Title

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Journal

Journal of Bacteriology, 174(10)

ISSN

0021-9193

Authors

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Publication Date

1992-05-01

DOI

10.1128/jb.174.10.3416-3421.1992

Peer reviewed

JOURNAL OF BACTERIOLOGY, May 1992, p. 3416–3421
0021-9193/92/103416-06$02.00/0

Vol. 174, No. 10

Phylogenetic Relationships of Chemoautotrophic Bacterial Symbionts of *Solemya velum* Say (Mollusca: Bivalvia) Determined by 16S rRNA Gene Sequence Analysis

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Received 4 November 1991/Accepted 9 March 1992

The protobranch bivalve *Solemya velum* Say (Mollusca: Bivalvia) houses chemoautotrophic symbionts intracellularly within its gills. These symbionts were characterized through sequencing of polymerase chain reaction-amplified 16S rRNA coding regions and hybridization of an *Escherichia coli* gene probe to *S. velum* genomic DNA restriction fragments. The symbionts appeared to have only one copy of the 16S rRNA gene. The lack of variability in the 16S sequence and hybridization patterns within and between individual *S. velum* organisms suggested that one species of symbiont is dominant within and specific for this host species. Phylogenetic analysis of the 16S sequences of the symbionts indicates that they lie within the chemoautotrophic cluster of the gamma subdivision of the eubacterial group *Proteobacteria*.

Procaryote-eucaryote associations in which marine invertebrates harbor chemoautotrophic bacteria as endosymbionts appear to be widespread in marine habitats such as deep-sea hydrothermal vents and coastal sediments (8, 15). In such symbioses, the procaryotes utilize the energy released by the oxidation of reduced inorganic substrates, such as hydrogen sulfide, to fix carbon dioxide via the Calvin-Benson cycle (7, 13). The hosts appear to derive nutrition from their endosymbionts and in turn provide the symbionts simultaneous access to the substrates from anoxic and oxic environments which are necessary for energy generation. Maintenance of such intracellular symbionts presents a novel metazoan acquisition of procaryotic energy generation and autotrophic carbon fixation.

While the existence of chemoautotroph-invertebrate symbioses is now generally accepted, little is actually known about the symbionts observed in the tissues of any of the hosts because none have been cultured. Comparison of rRNA sequences has greatly facilitated the identification of bacteria, including unculturable microorganisms, and the elucidation of their natural relationships (38). Phylogenetic analysis of 16S rRNA sequences enabled Distel et al. (12) to establish that the chemoautotrophic symbionts of the hydrothermal vent tubeworm and five species of bivalves of the subclass Lamellibranchia are related and cluster in the gamma subdivision of the *Proteobacteria* (formerly purple photosynthetic bacteria), one of the 11 major groups of eubacteria (30).

In this investigation we sought to establish the phylogenetic relationships and the species specificities of the symbionts of the protobranch bivalve *Solemya velum* Say, an Atlantic coast clam which has been studied as a shallow-water model of invertebrate-chemoautotroph associations (7, 9, 10). The phylogenetic placement of the *S. velum* symbionts, to date limited to sequence analysis of the 5S rRNA, indicates that these symbionts also fall in the *Proteobacteria* gamma subdivision (31). However, the small size of the 5S rRNA molecule (~120 bp) precludes resolution that can be attained with larger molecules such as 16S rRNA (~1,550 bp) (16). Species of the genus *Solemya* are, to date, the only bivalves of the subclass Protobranchia in which chemoautotrophic symbiosis has been documented. The protobranchs represent an important component of studies of chemoautotrophic symbioses, since they may be the closest living group to the ancestral bivalve condition, because they dominate the deep sea and are present along a gradient from the deep sea bottom to the shore (1).

