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Sulfur-Oxidizing Bacterial Endosymbionts: Analysis of Phylogeny and Specificity by 16S rRNA Sequences

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The 16S rRNAs from the bacterial endosymbionts of six marine invertebrates from diverse environments were isolated and partially sequenced. These symbionts included the trophosome symbiont of *Riftia pachyptila*, the gill symbionts of *Calyptogena magnifica* and *Bathymodiolus thermophilus* (from deep-sea hydrothermal vents), and the gill symbionts of *Lucinoma annulata*, *Lucinoma aequizonata*, and *Codakia orbicularis* (from relatively shallow coastal environments). Only one type of bacterial 16S rRNA was detected in each symbiosis. Using nucleotide sequence comparisons, we showed that each of the bacterial symbionts is distinct from the others and that all fall within a limited domain of the gamma subdivision of the purple bacteria (one of the major eubacterial divisions previously defined by 16S rRNA analysis [C. R. Woese, Microbiol. Rev. 51:221–271, 1987]). Two host specimens were analyzed in five of the symbioses; in each case, identical bacterial rRNA sequences were obtained from conspecific host specimens. These data indicate that the symbioses examined are species specific and that the symbiont species are unique to and invariant within their respective host species.

The recent discovery of the unique fauna of the hydrothermal-vent communities has brought to light an unexpected mode of animal nutrition in which chemoautotrophic bacterial symbionts are maintained within specialized cells of the host animal (4, 7, 8). The bacterial symbionts obtain energy by oxidizing reduced sulfur compounds from the environment (6, 17, 23) and can fix inorganic carbon (8, 21). A portion of this fixed carbon is utilized by the eucaryotic host (5, 18, 24). This type of symbiosis is now known to exist in members of a wide variety of marine invertebrate taxa encompassing at least four phyla. In addition to hydrothermal vents, a broad range of marine environments, from intertidal to deep sea, are now known to support symbioses of this kind (3, 19).

To date, the bacterial symbionts of all known sulfur-based invertebrate-bacterial endosymbioses have eluded cultivation. Thus, until recently there has been no satisfactory means of investigating many fundamental questions concerning the identity of these symbionts and the nature of the host-symbiont relationships. Among such questions are the following. Are the endosymbionts of an individual host organism monospecific or a mixed population? Does the composition of the endosymbiotic population vary among individuals of a given host species? Do the symbionts of different sulfur-based, animal-bacterial symbioses belong to a single, closely related phylogenetic group, or are they drawn from a variety of bacterial groups? How are these symbionts related to well-characterized, cultured bacterial species? Do the phylogenies of the bacterial symbionts parallel those of their respective hosts, or do their relationships reflect, for example, the geographic distribution or environment of the hosts? In this investigation we used newly developed methods of symbiont purification (D. L. Distel and H. Felbeck, J. Exp. Zool., in press) and rRNA sequence analysis (15, 16) to investigate these questions without having to cultivate the symbionts.

MATERIALS AND METHODS

Collection of animals. Animals were collected as follows: (i) *Lucinoma aequizonata* was collected by Otter trawl at a depth of 500 m on the sill of the Santa Barbara Basin off of the coast of California; (ii) *Lucinoma annulata* was collected by Van Veen grab at a depth of 100 m near the Hyperion sewage outfall in the Santa Monica Basin off of the coast of California; (iii) *Codakia orbicularis* was collected by hand from sea grass beds on Nassau Island, Bahamas; and (iv) *Riftia pachyptila*, *Calyptogena magnifica*, and *Bathymodiolus thermophilus* were collected from the Galapagos hydrothermal-vent site (0.5′ N) at a depth of 2,500 m by the submarine DSRV (deep-submergence research vehicle) Alvin.

Purification of bacterial symbionts. Bacterial symbionts were purified from homogenates of gill or trophosome tissue (Distel and Felbeck, in press) by centrifugation through Percoll (Sigma Chemical Co.) density gradients. All purifications were performed with fresh tissue immediately after animals were sacrificed. Purified bacterial preparations were obtained from *L. annulata*, *L. aequizonata*, *Codakia orbicularis*, *R. pachyptila*, and *Calyptogena magnifica*.

