyses. Phylogenetic and bootstrap analyses were performed as in ref. 16 by using the programs contained in the PHYLIP 3.4 package (21) implemented through the Genetic Data Environment (GDE) sequence editor (22). The distance programs used were DNADIST with Jukes and Cantor correction, SEQBOOT, and FITCH. The maximum parsimony programs we used were DNAPARS and DNABOOT. Trees were generated with global rearrangement and random sequence addition. Nucleotide positions which were undetermined or of ambiguous identity, insertions and deletions, alignment gaps, and sequence regions that could not be aligned with certainty in one or more taxa were eliminated from consideration. Analyses included 1018 nucleotide positions. Reference sequences were obtained from GenBank (M29021, M29022, M95651, M95656, M95658, M95662, M95665, M96398, X72772, X72775, and X72777) or from the Ribosomal Database Project (23). MAR-1 and MAR-2 consensus sequences are available from GenBank (U29163 and U29164).

***In Situ* Hybridization.** Design, synthesis, and purification of the DNA probes followed the methods described in ref. 19. Probes MAR-P1 (TCGCCACTAAGAGGTAAATCCT) and MAR-P2 (CCGCCACTAAGCCTATAAATAGA) hybridize specifically with *Escherichia coli* positions 838-859 of the MAR-1 and MAR-2 sequences, respectively, and contain at least three differences from all sequences published in the Ribosomal Database Project (23) and GenBank. Negative control probes LA-1 (CCGCCACTAAACCTGTATATAGG, *E. coli* positions 838-859) and SV-1 (CTAATAGCGCGAGGTCCGAA, *E. coli* positions 208-227) hybridize specifically with 16S rRNA target sequences in the Louisiana (Bush Hill) mytilid methanotrophic symbiont (19) and the *Solemya velum* chemoautotrophic symbiont (15) 16S rRNAs, respectively, but contain one and three mismatches to the appropriate target regions in the MAR sequences. Membrane hybridizations were performed to establish probe specificity and stringency conditions as in ref. 24 by using bulk RNA from symbiont-containing gill and symbiont-free foot tissues from the MAR mytilid, Louisiana mytilid, and *S. velum.*

Tissue preparation, fixation, sectioning, and *in situ* hybridization were performed as described (19), with hybridization and wash temperatures of 37°C and 42°C, respectively. Three separate specimens were used for *in situ* hybridization experiments. To achieve enhanced sensitivity, the *in situ* fluorescent signal was amplified by the method of Pinkel *et al.* (25) modified for use with oligonucleotide probes by replacement of the 50% formamide wash with two washes in PN buffer (a mixture of 0.1 M NaH_2PO_4 and 0.1 M Na_2HPO_4 to give pH 8/0.1% Nonidet P-40) at room temperature.

RESULTS AND DISCUSSION

An $\approx$1.5-kb segment representing 95% of the 16S rRNA gene was amplified. Unlike previously examined bivalve species (14–16), PCR products from MAR mytilid gills were heterogeneous and could not be sequenced directly. Thus, PCR products were cloned and examined individually. Only two insert sequences, designated MAR-1 and MAR-2, were identified among the clones from each of the two specimens in ratios of 24:1 and 37:1, respectively. Sequence variation among individual MAR-1 and MAR-2 clones did not exceed that expected due to the nucleotide incorporation error rates (2×10^{-4} misincorporations per nucleotide per cycle) (26) observed for *Taq* DNA polymerase.