PCR amplification. We used the polymerase chain reaction (PCR) (28) to amplify 16S rRNA coding regions from a mixture of procaryotic and eucaryotic DNA extracted from the symbiont-containing gills of *S. velum*. *S. velum* were collected from eelgrass beds near Woods Hole, Mass., and placed in filtered (passed through filters with a pore size of 0.2 μm) seawater to cleanse body surfaces prior to dissection. The gills, which contain ~10^9 bacterial symbionts per g (wet weight), and feet, in which symbionts have not been observed (7), were dissected, frozen in liquid nitrogen, and stored at −85°C. Frozen tissue was homogenized in lysis buffer, and DNA was isolated by using hexadecyltrimethylammonium bromide (4). DNA from *Escherichia coli* JM109, prepared by the miniprep method (4), was used as a positive control.

Amplification of 16S rRNA genes by PCR was carried out essentially by the method of Weisburg et al. (34) using eubacterial universal primers and 200 ng of template DNA. DNA products (Fig. 1) amplified from *S. velum* gill tissue (lane 1) and from the positive-control *E. coli* (lane 4) were prominent single bands of approximately 1,500 bp. Amplification was not detected when DNA template was not added (lane 2), nor when DNA from *S. velum* foot tissue was used as the template (lane 3).

The strong amplification from gill tissue DNA and lack of amplification from foot tissue DNA (Fig. 1) supports the conclusions from studies of enzyme activity, electron microscopy (9), and 5S rRNA sequences (31) that the bacteria are abundant within, and specific to, the gill tissue. This conclusion was further supported by lack of hybridization of

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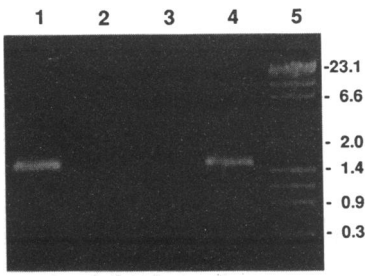

FIG. 1. PCR-mediated amplification of the 16S rRNA coding regions of the *S. velum* symbionts. DNA samples from symbiont-containing *S. velum* gill tissue (lane 1), *S. velum* foot tissue (lane 2), and *E. coli* (lane 4) as well as negative controls with no DNA template (lane 3) were submitted to PCR amplification primed by oligonucleotides complementary to conserved regions at opposite ends of the bacterial 16S rRNA gene (see "PCR amplification" section). Products were separated by electrophoresis in 1% agarose and stained with ethidium bromide. The sizes (in kilobases) of some of the DNA molecular weight standards (lane 5) are indicated to the right.

a 16S probe to genomic digests of foot DNA (data not shown).

16S rRNA gene sequence. Amplified DNA, purified using low-melting-point agarose (29), was sequenced directly using synthetic oligonucleotide primers (19, 36) and the U.S. Biochemical Sequenase Kit with glycerol (34% final concentration) added to the DNA denaturation step (37). Both strands of the amplified 16S rRNA gene were sequenced completely for one animal, a total of 1,460 bp. The 16S genes amplified from two other individual clams were partially sequenced (nucleotides 102 to 306, with numbers corresponding to those for *E. coli* [6]). The sequences of all three animals were identical in this region, which includes both variable and conserved portions of the molecule. Variability in the 16S rRNA sequences of symbionts from an individual, which would show up as either ambiguous sites or sites with high background, was not observed for any of the animals, indicating that only one bacterial rDNA was amplified. These results, coupled with the finding of identical partial sequence in three animals, indicated that the 16S rRNA gene was amplified from the *S. velum* symbionts and not from a free-living surface contaminant.

Southern blot and hybridization analysis. Restriction enzyme analysis of *S. velum* genomic DNA was conducted for three reasons: (i) to confirm that the 16S rRNA gene amplified was that of the gill endosymbionts, (ii) to examine restriction site variability between individual *S. velum* and (iii) to determine the number of rRNA gene copies in the symbiont genome. Enzymes were chosen on the basis of a restriction map predicted from the sequence of the 16S rRNA gene amplified from *S. velum* gills (Fig. 2). Restriction

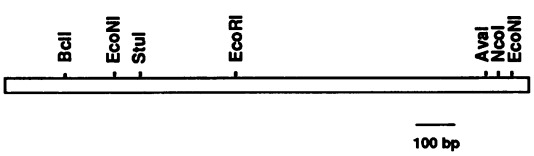

FIG. 2. Restriction map of *S. velum* symbiont 16S rRNA coding region based on the sequence of 16S rDNA (1,460 bp) amplified from *S. velum* gill tissue. Additional enzymes used in analyses predicted not to restrict the gene included *Bgl*II, *Pvu*II, and *Xho*I.