Extraction of RNA from bacterial preparations. Purified bacteria were washed in Mg- and Ca-free imidazole-buffered saline (0.49 M NaCl–0.003 M KCl–0.05 M imidazole [pH 8.0 at 22°C]), suspended in the same solution with 40 mM EDTA, and held on ice for 5 min. Lysozyme (5 mg/ml, Sigma) was added, samples were warmed and agitated at 37°C for 1 min, and cell debris was pelleted at 14,000 × g for 1 min on a Fisher microcentrifuge. Supernatants were rapidly transferred to tubes containing sodium dodecyl sulfate (0.1 volume of a 20% [wt/vol] solution), and RNA was

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TABLE 1. 16S rRNA sequence similarities of invertebrate-associated symbiotic bacteria and selected free-living bacteria[a]

Organism	16S rRNA sequence similarity[b] with:								
	Symbiont of:						*Thiomicrospira* strain L-12	*T. nivea*	*E. coli*
	L. aequizonata	*L. annulata*	*C. orbicularis*	*R. pachyptila*	*B. thermophilus*	*C. magnifica*			
Symbiont of:									
Lucinoma aequizonata	1.000	0.980	0.966	0.957	0.904	0.889	0.902	0.900	0.853
L. annulata		1.000	0.966	0.960	0.911	0.891	0.895	0.893	0.854
Codakia orbicularis			1.000	0.966	0.896	0.886	0.897	0.906	0.851
Riftia pachyptila				1.000	0.897	0.892	0.901	0.904	0.860
Bathymodiolus thermophilus					1.000	0.950	0.887	0.877	0.857
Calyptogena magnifica						1.000	0.867	0.878	0.843
Free-living bacteria									
Thiomicrospira strain L-12							1.000	0.852	0.851
Thiothrix nivea								1.000	0.864
Escherichia coli									1.000

[a] Because of the partial nature of the sequences derived by using reverse transcriptase and the omission of regions in which the identity of homologous residues is uncertain, the analysis was restricted to *E. coli* positions 240 to 336, 339, 341 to 451, 480 to 506, 669 to 732, 736 to 836, 850 to 867, 869 to 897, 1144 to 1205, and 1211 to 1375. Copies of the 16S rRNA sequences used in this study are available upon request to the authors.

[b] See Materials and Methods.

purified by phenol-chloroform extraction and salt precipitation (12). Samples which were processed on shipboard were stored over phenol at −20°C, and chloroform extraction was performed upon return to the laboratory about 4 weeks later. Final samples were stored as ethanol slurries at −80°C.

Symbiont-containing gill tissue from *B. thermophilus* was frozen on shipboard in liquid nitrogen. After disruption of the tissue by grinding under liquid nitrogen, bulk RNA was prepared from the frozen powder as described above. The bacterial 16S rRNA was purified from bulk RNA by electrophoresis on a 2.8% polyacrylamide gel (3 mm by 15 cm by 14 cm) containing 0.14% bisacrylamide, 8 M urea, 67 mM Tris base, 22.5 mM boric acid, and 1.25 mM EDTA. The 16S rRNA band (clearly resolved from the eucaryotic 18S rRNA) was visualized by UV shadowing (10) and recovered from the gel by excision and electroelution. Electroelution was performed with an electroelutor (model UEA; International Biotechnologies, Inc.) by following the recommended procedures of the manufacturer.

The quality and purity of symbiont rRNAs in all samples were checked by electrophoresis on 1.5% agarose gels containing 8 M urea, 67 mM Tris base, 22.5 mM boric acid, and 1.25 mM EDTA. Gels were stained with ethidium bromide, and RNA bands were visualized by transillumination at 302 nm. No contamination by host 18S or 28S rRNA was detected in the symbiont RNA samples.

Nucleotide sequences were determined by reverse transcription (12) with oligonucleotide primers specific to the small-subunit rRNA. The resulting sequences were aligned with currently available 16S rRNA sequences. For each pair of sequences, the evolutionary distance (defined as the average number of nucleotide substitutions per sequence position) was estimated as $-3/4 \times \ln[(4S-1)/3]$, where S is the observed fractional sequence similarity (15; G. J. Olsen, Methods Enzymol., in press). Fractional sequence similarity was defined as $M/(M + U + G/2)$, where M is the number of compared sequence positions with identical residues, U is the number of compared sequence positions with dissimilar residues, and G is the number of compared positions at which one of the sequences being compared contained an alignment gap. This treatment partially corrects the observed number of sequence differences for multiple and back mutations (11). For phylogenetic-tree construction, a distance matrix method (9) was employed to determine the optimal topology consistent with the pairwise evolutionary distance estimates (15; Olsen, in press).

