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Phylogenetic Diversity of Bacterial Symbionts of *Solemya* Hosts Based on Comparative Sequence Analysis of 16S rRNA Genes

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The bacterial endosymbionts of two species of the bivalve genus *Solemya* from the Pacific Ocean, *Solemya terraeregina* and *Solemya pusilla*, were characterized. Prokaryotic cells resembling gram-negative bacteria were observed in the gills of both host species by transmission electron microscopy. The ultrastructure of the symbiosis in both host species is remarkably similar to that of all previously described *Solemya* spp. By using sequence data from 16S rRNA, the identity and evolutionary origins of the *S. terraeregina* and *S. pusilla* symbionts were also determined. Direct sequencing of PCR-amplified products from host gill DNA with primers specific for *Bacteria* 16S rRNA genes gave a single, unambiguous sequence for each of the two symbiont species. In situ hybridization with symbiont-specific oligonucleotide probes confirmed that these gene sequences belong to the bacteria residing in the hosts gills. Phylogenetic analyses of the 16S rRNA gene sequences by both distance and parsimony methods identify the *S. terraeregina* and *S. pusilla* symbionts as members of the γ subdivision of the *Proteobacteria*. In contrast to symbionts of other bivalve families, which appear to be monophyletic, the *S. terraeregina* and *S. pusilla* symbionts share a more recent common ancestry with bacteria associating endosymbiotically with bivalves of the superfamily Lucinacea than with other *Solemya* symbionts (host species *S. velum*, *S. occidentalis*, and *S. reidi*). Overall, the 16S rRNA gene sequence data suggest that the symbionts of *Solemya* hosts represent at least two distinct bacterial lineages within the γ-*Proteobacteria*. While it is increasingly clear that all extant species of *Solemya* live in symbiosis with specific bacteria, the associations appear to have multiple evolutionary origins.

In the wake of discovering the extent to which chemoautotrophic symbioses occur in nature—more than 200 species representing six different host animal phyla have been described—has come the challenge to study and understand these organisms without the ability to culture or manipulate their bacterial partners outside of their natural habitats. Numerous investigators have followed the lead of C. Woese, N. Pace, and their associates (12, 43) and used the phylogenetic framework provided by rRNA gene sequences (19, 32) to characterize the bacteria involved in many chemoautotrophic associations (for a review, see reference 5). We have used this approach to study the evolutionary relationship of symbionts associated with bivalve hosts of the genus *Solemya*.

Solemyid clams are found throughout the world's oceans, in shallow water and deep-sea habitats, and in temperate and tropical regions (44). The family diverged between 435 and 500 million years ago, and its members maintain a primitive form even today (34). Their mode of nutrition has always been poorly understood, given that their protobranch gills and reduced palp proboscides prevent them from efficient suspension and deposit feeding, respectively. However, the discovery of chemoautotrophic symbionts in the gills of the coastal species *Solemya velum* provided the explanation (4). The symbionts supply the animal nutrition derived from the chemoautotrophic fixation of CO_2 fueled by the oxidation of reduced inorganic sulfur compounds from the *Solemya* habitat. Indeed, as other *Solemya* species are reexamined, they have all been found to harbor bacteria in their gill cells (see, e.g., references 8, 25, 30, and 38).

16S rRNA gene sequences from a limited number of *Solemya* symbionts indicate that the symbiotic bacteria are host species specific (12, 16, 25). Those studied to date are all members of the γ subdivision of the *Proteobacteria* and cluster loosely with other chemoautotrophic symbionts of marine invertebrate hosts, but their precise phylogenetic relationship is largely unresolved. The three known *Solemya* symbionts are separated by deep branches within the γ subclass of the class *Proteobacteria* phylogenetic tree (25). Although limited by the paucity of data, it has been inferred that the symbiosis was established early in the history of the genus *Solemya* with a single symbiont ancestor, and the observed relationship among the symbionts interpreted as reflective of the age of the host group (11, 16, 25). The symbionts of other bivalve lineages (i.e., Vesicomyidae and Lucinacea) appear to be monophyletic and display phylogenetic congruence with their host species (11). With the goal of understanding the origin and evolution of the *Solemya* symbionts, we expanded the 16S rRNA database to include the *S. pusilla* and *S. terraeregina* symbionts and unexpectedly uncovered a polyphyletic relationship among the five species.

MATERIALS AND METHODS

Organisms. Specimens of *S. pusilla* were collected in September 1995 from eelgrass beds in Sagami Bay, Central Japan, by S. Ohta (Ocean Research Institute of Japan). The clams were 4 to 8 mm long and were found in subtidal, silty sediments. Specimens of *S. terraeregina* (2 to 4 mm long) were collected in February 1994 from coral reef sediments off the coast of Lizard Island, Australia, by N. Dubilier (University of Hamburg, Hamburg, Germany). Unfortunately, no fresh or frozen specimens of either species were retained; so, studies were limited to tissue fixed for electron microscopy and DNA analysis (see below). Voucher specimens of both *S. pusilla* and *S. terraeregina* have been deposited in the Museum of Comparative Zoology (Harvard University) under catalog numbers 316095 and 316114, respectively.