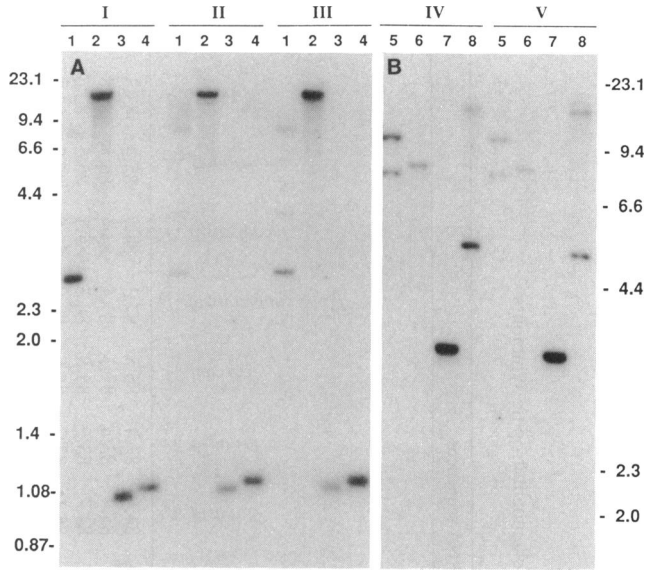

FIG. 3. Southern blot hybridization of restricted *S. velum* symbiont-containing gill genomic DNA with an *E. coli* 16S rRNA gene probe. DNA from different animals (I to V) was digested with individual or pairs of restriction enzymes and separated by electrophoresis on 1.3% (A) and 0.8% (B) agarose gels. Lanes: 1, *Ava*I; 2, *Bcl*I; 3, *Ava*I plus *Bcl*I; 4, *Eco*NI; 5, *Eco*RI; 6, *Xho*I; 7, *Pvu*II; 8, *Bgl*II. Enzymes were selected on the basis of the predicted restriction map of symbiont 16S rRNA coding regions (Fig. 2). The high-molecular-weight bands in *Ava*I and *Bgl*II digests are a result of partial digestion (data not shown). Numbers to the sides of the blots indicate molecular size standards (in kilobases).

fragment patterns resulting from this set of enzymes were unique to the sequence of the amplified gene, as determined by a search of all sequences of the 16S rRNA data base (24) and of the other chemoautotrophic symbionts (12).

Genomic DNA (5 μg) was digested with restriction enzymes according to the manufacturer's instructions. Gill tissue DNA from nine different animals was cut with three to nine enzymes, with at least three animals used for each enzyme. Southern blots were performed by the method of Maniatis et al. (21). The 16S rRNA gene of *E. coli*, amplified using PCR (see above), was used as a probe. The probe was radioactively labelled by using the Random Priming Labeling Kit (Bethesda Research Laboratory) and [α-^{32}P]dATP (6,000 Ci/mmol; New England Nuclear) according to the supplier's instructions and purified using a Sephadex G50 column (21). Blots were hybridized at 68°C and washed at 65°C in a solution of 0.1× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and 0.1% sodium dodecyl sulfate. *E. coli* genomic DNA, cut with *Pst*I, was used as a positive control producing expected rRNA gene fragment patterns as described (5) (data not shown).

The use of PCR of such a highly conserved bacterial gene with universal eubacterial primers (34) made it necessary to determine that the gene amplified was that of the endosymbionts and not other bacteria such as free-living surface contaminants. The fragment sizes produced by probing genomic *S. velum* DNA with *E. coli* 16S rDNA exactly matched those predicted by the sequence of the PCR product (Fig. 2 and 3). This suggested that the gene amplified was dominant in the *S. velum* gills. For example, enzymes predicted to cut twice within the gene produced the pre-

TABLE 1. Percent similarity and evolutionary distance matrix of 16S rRNA sequences of chemoautotrophic symbionts and selected free-living and endosymbiotic bacteria[a]