RESULTS

Approximately 1,000 bases of rRNA sequence was obtained from each of the 11 specimens examined. Samples of symbiont 16S rRNA were independently obtained from two specimens of each host species, with the exception of *Codakia orbicularis*, of which only one suitable specimen was available. Each of these symbiont 16S rRNA samples was then sequenced independently. In each case, 16S rRNA sequences obtained from the symbionts of different specimens of the same host species were identical at all positions determined. In each case, samples of 16S rRNA obtained from the symbionts from different host species differed in primary sequence. Each host specimen examined contained a symbiont population which was both unique to its species and invariant within the examined samples of that species.

The sequence data (Table 1) show that all of the symbionts examined fall within the gamma assemblage of the purple photosynthetic bacteria as previously defined by 16S rRNA analysis (25). Within this broad assemblage, the symbionts form a distinct and coherent group (Fig. 1). To date, no free-living bacterial species which fall clearly within the symbiont group are known; however, the hydrothermal-vent isolate *Thiomicrospira* sp. strain L-12 appears to be peripherally related. The symbiont group clearly comprises two distinct clusters, one of which contains the symbionts of the hydrothermal-vent tube worm *R. pachyptila* and of the lucinid species *L. aequizonata*, *L. annulata*, and *Codakia orbicularis*. The second cluster contains the symbionts of the hydrothermal-vent mollusks *B. thermophilus* and *Calyptogena magnifica*.

DISCUSSION

Recently, methods have become available which allow uncultured bacteria to be characterized on the basis of rRNA nucleotide sequences (12, 15). Stahl et al. (22) have sequenced the 5S rRNA of the symbionts from specimens of *R. pachyptila*, *Calyptogena magnifica*, and *Solemya velum*. The sequences were used to infer a 5S rRNA-based phylogenetic tree that included these symbionts and a number of