Electron microscopy. To establish the symbiotic condition of *S. pusilla* and *S. terraeregina*, transmission electron microscopy was used to examine their gills for the presence of bacterial symbionts. Three *S. terraeregina* clams from near Lizard Island in the Coral Sea were placed in TRUMPS fixative (29) immediately following collection. The gill tissues of individual clams were carefully removed

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TABLE 1. Bacterial species used in this study

Bacterium group	Host group	Species	Subdivision[a]	GenBank accession no.
Chemoautotrophic symbionts[b]	Bivalvia			
	Solemyidae	*Solemya terraeregina*	γ	U62131
		Solemya pusilla	γ	U62130
		Solemya velum	γ	M90415
		Solemya occidentalis	γ	U41049
		Solemya reidi	γ	L25709
	Lucinacea			
	Lucinidae	*Lucina floridana*	γ	L25707
		Codakia costata	γ	L25712
		Lucinoma aequizonata	γ	M99448
		Anodontia phillipiana	γ	L25711
	Thyasiridae	*Thyasira flexuosa*	γ	L01575
	Vesicomyidae	*Calyptogena elongata*	γ	L25718
	Mytilidae	*Bathymodiolis thermophilus*	γ	M99445
	Vestimentifera	*Riftia pachyptila*	γ	M99451
	Nematoda	*Laxus* sp.	γ	U14727
	Oligochaeta	*Inanidrilus leukodermatus*	γ	U24110
Other bacteria		*Escherichia coli*	γ	J01695
		Vibrio harveyi	γ	X56578
		Thiomicrospira strain L12	γ	L01576
		Agrobacterium tumefaciens	α	M11223
		Rickettsia rickettsii	α	M21293
		Pseudomonas testosteroni	β	M11224

[a] Subdivision within the *Proteobacteria*, a major lineage of the *Bacteria* as defined by Woese et al. (47).
[b] Chemoautotrophic symbionts listed by host species name.

following fixation. The gills from *S. pusilla* specimens were dissected away from the rest of the body after collection and fixed in a 2.5% paraformaldehyde–2.5% glutaraldehyde mixture in 0.1 M sodium cacodylate buffer (pH 7.4). All samples were postfixed with osmium tetroxide (4% in cacodylate buffer), stained with uranyl acetate (1%), dehydrated in an ethanol series, and embedded in Spurr's resin (42) by methods described previously (25). Ultrathin sections were stained with uranyl acetate (3%) and lead citrate (3%) and examined with a Hitachi H-7000 electron microscope.

DNA extraction. Immediately following collection, individual *S. pusilla* clams were dissected into gill and foot tissue with sterile dissection tools, taking care that the two tissue types never contacted one another. Excised tissues were immediately fixed in 70% ethanol (EtOH) for transport back to the laboratory and subsequent DNA extraction. Gill and foot tissues were treated separately because bacterial symbionts are abundant in solemyid gills whereas the foot tissue is symbiont free. Specimens of *S. terraeregina* were preserved whole in 80% EtOH, and gill and foot tissues were removed immediately prior to DNA extraction.

DNA was prepared from the tissues of four *S. pusilla* and three *S. terraeregina* individuals in the following ways. *S. terraeregina* tissues were homogenized in 5 M guanadinium isothiocyanate lysis buffer by the method of Ausubel et al. (2), while a 2% CTAB (hexadecyltrimethylammonium bromide) buffer containing 100 μg of proteinase K per ml was used on all *S. pusilla* specimens as described previously (46). Following lysis, phenol-chloroform and chloroform extractions were used to remove cellular proteins and other contaminants from the DNA, which was ultimately recovered by EtOH precipitation with ammonium acetate (2). DNA samples were stored frozen at −20°C.

PCR amplification and sequencing. Direct sequencing of the *S. terraeregina* and *S. pusilla* symbiont 16S rRNA genes recovered from host gills was carried out in one of two ways. For *S. terraeregina*, magnetic bead sequencing of PCR products generated with biotinylated primers 8F and 1492R, which universally recognize the 16S rRNA from species belonging to the domain *Bacteria* (45, 47), was carried out as described previously (25). For *S. pusilla*, PCR products amplified with nonbiotinylated PCR primers 8F and 1492R were sequenced automatically by the Dye-terminated cycle-sequencing method (ABI Prism sequencer; Perkin-Elmer). Reaction cocktails for PCR amplification of 16S rRNA genes consisted of either 1.25 U of Promega *Taq* polymerase, 1.5 mM $MgCl_2$, 0.2 mM deoxynucleoside triphosphates, 0.1 μM primers, and 1 to 10 ng of template in a final volume of 50 μl or 2.5 U of Gibco BRL *Taq* polymerase, 0.2 mM dioxynucleoside triphosphates, 1.5 mM $MgCl_2$, 0.5 μM primers, 0.05% Gibco BRL detergent, and 1 to 10 ng of template in a final volume of 100 μl. The PCR parameters were 94°C for 3 min followed by 35 cycles of 94°C for 1 min, 50°C for 1 min, and 72°C for 1.5 min. PCR with DNA extracted from host gills as the template resulted in a product of the expected size of ≈1,480 bp as determined by agarose gel electrophoresis, while DNA from host foot tissue failed to yield PCR products, indicating that the products obtained from host gill DNA originated from bacteria associated with the gill tissue. Prior to sequencing, the products of at least three independent amplifications per gill sample were pooled to account for possible sequence errors introduced by *Taq* polymerase. Partial sequences of PCR products from three individuals of each host species, which included both RNA-like and complementary strand sequences, were used to infer the entire 16S rRNA gene of each symbiont type. The 16S gene sequencing primers of Lare (26) were used in combination to obtain entire sequences. Overlapping hypervariable regions of sequence obtained from individual specimens of each species with various primers were identical in every case, indicating that each host species associates with a specific symbiont species.

In situ hybridization. To confirm that the 16S rRNA sequences originated from the *S. terraeregina* and *S. pusilla* symbionts, in situ hybridizations were performed on host gills by using oligonucleotide probes designed from and specific to the newly obtained sequences. The specificity of putative probe sequences chosen from highly variable regions was checked against sequences in GenBank by using BLAST (1) and against sequences in the Ribosomal Data Project (RDP) by using CHECK_PROBE (28). The probe made specific to the *S. terraeregina* symbiont (st1120) corresponds to *Escherichia coli* nucleotides 1129 to 1145, with the sequence 5′-TCCCATCCCGAAATGCTG. The st1120 probe has at least five mismatches with all known chemoautotrophic symbionts and three mismatches with 16S rRNA sequences in GenBank and the RDP. The probe made specific for the *S. pusilla* symbiont (sp1139) corresponds to *E. coli* nucleotides 1272 to 1291, with the sequence 5′-GCTTTCTCGGATTAGCTT CC. The sp1139 probe differs from all known chemoautotrophic symbionts by at least three nucleotide positions and from sequences in GenBank and the RDP by at least two nucleotide positions. As negative experimental controls, the sp1139 probe was used on *S. terraeregina* sections and the st1120 probe was used on *S. pusilla* sections. The st1120 probe contains six mismatches relative to the *S. pusilla* symbiont sequence in the corresponding region, and the sp1139 probe contains five mismatches relative to the *S. terraeregina* symbiont sequence in the corresponding region. The "universal" eubacterial probe Eub338 (5′-GCTGCC TCCCGTAGGAGT [20]) was also used on both species as a positive control. Following synthesis, probes were purified by silica gel thin-layer chromatography and prepared for hybridization experiments by enzymatically attaching a digoxygenin (DIG) molecule to the 3′ end of the probe with terminal transferase.