Organism	% Similarity[b]/evolutionary distance[c] of 16S rRNA sequences																			
	L. aequizonata sym	*L. annulata* sym	*C. orbicularis* sym	*R. pachyptila* sym	*S. velum* sym	*B. thermophilus* sym	*C. magnifica* sym	*Thiomicrospira* sp. strain L-12	*T. nivea*	*L. pneumoniae*	*C. burnetii*	*E. coli*	*A. pisum* S	*A. pisum* P	*V. harveyi*	*T. ferrooxidans* M612	*P. testosteroni*	*N. gonorrhoeae*	*R. rickettsii*	*A. tumefaciens*
Lucinoma aequizonata sym		98.0	96.6	95.6	92.8	90.6	88.9	89.9	90.6	90.2	91.8	84.7	84.6	83.4	86.5	87.3	84.7	85.4	83.3	86.8
Lucinoma annulata sym	2.1		96.7	95.8	92.9	91.5	89.5	89.5	90.2	90.0	90.8	85.0	85.4	83.4	87.1	87.4	84.4	86.5	82.8	87.1
Codakia orbicularis sym	3.5	3.3		96.5	94.0	90.3	89.2	89.7	90.9	90.6	91.7	84.9	84.7	83.5	85.8	88.3	85.1	86.5	82.6	87.0
Riftia pachyptila sym	4.5	4.3	3.6		93.5	90.2	89.5	90.2	90.1	90.1	91.1	85.4	85.1	83.7	86.6	88.0	86.9	86.9	83.8	88.3
Solemya velum sym	7.6	7.4	6.2	6.8		88.7	87.7	88.9	90.7	90.9	90.9	85.4	85.2	84.4	86.7	87.5	85.5	86.5	84.3	86.0
Bathymodiolus thermophilus sym	10.1	9.0	10.4	10.5	12.3		94.9	89.3	88.4	87.7	88.5	85.4	85.2	84.2	86.9	84.9	82.9	84.4	83.0	84.5
Calyptogena magnifica sym	12.0	11.3	11.6	11.3	13.4	5.2		87.1	88.4	87.6	88.2	84.0	84.7	83.5	85.0	83.1	82.1	83.7	83.4	84.5
Thiomicrospira sp. strain L-12	10.9	11.4	11.0	10.6	12.0	11.5	14.1		86.3	87.7	87.8	84.7	84.2	83.7	85.9	84.8	84.2	84.4	81.1	84.1
Thiothrix nivea	10.0	10.5	9.7	10.6	9.9	12.6	12.6	15.1		90.1	89.9	86.5	86.2	84.4	86.5	86.9	82.7	85.8	83.8	85.9
Legionella pneumophila	10.5	10.7	10.0	10.7	9.7	13.4	13.6	13.5	10.7		91.8	86.2	86.5	84.7	88.1	88.0	83.8	84.8	84.3	85.6
Coxiella burnetii	8.7	9.8	8.8	9.4	9.7	12.5	12.8	13.3	10.8	8.7		85.2	85.0	84.6	86.1	87.6	85.2	85.4	84.3	86.3
Escherichia coli	17.1	16.7	16.9	16.2	16.2	16.3	17.9	17.1	14.9	15.2	16.4		96.6	91.7	92.5	86.8	82.4	83.9	81.2	82.8
Acyrthosiophon pisum S	17.2	16.2	17.1	16.5	16.5	16.4	17.1	17.8	15.2	14.9	16.8	3.5		89.5	92.0	85.1	81.2	84.2	81.6	83.4
Acyrthosiophon pisum P	18.8	18.8	18.6	18.4	17.5	17.8	18.6	18.3	17.5	17.1	17.3	8.8	11.3		87.3	83.5	80.6	81.3	81.2	81.1
Vibrio harveyi	14.9	14.2	15.7	14.7	14.7	14.4	15.6	15.6	14.9	12.9	15.4	7.9	8.5	13.9		85.5	82.9	82.6	82.0	82.5
Thiobacillus ferrooxidans M612	13.9	13.7	12.7	13.0	13.7	16.8	19.1	17.0	14.4	13.0	13.6	14.5	16.6	18.6	16.1		85.5	85.5	83.5	84.2
Pseudomonas testosteroni	17.1	17.5	16.5	14.4	16.1	19.4	20.5	17.7	19.7	18.3	16.4	20.1	21.7	22.4	19.4	16.1		87.5	81.7	81.8
Neisseria gonorrhoeae	16.3	14.9	14.9	14.4	14.9	17.5	18.4	17.5	15.7	16.9	16.3	18.1	17.8	21.6	19.8	16.1	13.7		81.8	85.1
Rickettsia rickettsii	18.8	19.6	19.9	18.3	17.6	19.3	18.7	21.8	18.3	17.6	17.6	21.7	21.1	21.7	20.6	18.7	21.0	20.8		87.5
Agrobacterium tumefaciens	14.5	14.1	14.3	12.8	15.4	17.4	17.4	17.9	15.6	15.9	15.1	19.6	18.7	21.7	20.0	17.7	20.8	16.6	13.7	