Phylogenetic analyses comparing the two cloned sequences and those from a variety of reference bacteria demonstrate that the MAR sequences are derived from members of the gamma-subdivision of the Proteobacteria bacteria and are most closely related to previously examined methanotrophic and thioautotrophic symbionts found in mytilids (Fig. 2). The MAR-1 sequence represents a new species closely related to the thioautotrophic symbiont of the Galapagos hydrothermal vent mytilid, *Bathymodiolus thermophilus* (14). Bootstrap analyses using either evolutionary distance or maximum parsimony methods strongly support the placement of the MAR-1 and the *B. thermophilus* symbionts within a single monophyletic group, which falls within a larger clade containing only thioautotrophic symbionts of bivalves. The same analyses demonstrate that the MAR-2 sequence represents a new bacterial species closely related to the methanotrophic symbiont of an unnamed mytilid species collected from a cold seep site off the coast of Louisiana (19). Bootstrap analyses also show these two sequences form a monophyletic group which falls within a larger clade, composed exclusively of 16S rRNA sequences of free-living and cultivable type I and type X methanotrophic bacteria. These results confirm the close phylogenetic relationship of the MAR sequences and the 16S rRNA sequences of known thioautotrophic and methanotrophic symbionts.

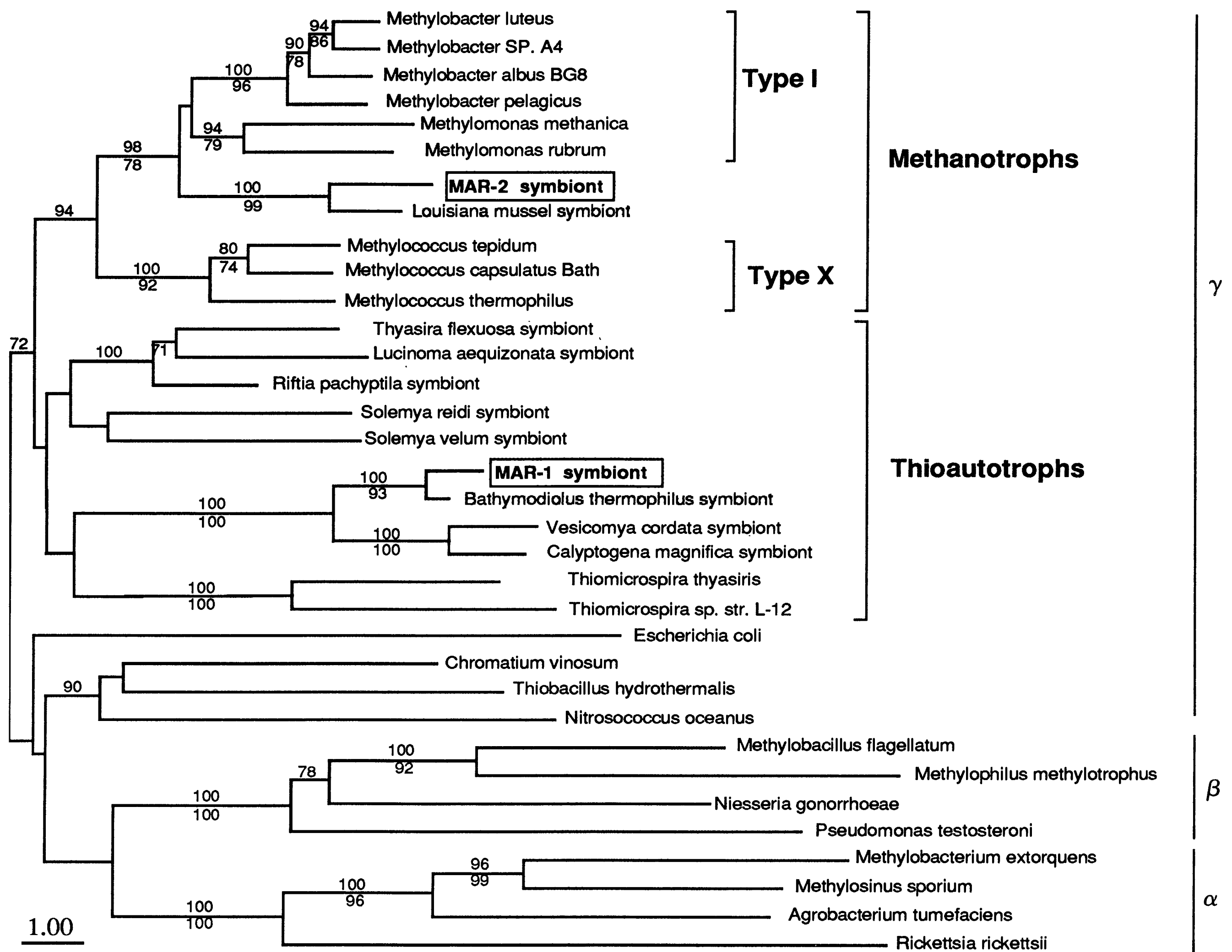

FIG. 2. Evolutionary distance phylogenetic tree based on 16S rRNA sequences of MAR mytilid symbionts and representative Proteobacteria (α, β, and γ subdivisions). MAR mytilid symbionts are highlighted. Parsimony analysis yielded a single shortest tree (not shown) of 1837 steps which is identical in topology to the distance tree shown at all significant nodes. Bootstrap values for nodes supported in greater than 70 of 100 trees by evolutionary distance (upper) and maximum parsimony (lower) analyses are shown. Scale bar = 1 substitution per 100 nucleotide positions.

Fluorescent *in situ* hybridization (FISH) with oligonucleotide probes was used to confirm that the cloned sequences originate from the two symbiont morphotypes rather than from a chance contaminant or surface-associated bacterium. Specific probes, designated MAR-P1 and MAR-P2, were designed to hybridize with unique regions of the RNA-like strand of the MAR sequences. While hybridization of MAR-P1 was readily detectable in tissue sections by using conventional techniques (24, 27, 28), these methods were not sufficiently sensitive to detect hybridization of MAR-P2.