Four specimens each of *S. pusilla* and *S. terraeregina* were prepared for in situ hybridizations following fixation in Bouin's solution and formalin, respectively. Fixed samples were dehydrated in an ethanol series (70, 80, 90, 95, and 100%) followed by three washes in xylene and infiltration with paraffin wax. Wax-embedded specimens were then sectioned (≈6 μm) and mounted on 3-aminopropyltriethyloxysilane (APTS)-coated slides and baked at 55°C for at least 3 h.

In situ hybridizations were carried out with the Genius III nonradioactive DNA-labeling and detection kit (Boehringer Mannheim). Briefly, sections were

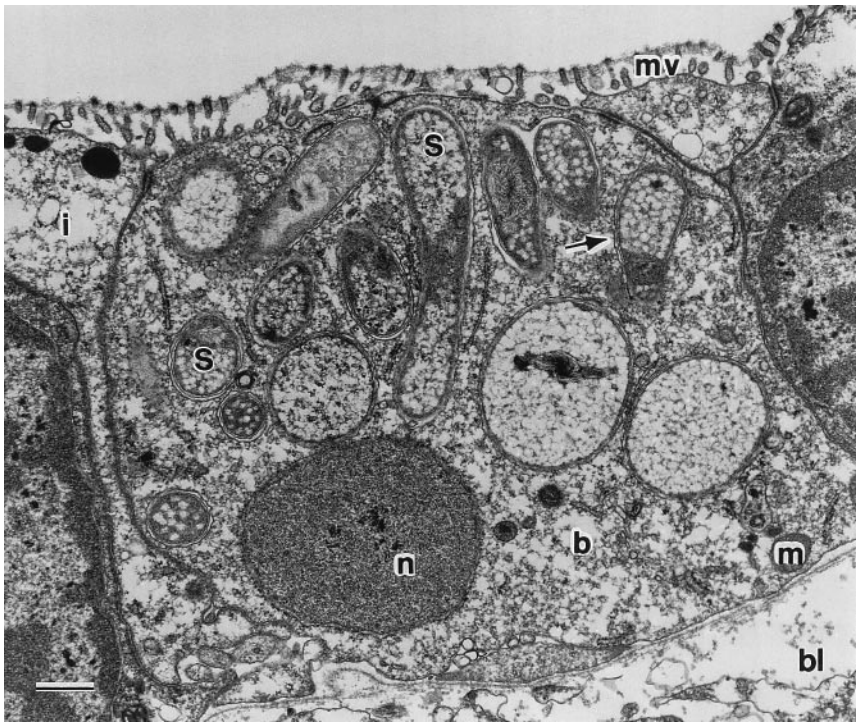

FIG. 1. *S. pusilla* transmission electron micrograph. The figure shows a transverse section through a single bacteriocyte (b) containing bacterial symbionts (s) flanked by symbiont-free intercalary cells (i). The gill cells are bordered by a microvillar surface (mv) on the outer edge and by the blood space (bl) on the inner surface. Other host cell inclusions include a nucleus (n) and mitochondria (m). Bar, 1 μm.

deparaffinized in xylene, rehydrated in a reverse ethanol series (100, 95, 90, 80, and 70%), and neutralized in 2× SSPE buffer (0.3 M NaCl, 20 mM $NaH_2PO_4 \cdot H_2O$, 20 mM EDTA). The sections were incubated for 10 min in 0.1 M triethanolamine (pH 8)–0.25% acetic anhydride and rinsed for 10 min in 2× SSPE. The sections were prehybridized with 0.5 mg of salmon sperm DNA per ml–0.25 mg of yeast tRNA per ml–5× Denhardt's reagent in 2× SSPE in a moist chamber at 42°C for 30 min. Then, approximately 100 pmol of DIG-conjugated probe was added to each section, and the sections were incubated for an additional 4 to 12 h at 42°C. Unbound probe was removed by washing for 1 h each in 2× SSPE, 1× SSPE, and 0.2× SSPE. The bound probes were detected, as specified by the manufacturer, with alkaline phosphatase-conjugated anti-DIG Fab fragment. Following treatment with antibody (1:500 dilution), the sections were incubated with a solution of 4-nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl phosphate in the dark for 4–12 h at room temperature. The labeling reaction was stopped by dipping the slides in distilled water, followed by counterstaining of the tissue with eosin stain, dehydration in an ethanol series (see above) and xylene, and mounting with Permount (Sigma) mounting medium. Hybridization results were viewed on a Zeiss Axioskop compound microscope equipped with an Optronix 1 chip cooled charge-coupled device camera. Photographs were printed on a Sony color video UP5200MD video printer.