[a] Portions of the 16S rRNA molecules that were not included in analysis included all sites for which nucleotides were unavailable or ambiguous in any of the organisms and regions that were difficult to align or highly variable. The analysis was restricted to *E. coli* nucleotide positions (6) 219 to 334 (but not 226, 234, 262, 292, 312, 313, and 329), 337, 339 to 419 (but not 388 and 411), 422 to 450 (but not 437), 481 to 484, 486 to 501, 664 to 835 (but not 718, 720, 721, 732, and 733), 851 to 856, 858, 863 to 875, 877, 1047 to 1133 (but not 1049, 1051, 1066, 1071, 1125, and 1129), 1139 to 1182, 1184 to 1205, 1209 to 1376 (but not 1261, 1265, and 1295). The sources for sequences are as follows: *S. velum* symbiont (sym) (this study); all other chemoautotrophic symbionts, *Thiomicrospira* sp. strain L-12, and T. *nivea* (12); *A. pisum* primary (P) and secondary (S) symbionts (33); *C. burnetii* and *R. rickettsii* (35); *L. pneumophila* (24); *E. coli* (6); *A. tumefaciens* and *P. testosteroni* (39); *N. gonorrhoeae* (25); and *V. harveyi* (26; GenBank accession no. M58172).

[b] Percent similarity matrix shown in the upper right-hand triangle. Percent similarity (S) was calculated by the method of Olsen (23) as follows: $S = [M/(M + U + G/2)] \times 100$, where M is the number of compared positions that are identical, U is the number of positions that are different, and G is the number of positions for which one organism has a base pair and the other organism does not.

[c] Evolutionary distance matrix is shown in the lower left-hand triangle. Pairwise evolutionary distance (D), which is an estimation of mutations per nucleotide position, was computed from percent similarity, using a method which partially corrects for multiple and back mutations: $D = 3/4 \ln [(4S - 1)/3]$ (17).