To detect *in situ* probe hybridization with enhanced sensitivity, three successive rounds of alternating incubation with fluorescein-avidin and biotinylated anti-avidin Fab were used to construct a multilayered "sandwich" of fluor and antibody at the binding site of a biotinylated probe (25). This "FISH sandwich" method allowed the hybridization of both probe MAR-P1 and MAR-P2 to be detected in the symbiont-containing apical region of the gill bacteriocytes (Fig. 3). The patterns of hybridization observed with the two probes are distinctly different: probe MAR-P2 hybridizes with sparsely distributed spherical to ovoid structures, $\approx$1.5–2 μm in diameter (Fig. 3*A*), while probe MAR-P1 hybridizes with structures that are small, numerous, and evenly distributed throughout the apical region (Fig. 3*B*). These patterns are consistent with the size, shape, relative abundance, and distribution of the large and small symbiont morphotypes, respectively. No detectable hybridization was observed with the negative control probes (Fig. 3*D*), while a positive control, complementary to a region conserved in all eubacterial 16S rRNAs (Fig. 3*C*), stained in a pattern consistent with hybridization to both large and small morphotypes. These results clearly demonstrate that the two morphotypes observed in the MAR mytilid bacteriocytes are phylogenetically distinct bacterial species.

Although 16S rRNA phylogeny alone often fails to predict physiological or metabolic capability, two observations strongly suggest that these two symbiont species are thioautotrophs and methanotrophs, respectively: (*i*) the two symbionts are most closely related to thioautotrophic and methanotrophic symbionts found in monospecific associations with closely related mytilid species (16, 19, 29), and (*ii*) these symbionts fall within separate clades composed exclusively of either thioautotrophic or methanotrophic bacteria. This dual symbiont population, therefore, appears to provide the symbiosis with the unique capability of utilizing either reduced sulfur compounds or methane as a source of metabolic energy and either carbon dioxide or methane as a primary source of biomass carbon. Presumably, these carbon and energy pathways, which are normally unavailable to animals, afford this symbiosis extraordinary versatility to thrive in otherwise hostile environments.