Phylogenetic analysis. The 16S rRNA gene sequences of the *S. terraeregina* and *S. pusilla* symbionts were compared with the *S. velum* (16), *S. reidi* (12), and *S. occidentalis* (25) symbiont sequences, as well as with a variety of published symbiotic and free-living bacterial 16S rRNA gene sequences available from RDP (33) (Table 1). The sequences were aligned manually with the Genetic Data Environment sequence editor (40) according to conserved regions of sequence and the predicted secondary structure of eubacterial 16S rRNAs (48). The phylogenetic positions of the *S. terraeregina* and *S. pusilla* symbionts were determined by both distance and parsimony phylogenetic methods. A total of 899 characters (excluding ambiguous nucleotides and alignment gaps) were included in the mask. Phylogenetic distances were calculated by using the Jukes and Cantor correction and the DeSoete tree-fit program (9, 10). Bootstrap values were calculated with both distance (DNADIST, SEQBOOT, FITCH) and parsimony (DNAPARS, DNABOOT) algorithms contained in the Phylip 3.5 program (17). Bootstrap values of >70% (out of 100 replicates) are considered to support significantly the grouping of organisms in an associated node (24).

Nucleotide sequence accession numbers. The *S. terraeregina* and *S. pusilla* symbiont 16S rRNA gene sequences have been deposited in GenBank under accession numbers U62131 and U62130, respectively.

RESULTS

Electron microscopy. Inclusions resembling gram-negative bacteria were seen in the gill cells of both *S. pusilla* and *S. terraeregina* by transmission electron microscopy (Figs. 1 and 2). The ultrastructure is remarkably similar to that described for other species of *Solemya* (4, 8, 36). The symbionts are rod shaped but pleomorphic, with some cells being rounder and fatter than others and apparently filled with glycogen. The bacteria occur within the cytoplasm of host cells and are contained within host-derived membranes. Host cells resembling "bacteriocytes," described previously (4), were visible and contained numerous symbionts concentrated in the apical region of the cell just below the microvillar surface. The microvilli covering the length of the filament are extensions of the goblet-shaped, symbiont-free host cells ("intercalary cells") which alternate with the bacteriocytes along the length of the filaments. The ciliated host cells at the tips of the filaments were also characteristically symbiont free, but contained numerous mitochondria.

16S rRNA gene sequences. Single bands of amplified DNA, ≈1,480 bp long as determined by agarose gel electrophoresis, resulted from PCRs with *Bacteria* 16S rRNA primers and DNA extracted from *S. pusilla* and *S. terraeregina* gills as the template. No product was obtained when foot DNA was used. Direct sequencing of the gill PCR products, which included overlapping partial sequences from three individuals of each species, yielded single, host-specific sequences. A total of 1,505 and 1,282 nt were sequenced from the *S. terraeregina* and *S. pusilla* symbiont 16S rRNA genes, respectively. Together with in situ hybridizations with symbiont-specific probes designed from these sequences (see below), these results indicate that the 16S rRNA genes we amplified and sequenced belong to the

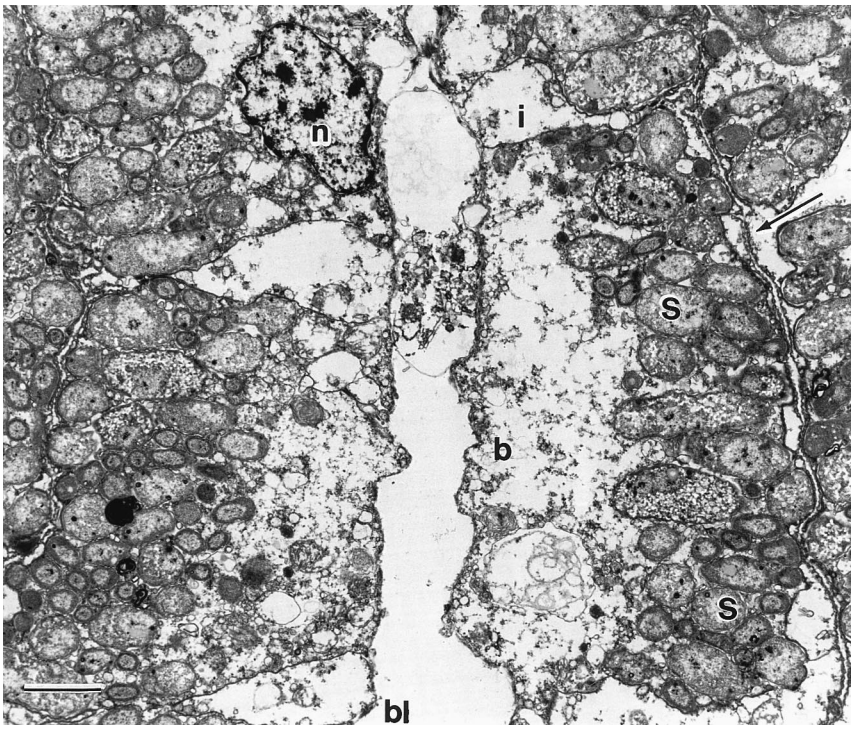

FIG. 2. *S. terraeregina* transmission electron micrograph. The figure shows a transverse section through gill filaments, showing alternating symbiont-free intercalary cells (i) and symbiont-containing bacteriocytes (b); the blood space that separates the filament is visible in the center of the micrograph (bl). Intracellular inclusions resembling prokaryotic cells located in the apical region of each bacteriocyte are identified as bacterial symbionts (s). The outer surface of the filament is covered with microvilli (arrow). n, host cell nucleus. Bar, 5 μm.

S. terraeregina and *S. pusilla* symbionts and not to surface contaminants.

In situ hybridization. In situ hybridization experiments with oligonucleotide probes designed to be specific for each new bacterial sequence demonstrate that the 16S rRNA genes amplified from *S. terraeregina* and *S. pusilla* gills are representative of the symbiotic bacteria associated with these hosts. Initial experiments with the positive control Eub338 probe resulted in labeling of the symbionts in the gills of both *S. pusilla* and *S. terraeregina* (data not shown). The *S. pusilla* symbiont-specific probe sp1139 resulted in strong labeling of the bacteria in *S. pusilla* gills, and, although relatively weaker, the *S. terraeregina* symbiont-specific probe st1120 resulted in labeling of the symbionts in *S. terraeregina* gills (Fig. 3). Very little labeling was observed in either species with the negative controls, i.e., sections hybridized with the probe specific for the symbiont of the other species (Fig. 3) and sections not treated with a probe (data not shown).