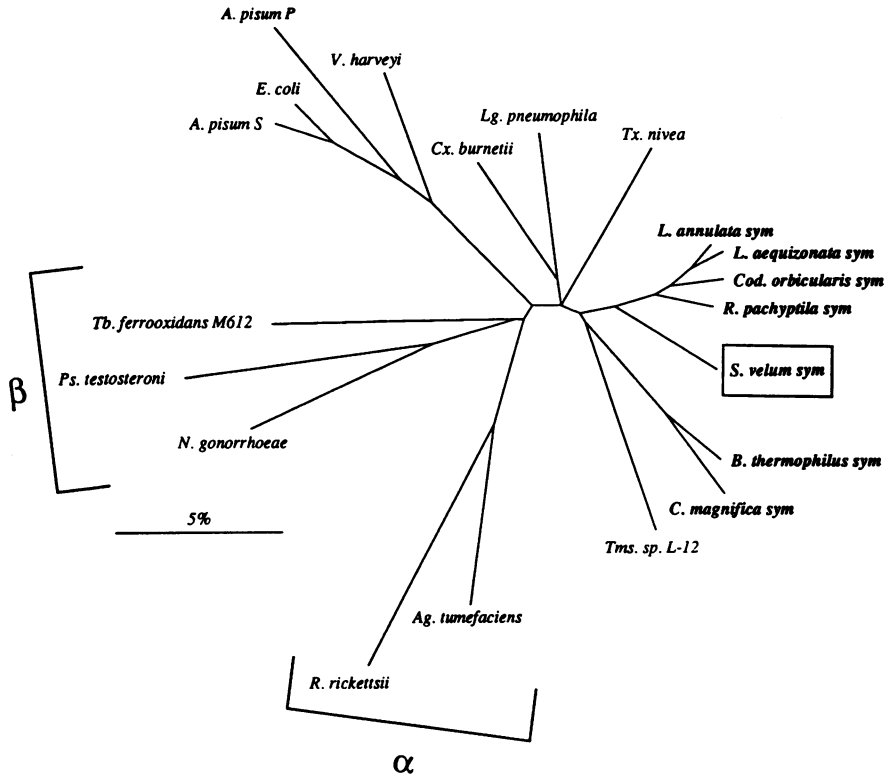

FIG. 4. Unrooted phylogenetic tree showing the position of the *S. velum* symbionts in relation to that of other *Proteobacteria* species on the basis of 16S rRNA gene sequences. The tree was constructed from evolutionary distances in Table 1. Members of the alpha and beta subclasses of the *Proteobacteria* are bracketed; all others are of the gamma subclass. Chemoautotrophic symbionts (sym) are listed in boldface type. Full species names listed in Table 1. Scale bar represents percent similarity.

dicted size bands for *S. velum* genomic DNA (Fig. 3A): *Ava*I and *Bcl*I, 1,080 bp; *Eco*NI, 1,109 bp; and *Nco*I and *Stu*I, 998 bp (data not shown). We suggest that this technique is generally useful for the confirmation of the presence of PCR-generated sequences in cells with multiple types of DNA.

The restriction patterns of 16S rRNA coding regions for DNA extracted from *S. velum* gills were identical for all nine clams examined; representative results are shown in Fig. 3. This, along with the lack of variability in the partial sequence of 16S rDNA for three individuals, suggests that there is a single dominant bacterial species within *S. velum* and that the host-symbiont association is species specific. This result is in agreement with the findings of Distel et al. (12) for lamellibranch bivalve and tubeworm chemoautotrophic symbionts.

Single bands were evident for all enzymes predicted to cut outside or near the ends of the gene such as *Ava*I, *Bcl*I, *Eco*NI, *Pvu*II, *Xho*I (Fig. 3), and *Nco*I (band size, 9,600 bp; data not shown). Some of these enzymes generated restriction fragments larger than that of a typical bacterial ribosomal operon (which includes the 5S, 16S, and 23S rRNA genes [~5 kb]), indicating that the single bands observed were not generated by double cuts within multiple operons. Furthermore, only two bands were observed for enzymes predicted to cut near the middle of the 16S rRNA gene such as *Eco*RI (Fig. 3B) and *Stu*I (bands of 4,400 and 19,500 bp; data not shown). Thus, all enzymes in all animals generated patterns consistent with the presence of only one copy of the 16S rRNA gene in the symbiont genome (Fig. 3). However, it should be noted that a large duplication of the region containing the rRNA operon with no subsequent changes at any of the nine restriction sites could escape detection by this analysis.

These results suggest that the symbiont genome contains but a single rRNA operon. Bacterial rRNA operons (*rrn*), which include the 5S, 16S, and 23S rRNA genes, vary considerably in number among bacteria. In contrast to free-living species of *Proteobacteria*, which have 4 to 7 *rrn* loci (18), only one copy has been detected in other endosymbionts including both the primary (P) and secondary (S) symbionts of the pea aphid, *Acyrthosiphon pisum* (33) (included in Fig. 4). Multiple rRNA operons have generally been thought necessary to support a high rate of rRNA synthesis in rapidly dividing cells (3, 22). Unterman and Baumann (32) suggested that the aphid symbionts therefore grow slowly, with doubling times of 2 days to parallel the growth rate of the aphid host. They further speculated that the single rRNA operon in the aphid symbiont genome is a consequence of the adaptation to a symbiotic existence, which necessitates a slow growth rate. Although the division rate of *S. velum* symbionts is not known, it is unlikely that they grow slowly, since they must produce all of the biomass for their invertebrate host. Studies of *rrn* copy number and growth rates of endosymbionts and their free-living relatives from a variety of phylogenetic groups may help resolve the significance of rRNA operon redundancy.