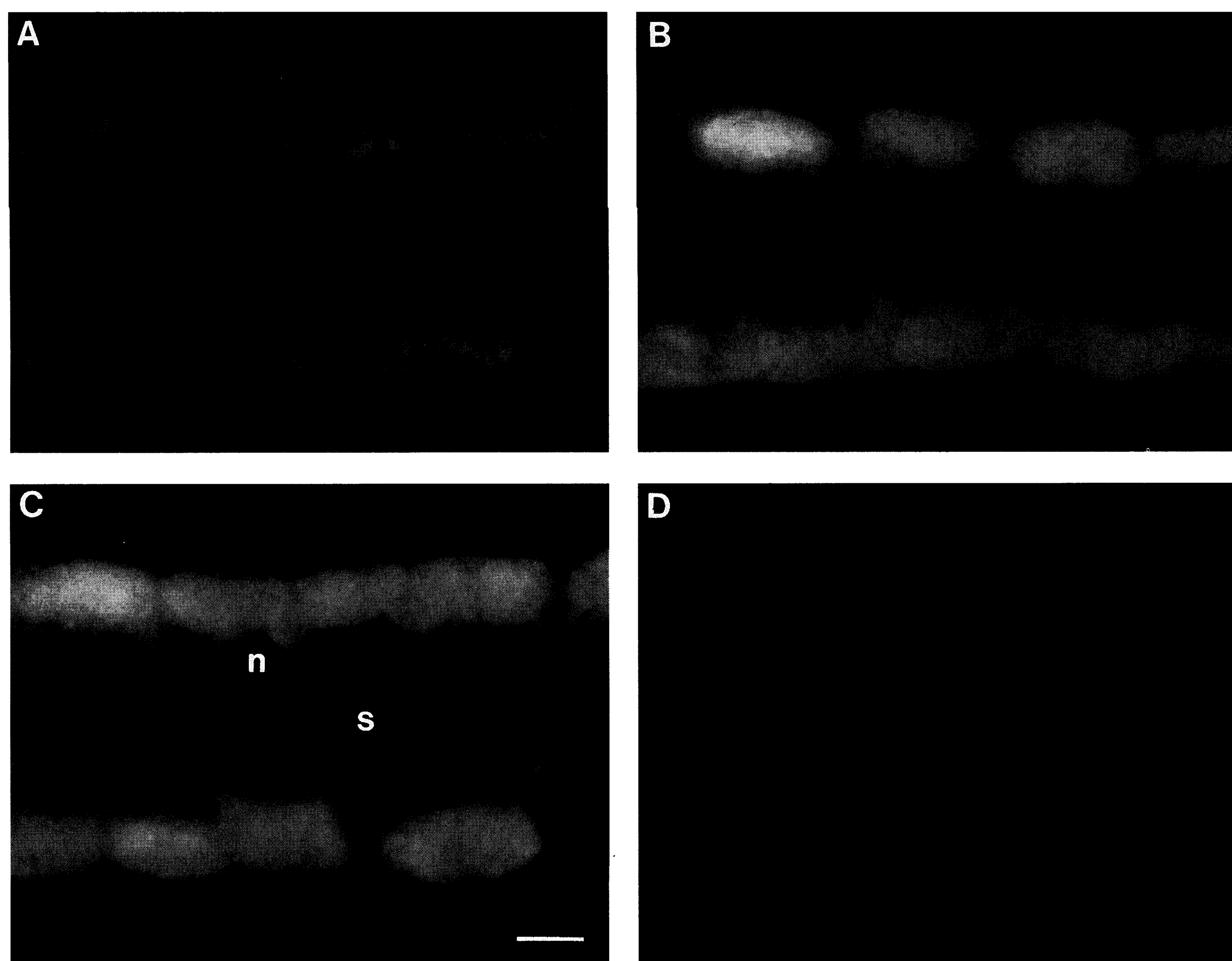

FIG. 3. The large and small MAR mytilid symbionts are phylogenetically distinct bacterial species. Epifluorescence micrographs of consecutive transverse sections of MAR mytilid gill tissue show *in situ* hybridization of the following fluorescein-labeled oligonucleotides complementary to 16S rRNA sequences: symbiont-specific probes MAR-P2 (*A*) and MAR-P1 (*B*), universal eubacterial probe (*C*), and negative control probe LA-1 (*D*). MAR-P1 and MAR-P2 are specific for the phylogenetically thioautotroph-like MAR-1 and methanotroph-like MAR-2 16S rRNA sequences cloned from the MAR mytilid. n, Bacteriocyte nucleus; s, blood sinus. (Bar = 10 μm.)