Symbiont phylogeny. Phylogenetic analyses performed by both distance and parsimony methods placed the *S. terraeregina* and *S. pusilla* symbionts within the γ subdivision of the *Proteobacteria*, the lineage of *Bacteria* containing all previously studied *Solemya* symbionts and the majority of other chemoautotrophic symbionts associated with marine invertebrate hosts (5, 25). Trees of similar topology resulted from both distance (Fig. 4) and parsimony (results not shown) analyses. The symbionts of *S. terraeregina* and *S. pusilla* are closely related to one another (95.7% sequence identity) but are only distantly related to the *S. velum*, *S. occidentalis*, and *S. reidi* symbionts (Table 2). The symbionts of *S. pusilla* and *S. terraeregina* are instead associated with the symbionts of bivalve hosts belonging to the superfamily Lucinacea (Fig. 4). The *S. terraeregina* and *S. pusilla* symbionts form a monophyletic group with the *Thyasira flexuosa* and *Anodontia phillipiana* symbionts, supported by bootstrap values of 93% (distance) and 70.7% (parsimony). The *S. pusilla* and *T. flexuosa* symbionts (96.2% identity) have apparently diverged more recently from a common ancestor within this group, as indicated by bootstrap values of 91% (distance) and 71.2% (parsimony) at their ancestral node. Similarly, the *S. terraeregina* and *A. phillipiana* symbionts (96.9% identity) may have diverged more recently within the group, although such a relationship is only weakly supported by bootstrap values of 73% (distance) and 52.3% (parsimony). The previously reported monophyletic relationship of the *S. velum* and *S. occidentalis* symbionts (25) is supported in the present analysis, albeit weakly, as indicated by bootstrap values of 77% (distance) and 46% (parsimony) at the base node. Also as in previous studies, the relationship of the *S. reidi* symbiont and the *S. velum* and *S. occidentalis* symbiont cluster to other members of the γ subdivision of the *Proteobacteria* remains unresolved.

DISCUSSION

Like all other solemyid clams examined to date, *S. terraeregina* and *S. pusilla* harbor endosymbiotic bacteria in their gills. These symbionts resemble the chemoautotrophic symbionts of other host species based on ultrastructure and their 16S rRNA gene sequences. Surprisingly, the *S. terraeregina* and *S. pusilla* symbionts have a different phylogenetic origin from that of other *Solemya* symbionts. Such diversity among chemoautotrophic symbionts is unprecedented within a single bivalve host genus and argues against a hypothesis of host-symbiont cospe-

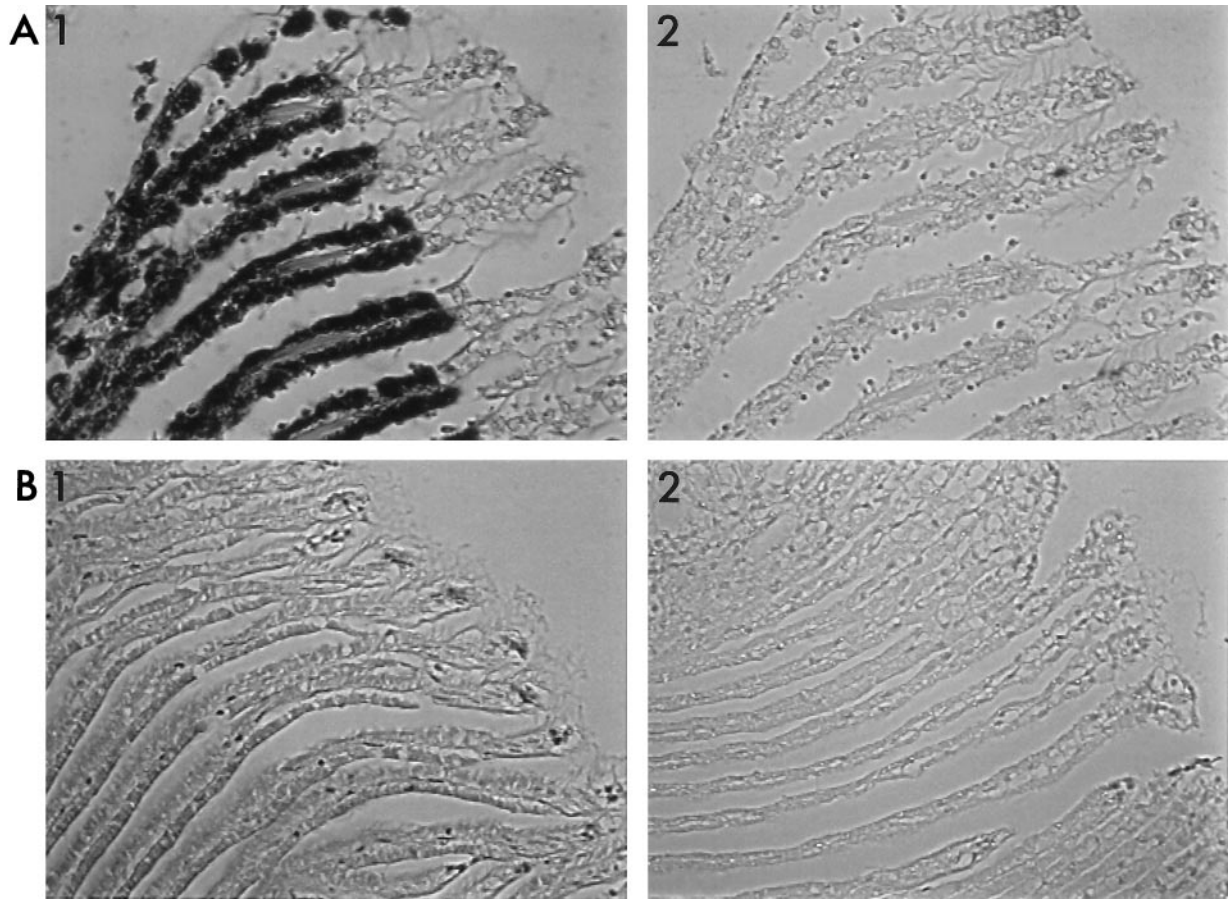

FIG. 3. In situ hybridization with DIG-labeled oligonucleotide probes on sections of *S. pusilla* (A) and *S. terraeregina* (B) gills. The figure shows labeling of *S. pusilla* gills after hybridization with the *S. pusilla* symbiont-specific probe sp1139 (A1) and the *S. terraeregina* symbiont-specific probe st1120 (A2) and labeling of *S. terraeregina* gills after hybridization with *S. terraeregina* symbiont-specific probe st1120 (B1) and the *S. pusilla* symbiont-specific probe sp1139 (B2). Bacteriocytes are located in the gill filaments to the left of the symbiont-free ciliated edges.

