Analysis of Hydrothermal Vent–Associated Symbionts by Ribosomal RNA Sequences

Abstract. *Ribosomal RNA (rRNA) sequences were used to establish the phylogenetic affiliations of symbioses in which prokaryotes appear to confer sulfur-based chemoautotrophy on their invertebrate hosts. Two submarine hydrothermal vent animals, the vestimentiferan tube worm* Riftia pachyptila *and the clam* Calyptogena magnifica, *and a tidal-flat bivalve,* Solemya velum, *were inspected. 5S rRNA's were extracted from symbiont-bearing tissues, separated into unique forms, and their nucleotide sequences determined and related to other 5S rRNA's in a phylogenetic tree analysis. The prokaryotic symbionts are related to one another and affiliated with the same narrow phylogenetic grouping as* Escherichia coli *and* Pseudomonas aeruginosa. *The sequence comparisons suggest that* Riftia *is more closely related to the bivalves than their current taxonomic status would suggest.*

Evidence has accumulated that sulfur-oxidizing microbes can establish symbiotic relationships with certain invertebrates, producing "chemoautotrophic animals" (*1*). The putative symbionts were identified histologically and by the presence of high levels of certain Calvin cycle and sulfur-oxidative enzymes in the hydrothermal vent tube worm *Riftia pachyptila* (*2*), in which the bacteria fill a specialized organ, the trophosome. Similar associations were noted in the gill tissues of vent clams, *Calyptogena* spp. (*3*) and in the bivalve *Solemya* (*4*), which inhabits sulfide-rich tidal flats. However, efforts to characterize further the chemoautotrophic symbionts have been hampered by their resistance to cultivation.

One approach to characterizing uncultivable organisms is to establish their phylogenetic relationships to better-known organisms by appropriate macromolecular sequence comparisons (*5*). Ribosomal RNA's (rRNA) seem well-suited among cellular macromolecules for such analyses because of their ubiquitous distribution, functional constancy, high conservation of primary structure, and apparent freedom from artifacts of lateral transfer (*6*). We have characterized the symbioses mentioned above; 5*S* RNA was used because it is relatively easily isolated and analyzed and because its sequence for some 200 organisms and organelles are available for comparison.

Frozen samples of *Riftia pachyptila* (trunk wall and trophosome) and *Calyptogena magnifica* (gill tissue) and live specimens of *Solemya velum* were obtained (*7*); gill and foot tissues were excised and frozen immediately upon receipt. Total RNA was isolated from homogenized tissues extracted with hot phenol and sodium dodecyl sulfate and fractionated by polyacrylamide gel electrophoresis (Fig. 1A). After elution, the mixtures of 5*S* rRNA's (host and symbiont) were labeled at their 5′ termini with [γ-^{32}P]ATP (adenosine triphosphate) and polynucleotide kinase or at their 3′ termini with [5′-^{32}P]pCp (C, cytosine) and RNA ligase and were resolved by electrophoresis on 8 percent polyacrylamide sequencing gels (Fig. 1B). All 5*S* rRNA's were sequenced from both termini by enzymatic and chemical partial digestions (Fig. 1C). The derived sequences and the alignments used for phylogenetic analysis are shown in Fig. 2.

The relation of the symbiont 5*S* rRNA's to those of better-known organisms is best understood as a phylogenetic tree (Fig. 3). The branch lengths are proportional to evolutionary distance as estimated from sequence divergence (legend to Fig. 3). The prokaryotic symbionts fall into the "purple photosynthetic bacteria" grouping, so named because the deepest branchings in the group involve the purple photosynthetic pheno-