Fluorescent and electron micrographic observations, as well as the relative abundance of clones recovered from gill tissues, support the conclusion that the thioautotrophic symbiont type is considerably more abundant in the MAR mussel specimens examined. Additionally, the thioautotrophic symbionts stain with considerably greater intensity when using ribosome-specific probes (Fig. 3 *A* and *B*), indicating a higher rRNA content and, therefore, a higher ribosome copy number per cell (30). Taken together, these observations suggest a higher growth rate for the thioautotrophic symbionts in these specimens. While this is consistent with the relative abundance of sulfide in comparison with methane observed in MAR vent effluents (up to 5.9 mM and 100 μM, respectively, in end-member fluids) (31, 32), it is not yet established that these observations indicate greater production by thioautotrophic symbionts or greater dependence of this symbiosis on thioautotrophically fixed carbon. Although stable carbon isotope (δ^{13}C) ratios for the MAR mytilid tissues are typical of thioautotroph–bivalve symbioses (8), these results may be coincidental, since d^{13}C values for source methane have not yet been determined for the MAR hydrothermal vent site. Currently, we are examining other "dual-symbiont" mytilids from sites with different ambient methane and sulfide concentrations to resolve whether symbiont population composition is genetically predetermined in an individual host species or is a response to environmental conditions.

As observed in other families of symbiont-bearing bivalves (16), each mytilid host species draws its specific symbionts from monophyletic bacterial groups specifically associated with the host family (16). Such cospeciation of host and symbionts is an indication of the antiquity of these symbioses. Unlike other bivalves, however, the mytilids are associated with two, rather than one, monophyletic bacterial group. While the order in which these two bacterial groups became established as mytilid symbionts is not yet known, comparative analyses of host and symbiont phylogeny will help to determine the combination of acquisitions and losses necessary to explain the modern existence of mytilid species which contain one or both symbiont types.

It is not clear why the stable intracellular coexistence of multiple symbiont species has evolved and persisted in the MAR hydrothermal vent mytilid or why this unusual condition has not yet been observed in other metazoans. Competition between symbionts for intracellular space and resources; the increased evolutionary cost of developing recognition, regulation, maintenance, and transmission mechanisms for two symbionts; and the dependence of the system on the survival of three separate organisms are factors which may limit the success of multiple intracellular symbioses. Distinct physiological roles for these two symbiont species, however, may be an important feature in their successful co-colonization of the MAR mytilid bacteriocytes. Niche differentiation by physio-

logically distinct bacteria within the host cells may minimize intersymbiont competition while providing the host with greater flexibility to exploit carbon and energy sources in the environment. Prior association of these two bacteria may also have been a factor. Methanotrophic bacterial species commonly occur in consortia with bacteria able to metabolize their toxic metabolic end products (33). Thus, co-colonization may have arisen through the uptake of a preexisting bacterial consortium. In such a "symbiosis within a symbiosis," physiological interaction between the two symbionts may be as significant as the interaction of either symbiont with its host.

We thank the R/V *Atlantis II* and DSRV *ALVIN* crews for their skillful help in obtaining samples, M. F. Polz and J. J. Robinson for fixing specimens, J. P. Bowman, L. I. Sly, and E. Stackebrandt for prepublication sequences, and L. A. Claussen for manuscript preparation. This work was supported by grants from the Office of Naval Research and the National Science Foundation to C.M.C., which we gratefully acknowledge.

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