ciation for *Solemya*, in contrast to the Vesicomyidae and Lucinacea.

The morphological uniformity among clams of the genus *Solemya* is striking. Apart from relatively large variations in size, all *Solemya* spp. closely resemble one another with respect to external morphology (3, 31). All solemyids have notably enlarged protobranch gills, reduced palp proboscides, a reduced or absent digestive system, and symbiotic gill bacteria (18, 37). Including the present study, the gills of six species have now been studied at the ultrastructural level. In all of these solemyids, the symbionts are contained within specialized gill cells known as bacteriocytes, which are distributed along the length of the filaments in an alternating pattern with symbiont-free intercalary cells (Fig. 1 and 2). Electron micrographs and ultrastructural descriptions of the gills of *Solemya* species so closely resemble one another that it is impossible to tell them apart! These observations suggest that the symbionts may be closely related.

Although the metabolic capabilities of the *S. terraeregina* and *S. pusilla* symbionts could not be established in the current studies due to limited specimen availability (see Materials and Methods), the morphological similarities of both hosts and symbionts and the character of the symbionts inferred from their 16S rRNA gene sequences suggest that these symbioses are chemoautotrophic. Furthermore, their placement within a cluster exclusively containing sulfur-oxidizing, chemoautotrophic bacterial species of the γ subdivision of the *Proteobacteria* suggests that they have a similar metabolism. In addition, both hosts occupy habitats where chemoautotrophic associations have the potential to occur. *S. pusilla* is found near seagrass beds in shallow coastal waters in the eastern Pacific, a habitat closely resembling that of the congener species *S. velum* in the eastern Atlantic (4). Similarly, the calcareous sand habitat of *S. terraeregina* in the tropical Pacific closely resembles that of *S. occidentalis*, which lives throughout the Caribbean in coral reef environments (25). Both *S. terraeregina* and *S. pusilla* harbor bacteria in their gills, inhabit reducing environments, and display adaptations such as hypertrophied ctenidia and tiny palp proboscides identical to *Solemya* species known to derive nutrition from chemoautotrophic symbionts, suggesting that they have a similar biology.

The morphological and ecological uniformity, in combination with the inferred physiological uniformity, among the five *Solemya* species in the current study implies that the symbiosis has a long evolutionary history within this host group. As in lucinid and vesicomyid clams, the symbiosis in the *Solemya* species could have been established in an ancestral host, prior to diversification of the modern species. Such a history would suggest that the symbionts of these species are phylogenetically closely related. Surprisingly, the symbionts of *S. terraeregina* and *S. pusilla* are more closely related to bacteria which associate with bivalves from the superfamily Lucinacea (containing

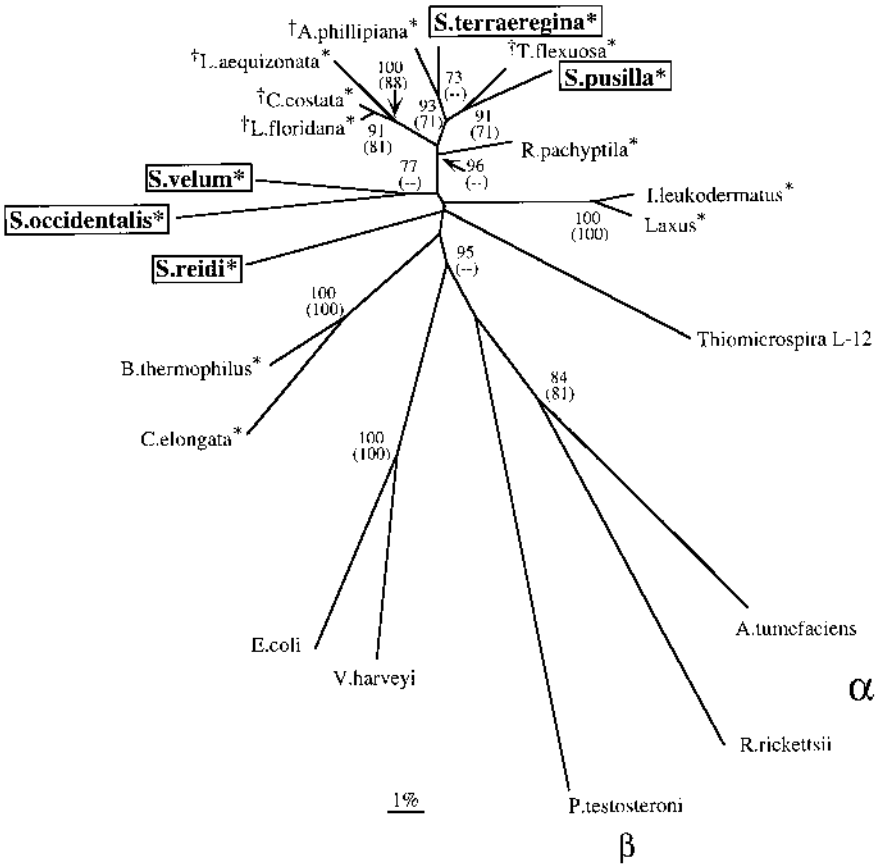

FIG. 4. Unrooted evolutionary distance tree (Jukes and Cantor correction) of selected members of the *Proteobacteria* based on 16S rRNA gene sequences. All bacteria shown are members of the γ subdivision, except *Agrobacterium tumefaciens* and *Rickettsia rickettsii* (α subdivision) and *Pseudomonas testosteroni* (β subdivision). A total of 899 nucleotide positions were included in the distance analyses. Parsimony methods produced a tree with similar topology. Bootstrap values out of 100 repetitions resulting from distance and parsimony (in parentheses) methods are shown at their corresponding nodes. Chemoautotrophic symbionts are identified by an asterisk. *Solemya* symbionts are shown in boldface type and boxed. †, symbionts of hosts of the bivalve superfamily Lucinacea. See Table 1 for further information on host classifications. The scale bar denotes one change per 100 nucleotides.