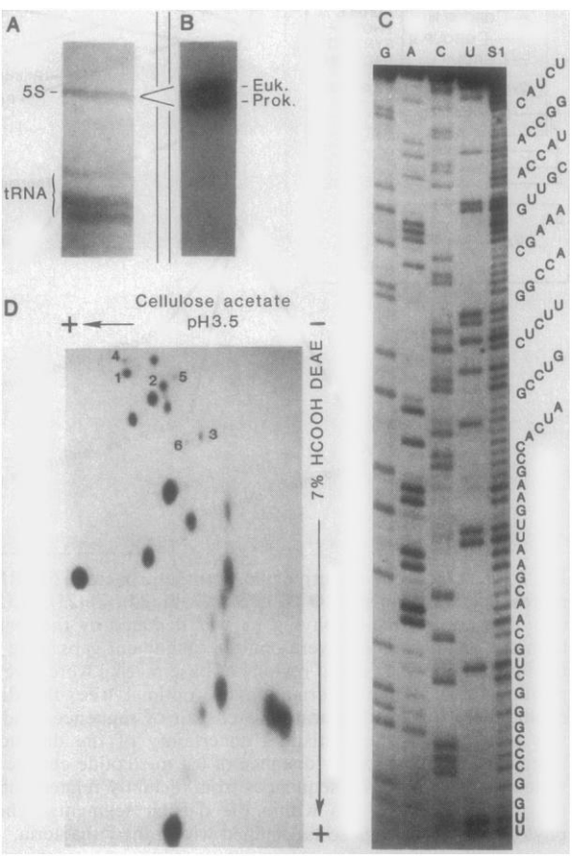

Fig. 1. *Solemya velum* 5*S* rRNA sequence and abundance analysis. Total RNA from *S. velum* gill tissue was fractionated on a preparative, 8 percent polyacrylamide gel containing 7*M* urea. RNA bands were visualized by ultraviolet shadow (A). The 5*S* rRNA zone was excised and eluted from the gel slice, and the rRNA's were recovered by precipitation. The mixture of 5*S* rRNA's was treated with alkaline phosphatase and labeled at the 5′ ends with polynucleotide kinase and [γ-^{32}P]ATP and at the 3′ termini with RNA ligase and [5′-^{32}P]pCp, as described (*17*). The end-labeled 5*S* rRNA's were then purified on 80-cm, 8 percent polyacrylamide sequencing gels (B). Individual 5*S*-sized bands were located by autoradiography, excised, and eluted from the gel slices. Sequence analysis was performed on the 5′ and 3′ end-labeled RNA's by enzymatic (*18*) and chemical (*19*) partial digestion. Terminal nucleotide analyses were performed by thin-layer chromatography or paper electrophoresis of nuclease P1 (5′-labeled) or alkali (3′-labeled) RNA hydrolyzates (*20*). (C) A representative autoradiograph of a portion of the *S. velum* host (eukaryotic) 5*S* rRNA was subjected to chemical sequencing. Measurement of the relative in vivo abundance of host and symbiont 5*S* rRNA's was performed by two-dimensional electrophoretic analysis of in vitro–labeled oligonucleotides resulting from complete digestion of total 5*S* rRNA (A) with either ribonuclease A (D) or T_1 (not shown). Total *S. velum* gill 5*S* rRNA was completely digested with ribonuclease A end-labeled with [γ-^{32}P]ATP and polynucleotide kinase. After labeling, an excess of 3′-uridylic acid and an additional 0.7 U of polynucleotide kinase were added, and the incubation was continued to remove the remaining [γ-^{32}P]ATP, which otherwise obscures the electropherogram. The end-labeled oligonucleotides were resolved by two-dimensional electrophoresis (*21*) and located by autoradiography (D). Spots 1 (GGGU), 2 (GAAGU), and 3 (GAAAGC) contain oligonucleotides unique to the *S. velum* host (eukaryotic) 5*S* rRNA, while spots 4 (GGGU), 5 (AGAAGU), and 6 (GGAAC) are unique to the symbiont (prokaryotic) 5*S* rRNA sequence (G, guanine; U, uracil; A, adenine). All spots were excised, and the radioactivity was determined by scintillation counting. Least squares analysis yielded the estimate that 19 percent of the 5*S* rRNA recovered from the *S. velum* gill tissue is of the prokaryotic type.