Phylogenetic analysis of the *S. velum* symbionts. Phylogenetic analysis was conducted using the Genetic Data Environment program (Steve Smith, Harvard Genome Laborato-

ry). Homologous nucleotide positions of 16S rRNA sequences of selected bacteria were aligned with that of the *S. velum* symbiont using conserved regions of sequence and secondary structure as a guide. Percent similarities and evolutionary distances of 16S rRNA for *S. velum* and selected free-living and symbiotic bacteria are shown in Table 1. Phylogenetic trees were constructed from evolutionary distances by the least-squares method of De Soete (11) and by parsimony analysis with bootstrapping (14), using the Treetool computer program (20).

A phylogenetic tree based on evolutionary distances (Fig. 4) indicates that the symbionts fall within the gamma subdivision of the *Proteobacteria* (30) and is consistent with earlier results based on comparison of 5S rRNA (31). This topology is also supported by parisimony analysis with bootstrapping. The 16S rRNA gene sequence indicates that the *S. velum* symbionts fall between the two branches of the chemoautotrophic symbiont cluster (Fig. 4). One branch includes the vent tubeworm *Riftia pachyptila*, and three lucinid clams, *Lucinoma annulata*, *Lucinoma aequizonata*, and *Codakia orbicularis*, and the other branch includes the symbionts of the vent clam *Calyptogena magnifica*, the vent mussel *Bathymodiolus thermophilus*, and the free-living chemoautotroph *Thiomicrospira* sp. strain L-12. Alterations of the positions analyzed and variations in order of sequence addition always resulted in the same topology of branch points of the chemoautotrophic symbionts. While the *Proteobacteria* class includes many species found in association with eucaryotes, including both parasitic and mutualistic symbionts of plants (e.g., agrobacteria and rhizobia) and animals (e.g., enteric organisms and rickettsiae) (30), the positions of *S. velum* symbionts in the tree supports the conclusion of Distel et al. (12) that the chemoautotrophic symbionts are from an evolutionarily distinct group of bacteria.

While the symbionts as a whole appear to have a common origin, the evolution within the cluster is complex. Distel et al. (12) suggested that the symbiosis has evolved separately in many host lineages, because the symbiont trees do not parallel the host classifications which are, unfortunately, not based on explicit phylogenies. The evolutionary separation between the bivalve subclasses Lamellibranchia and Protobranchia, which are considered to be closest to the ancestral bivalve condition, is well characterized morphologically and in the fossil record (2, 27). If lamellibranchs truly arose from protobranchs, then the topology of this tree placing the symbionts of a protobranch between two branches of lamellibranchs (Fig. 4) lends strong support to the idea of multiple origins of the symbioses. Further studies of host invertebrate evolutionary histories are needed so that the phylogenetic relationships of symbionts may be compared with those of their hosts, enabling us to resolve the possibility of polyphyletic origin of chemoautotrophic symbioses. In addition, future characterization of the 16S rRNA of symbionts from several species within a given genus such as *Solemya*, will help determine whether the symbiosis has evolved a single time within a particular lineage, as suggested for the lucinid bivalves (12).

Nucleotide sequence accession number. The 16S rRNA sequence reported here has been submitted to GenBank and given accession number M90415.

We thank Dave Lane and Will Weisburg for providing PCR primers and advice; Sabrina Drill and Michael Eisen for skilled technical assistance; Rob Dorit, Barbara Bowen, and Adam Marsh for helpful advice and discussion; and Wally Gilbert for the use of equipment.

This work was supported by National Science Foundation grant DCB 8718799.

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