the thyasirid and lucinid bivalves) than those found associated with *Solemya*. These two solemyid symbionts are more closely related to and form a monophyletic group with the chemoautotrophic symbionts of *A. phillipiana* and *T. flexuosa* (Table 2; Fig. 4). The placement of the other *Solemya* symbionts within the tree, however, is consistent with all previous phylogenetic studies of chemoautotrophic symbionts (see, e.g., reference 25). That is, the present analyses show that the *S. velum* and *S. occidentalis* symbionts are still the two most closely related species and evolved from a common ancestor. Their position relative to other bacteria, including other *Solemya* symbionts, however, is unresolved (Fig. 4).

The polyphyletic origin of the *Solemya* symbionts is in contrast to the origins of all chemoautotrophic symbionts associating with bivalve hosts that have been studied to date. That is, monophyly has been demonstrated for the bacterial symbionts of the bivalve family Vesicomyidae and the superfamily Lucinacea. Furthermore, host-symbiont cospeciation has been inferred for these groups based on fossil (hosts) and molecular sequence (symbionts) data (11). The symbionts associated with solemyid clams, however, belong to at least two and possibly three distinct phylogenetic groups (Fig. 4). The solemyid host species, on the other hand, are assumed to be monophyletic, given their remarkable morphological similarity and classical grouping into a single genus (3, 49). Thus, it appears that the symbiosis pervasive in *Solemya* did not arise from parallel diversification of host and symbiont lineages. Rather, the observed associations could have arisen from multiple symbiotic events in which different bacteria became symbionts of one or more *Solemya* species. Alternatively, an ancestral symbiosis could have established the symbiotic condition in the genus, with shifts in symbiont populations occurring in some host species after or during diversification.

The first scenario is supported by the observation that numerous chemoautotrophic bacteria formed symbioses with marine invertebrates in the past. The sulfur-oxidizing chemoautotrophic bacterial symbionts of hosts from a range of phyla show diverse origins within the γ subdivision of the *Protobacteria* (11, 12), and there are recent reports of chemoautotrophic symbionts having close phylogenetic affinity with members of the ε subdivision of the *Protobacteria* (22, 35). Methanotrophic bacteria also occur as symbionts of marine invertebrate hosts (6, 7, 39), and the extreme example of hosts having stable symbiotic associations with both sulfur-oxidizing chemoautotrophic and methanotrophic bacteria has recently been described for two species of deep-sea mussels (13). It has also been observed that certain sulfur-oxidizing chemoautotrophs may have the ability to associate with more than one kind of host. For example, the bacterial symbionts of nematode and oligochaete hosts are closely related, while their hosts belong

TABLE 2. Evolutionary distance and percent similarity matrices constructed from 16S rRNA gene sequences of the *S. pusilla* and *S. terraeregina* symbionts and selected endosymbiotic and free-living bacteria[a]