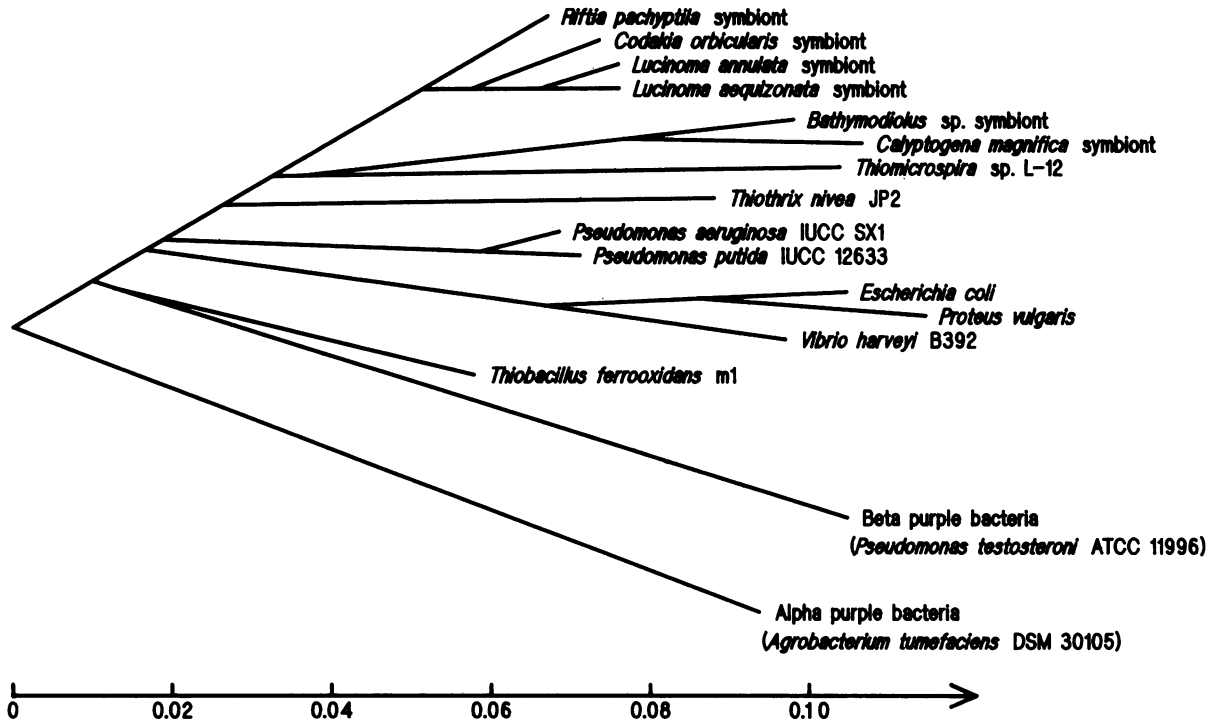

FIG. 1. 16S rRNA-based phylogeny of invertebrate-associated symbiotic bacteria. All of the bacteria represented belong to the purple bacteria (25). This tree was abstracted from a larger one that included additional, unpublished sequences. The horizontal components of the tree branches represent the estimated number of nucleotide substitutions per sequence position (see scale on horizontal axis).

better characterized, free-living bacteria. The present study substantially expands on these earlier observations in a number of important respects. First, the use of the larger 16S rRNA molecule (ca. 1,500 nucleotides) provided a larger statistical sampling of rRNA sequence differences between the inspected populations than can be obtained with the 5S rRNA molecule (ca. 120 nucleotides). Second, each symbiont 16S rRNA sample examined in this investigation was prepared from a single host specimen, and two specimens of each host species were sampled whenever possible. This allowed comparison of symbiont populations between individual specimens of the same host species as well as among different host species. Third, symbiont populations from a number of additional host species were inspected. Host species were chosen to represent the broadest possible range of host taxonomic relationships, geographical locations, and environments within the context of this type of symbiosis.

The methods applied here used the entire recoverable pool of microbial 16S rRNA molecules from each host specimen as a sequencing template. Therefore, unambiguous nucleotide sequence data are expected only if the symbiont populations of each host specimen are monospecific or are vastly dominated (>90%) by a single symbiont type. An unambiguous sequence was obtained in each case, indicating that the symbiont population of each host specimen is indeed predominantly or entirely composed of a single symbiont type. This agrees with the findings of Stahl et al. (22) that the symbionts of *R. pachyptila*, *Calyptogena magnifica*, and *S. velum* are monospecific in terms of the 5S rRNA content of the tissue samples. The results of thermal denaturation studies (14) performed on bulk DNA obtained from the trophosome of *R. pachyptila* are also consistent with the existence of a single, homogeneous symbiont population in that host organism.

It should also be noted that in the current investigation, samples of *R. pachyptila* trophosome contained tissue from the top, center, and base of the trophosome in approximately equal quantities. Therefore, our results indicate that the symbiont population of *R. pachyptila* constitutes a single pleomorphic species (3) rather than a mixed population distributed unequally between the top and base of the trophosome (1, 2).

Not only were the bacterial 16S rRNAs homogeneous within the individual animals, but no specimen-to-specimen variation in symbiont 16S rRNA sequence was found in the five cases in which two animals of a given species were analyzed independently. That is, the symbionts appeared to be unique to, and invariant within, the respective host species. Also, analysis of the *S. velum* symbiont 5S rRNA in gills pooled from several specimens showed the presence of a single symbiont 5S rRNA sequence (unpublished observations). The consistency of these findings in six different symbioses (including the 5S rRNA analysis) suggests that the association of one specific symbiont with each host species is a general rule in this type of symbiotic system.

The similarities of the symbiont rRNA sequences to one another and to some related free-living bacterial species are listed in Table 1. A phylogenetic tree summarizing these data is presented in Fig. 1. The symbionts examined form a coherent group which falls within a limited domain of the gamma subdivision of the purple bacteria, according to the nomenclature of Woese (25). The symbionts make up two rather distinct phylogenetic clusters (Fig. 1). The first cluster contains the symbionts of the three lucinid clams and of the hydrothermal-vent tube worm, and the second contains the symbionts of the hydrothermal-vent bivalves. *Thiomicrospira* sp. strain L-12 (20), a free-living, sulfur-oxidizing obligate chemolithotroph which was isolated from the Gala-

pagos hydrothermal vents, appears to be peripherally related to the cluster containing the *B. thermophilus* and *Calyptogena magnifica* symbionts. This is in accord with the previous assignment of the *Calyptogena magnifica* symbiont by 5S rRNA analysis (13). More distantly related are the free-living bacteria *Thiothrix nivea* and the fluorescent pseudomonads (represented in Fig. 1 by *Pseudomonas aeruginosa* and *Pseudomonas putida*), also in accordance with 5S rRNA sequence analysis.