Species	Evolutionary distance (lower left) and % similarity (upper right) for:																				
	S. pusilla	*S. terraeregina*	*S. reidi*	*S. occidentalis*	*S. velum*	*T. flexuosa*	*A. phillipiana*	*L. aequizonata*	*C. costata*	*L. floridana*	*R. pachyptila*	*B. thermophilus*	*C. elongata*	*Laxus* sp.	*I. leukodermatous*	*Thiomicrospira* strain L12	*E. coli*	*V. harveyi*	*R. rickettsii*	*A. tumefaciens*	*P. testosteroni*
S. pusilla[b]		95.7	88.3	89.8	91.1	95.9	94.5	92.1	93.2	93.5	93.5	89.2	88.1	89.3	89.0	88.3	83.3	83.9	80.8	82.4	81.5
S. terraeregina[b]	4.3		90.0	89.9	91.8	96.3	97.2	93.8	95.4	95.8	94.8	89.0	87.9	91.1	91.2	89.2	83.9	84.1	80.5	83.8	82.4
S. reidi[b]	11.7	10.0		88.2	90.3	89.5	90.1	89.3	91.1	91.4	91.4	88.0	86.7	90.1	90.0	88.1	84.5	84.2	81.6	81.8	81.1
S. occidentalis[b]	10.2	10.1	11.8		90.1	89.2	89.5	87.8	89.1	89.2	89.7	87.2	85.0	87.8	88.3	86.1	82.2	81.8	80.1	81.1	80.4
S. velum[b]	8.9	8.2	9.7	9.9		91.4	92.5	89.8	92.1	92.2	92.5	88.1	85.8	90.1	90.5	87.2	84.5	84.2	80.6	83.1	81.1
T. flexuosa[b]	4.1	3.7	10.5	10.8	8.6		95.1	93.3	94.8	95.1	95.3	90.2	89.5	90.5	90.7	88.9	83.5	84.0	81.6	83.1	82.0
A. phillipiana[b]	5.5	2.8	9.9	10.5	7.5	4.9		93.8	95.9	95.8	95.3	89.6	87.6	90.1	90.5	88.8	84.3	83.4	80.7	84.2	82.5
L. aequizonata[b]	7.9	6.2	10.7	12.2	10.2	6.7	6.2		96.8	96.6	94.4	89.8	87.9	90.8	90.4	88.1	82.8	83.5	81.0	84.0	81.0
C. costata[b]	6.8	4.6	8.9	10.9	7.9	5.2	4.1	3.2		99.2	95.8	90.5	88.9	91.7	91.8	88.4	84.0	84.3	80.9	84.4	81.5
L. floridana[b]	6.5	4.2	8.6	10.8	7.8	4.9	4.2	3.4	0.8		95.9	91.1	88.3	91.8	91.9	88.5	84.3	84.4	80.4	84.0	81.5
R. pachyptila[b]	6.5	5.2	8.6	10.3	7.5	4.7	4.7	5.6	4.2	4.1		90.9	89.3	92.2	92.0	90.2	84.8	84.6	81.6	85.2	82.3
B. thermophilus[b]	10.8	11.0	12.0	12.8	11.9	9.8	10.4	10.2	9.5	8.9	9.1		93.7	87.9	87.5	87.3	83.4	85.2	80.8	81.9	80.1
C. elongata[b]	11.9	12.1	13.3	15.0	14.2	10.5	12.4	12.1	11.1	11.7	10.7	6.3		86.2	86.2	85.2	82.5	83.4	80.9	82.0	78.8
Laxus sp.[b]	10.7	8.9	9.9	12.2	9.9	9.5	9.9	9.2	8.3	8.2	7.8	12.1	13.8		97.8	87.7	85.0	85.0	80.1	83.2	80.4
I. leukodermatus[b]	11.0	8.8	10.0	11.7	9.5	9.3	9.5	9.6	8.2	8.1	8.0	12.5	13.8	2.2		87.7	85.4	85.4	80.3	83.1	79.8
Thiomicrospira strain L12	11.7	10.8	11.9	13.9	12.8	11.1	11.2	11.9	11.6	11.5	9.8	12.7	14.8	12.3	12.3		82.0	82.3	79.2	80.9	78.9
E. coli	16.7	16.1	15.5	17.8	15.5	16.5	15.7	17.2	16.0	15.7	15.2	16.6	17.5	15.0	14.6	18.0		89.3	76.9	79.5	79.5
V. harveyi	16.1	15.9	15.8	18.2	15.8	16.0	16.6	16.5	15.7	15.6	15.4	14.8	16.6	15.0	14.6	17.7	10.7		78.0	78.5	77.9
R. rickettsii	19.2	19.5	18.4	19.9	19.4	18.4	19.3	19.0	19.1	19.6	18.4	19.2	19.1	19.9	19.7	20.8	23.1	22.0		83.2	79.5
A. tumefaciens	17.6	16.2	18.2	18.9	16.9	16.9	15.8	16.0	15.6	16.0	14.8	18.1	18.0	16.8	16.9	19.1	20.5	21.5	16.8		77.3
P. testosteroni	18.5	17.6	18.9	19.6	18.9	18.0	17.5	19.0	18.5	18.5	17.7	19.9	21.2	19.6	20.2	21.1	20.5	22.1	20.5	22.7	

[a] A total of 899 nucleotide positions of the 16S rRNA genes were used in the analyses, with values reported as percentages. The evolutionary distance was calculated by using the Jukes and Cantor correction (see Materials and Methods). Sequences other than the *S. pusilla* and *S. terraeregina* symbionts were obtained from the RDP (33).

[b] Chemoautotrophic symbionts, listed under the host name.

to distantly related animal phyla (15). Thus, there is apparently great potential for a wide range of symbiotic associations between bacteria and marine invertebrates occupying reducing habitats.

There is also evidence to support the replacement hypothesis for the evolution of the symbiosis in modern *Solemya* species. According to the current 16S rRNA data, the symbionts associated with bivalve hosts of the superfamily Lucinace and certain *Solemya* species are derived from a common ancestor (Fig. 4). Many lucinacean species form chemoautotrophic symbioses similar to those observed in *Solemya*, and lucinids cooccur with *Solemya* in both the modern and fossil ocean environments (14, 23). Furthermore, a recent study of the tropical lucinid *Codakia orbicularis* provides convincing evidence that its endosymbionts are obtained from the environment (21). Thus, there apparently is and was the opportunity for symbiont exchange between lucinid and solemyid hosts. While mechanisms of lateral symbiont transfer have not been directly studied for chemoautotrophic bivalves, gill epithelium has been observed to be microphagous, most notably in *Thyasira* species (41). For example, the gills of *T. flexuosa* contain masses of bacteria under a bulging cuticle, which are ultimately digested in large phagocytic vacuoles by the host cells. New symbionts are thought to be obtained through endocytosis (41). This observation also implies an environmental stock population of free-living symbionts in the habitat. It is conceivable, therefore that cooccurring lucinid, solemyid, and thyasirid clams encountered and endocytosed identical or closely related bacteria, which may have replaced an existing symbiont population within certain species or individuals. The symbiosis in previously symbiotic clams would remain essentially undisturbed, and any adaptations to the symbiotic condition on the part of the host (such as hypertrophied gills or a reduced digestive system) would be maintained. The phylogenetic affinity of the new symbiont population, however, could be different relative to that of the previous symbiont population.

Either of these two scenarios could explain the observed phylogenetic relationships among the five *Solemya* symbionts included in this study. The fundamental assumption in developing these arguments, however, has been that the five host species form a monophyletic group. Apart from variations in size, the solemyids are morphologically identical, but their classification has historically undergone many revisions. Their higher-level taxonomy (i.e., where the Protobranchia fit within the Bivalvia) is still debated in the literature (27, 34), and a new classification scheme for the genus *Solemya* (i.e., how the genus should be divided into subgenera) was suggested as recently as 1980 (3). Thus, we are currently testing the hypothesis of monophyly among these five hosts as the next step in understanding how the *Solemya* symbiosis evolved. If it is true that the *Solemya* symbionts have diverse origins relative to their hosts, we will be challenged to reconsider the issues of host-symbiont specificity and stability in chemoautotrophic associations and the role that these symbioses may play in evolution.

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