Where direct comparison is possible, 16S rRNA and 5S rRNA sequence analyses are consistent in placing the sulfur-metabolizing endosymbionts within the gamma subdivision of the purple bacteria. The 16S rRNA analysis, however, allows greater resolution of relationships within the symbiont group. For example, 16S rRNA analysis differs from 5S rRNA analysis in clearly demonstrating a close relationship between the symbionts of *R. pachyptila* and *Calyptogena magnifica*. We believe that these repositionings reflect the better definition of relationship achieved by using the larger (and more information-rich) 16S rRNA sequences.

The sequence data presented here show clearly that the presence of a particular symbiont population in a given host does not reflect the geographical location or environment in which the host animal is found. For example, the symbionts of *R. pachyptila* are not related most closely to the symbionts of the neighboring, vent-associated bivalves *Calyptogena magnifica* and *B. thermophilus*. Rather, the *R. pachyptila* symbionts are more closely related to those of the lucinid clams, which are found at great distances from the Galapagos vent sites. Similarly, the symbionts of *L. aequizonata* are closely related to the symbionts of *Codakia orbicularis*, even though the former is found at depths of about 500 m and temperatures of about 6°C in Pacific waters while the latter is found at depths from one to a few meters and temperatures that may reach 30°C in the tropical Atlantic.

The sulfur-based symbioses examined appear to be stable and species-specific relationships, and the symbionts of each are drawn from a restricted phylogenetic grouping. This suggests that these host and symbionts may have coevolved from one or a few common ancestral host-symbiont systems. The relationships among the symbionts of the three lucinid clam species examined parallel the taxonomic relationships of their respective hosts. That is, the 16S rRNAs of the symbionts of *L. aequizonata* and *L. annulata* are more similar to one another than either is to the 16S rRNA of the *Codakia orbicularis* symbiont; correspondingly, these two host *Lucinoma* species are believed to be more closely related to each other than either is to *Codakia orbicularis*. Similarly, the symbionts of all three lucinid clams are more closely related to one another than to the symbionts of *Calyptogena magnifica* or *B. thermophilus*. This again corresponds to the classification of the hosts; *Calyptogena magnifica* is usually placed in a different superfamily (Carditacea or Glossacea) than the lucinids (Lucinacea), and *B. thermophilus* is placed in a different order (Mytiloida) than the lucinids and *Calyptogena magnifica* (Veneroida). These observations are consistent with (although not proof of) the possibility that the lucinid hosts and their respective symbionts have coevolved from a common ancestral host-symbiont system. These findings are also in agreement with suggestions (19), based on the morphology of the lucinids, that sulfur-based symbiosis is indeed an ancient mode of nutrition that has played a critical role in the evolution of these mollusks.

Although common ancestry is suggested for the lucinid symbioses, neither the grouping of the symbionts of *R. pachyptila* with the symbionts of the lucinids (in which the hosts are classified in different phyla) nor the grouping of the symbionts of *Calyptogena magnifica* with the symbionts of *B. thermophilus* (in which the hosts are classified in different orders) can be easily explained in terms of coevolution from a common ancestral symbiosis. This suggests an independent origin of symbiosis in two or more of these host lines. Alternatively, some of these symbioses may have arisen in separate but nonindependent events by lateral transfer of symbionts between different host taxa. Comparisons of symbiont sequences from additional host species which are members of these host taxa and of 18S rRNA (eucaryotic) sequences from these and other host species should help resolve these questions.

If, as these data indicate, each host animal contains only one type of symbiont, and this type of symbiont is possessed uniformly throughout the host species, it follows that the mechanism for selection or transmission of the symbionts must be specific. The symbiont could be passed directly to the egg during spawning, or the symbiont could be selected by the host at some early stage in development. Alternatively, the symbionts might use a host-specific "infection" mechanism. The first possibility would suggest that the entire life cycle of the symbiont occurs within the host tissues. However, there has been no positive identification of symbionts within the gametes of these host species. The latter two possibilities suggest that the symbionts have free-living forms. It may now be possible to distinguish between the former and latter possibilities by using 16S rRNA sequence analysis. This technique can be used to verify or refute the identity of putative bacterial symbiont cultures isolated from the environment of the host or from its gametes. Additionally, unique sequences within the 16S rRNA molecule may be useful as targets for organism-specific hybridization probes, thus facilitating the search for symbionts in gametes, early larval stages, environmental samples, or enrichment cultures.

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