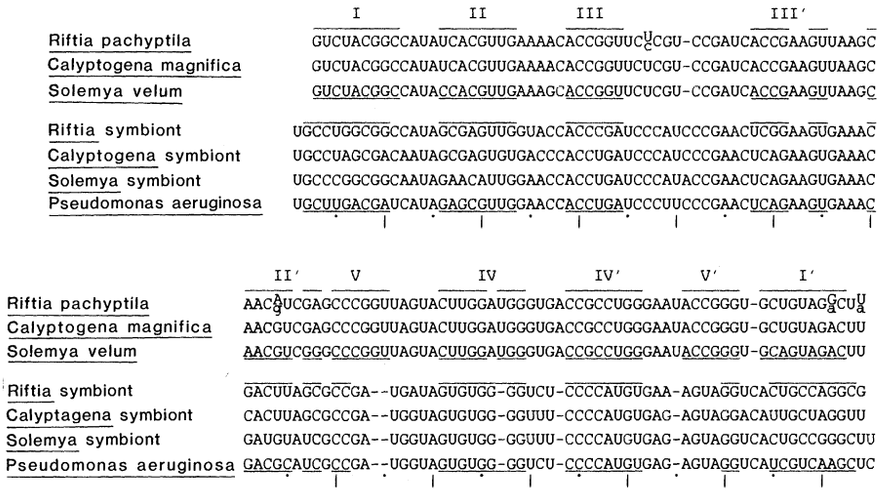

Fig. 2. Alignment of the 5*S* RNA sequence from *R. pachyptila, C. magnifica*, and *S. velum* and those of their respective symbionts with that of *P. aeruginosa* (*22*). Four minor sequence variants observed in the *Riftia* 5*S* rRNA are indicated by lower case letters. Regions of base pairing as defined by the 5*S* rRNA consensus secondary structure (*23*) are indicated by horizontal bars and labeled (*17*).

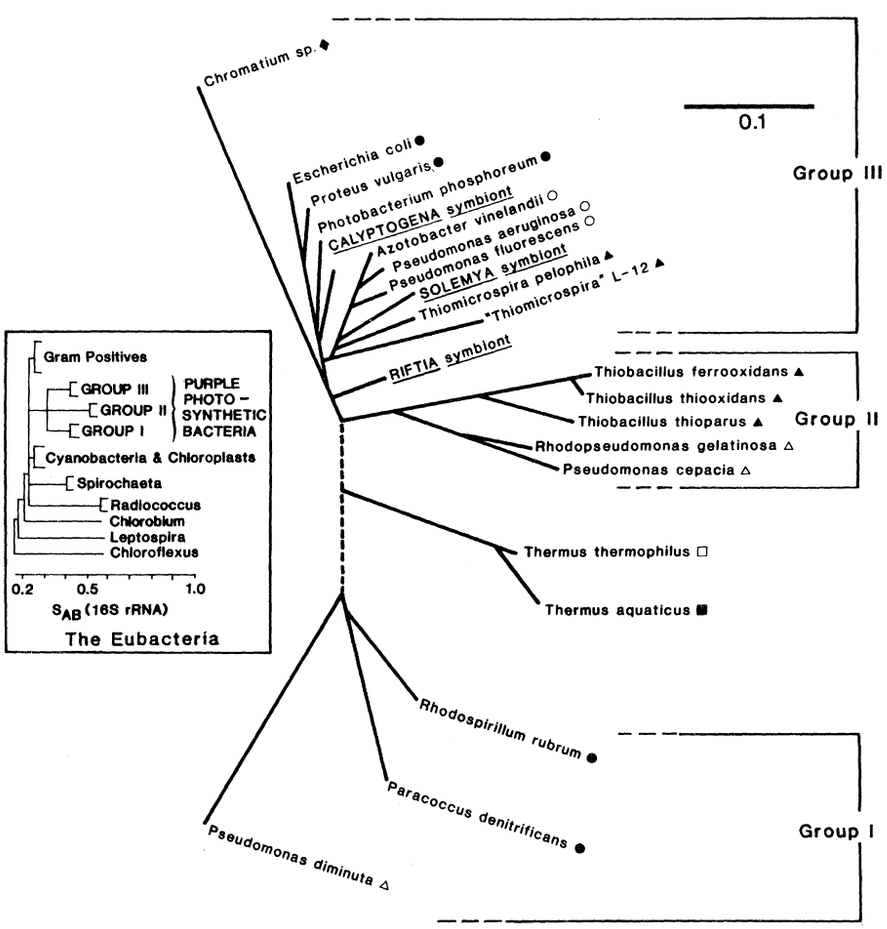

Fig. 3. The phylogeny of purple photosynthetic bacteria 5*S* rRNA's. The 5*S* rRNA sequences of representative organisms [● (*12*), ○ (*22*), ■ (*24*), □ (*25*), ▲ (*26*), △ (*27*), ◆ (*28*)] were aligned (*12*). The corresponding phylogeny was deduced by the matrix method (*12, 29*). Regions of terminal length variation were omitted, alignment gaps were treated as one-half the weight of the unpaired positions, and regions of base pairing were given half the weight of the unpaired positions (*30*). While determining the optimal tree, the difference between the estimated evolutionary distance separating each pair of sequences and the corresponding tree distance was weighted by the statistical uncertainty of the distance estimate (*12*). The scale bar represents an evolutionary distance of 0.1 nucleotide change per sequence position. Analyses which include 5*S* rRNA sequences from distantly related eubacteria (not shown) suggest that the root of the tree lies within the dashed segments. The inset (*6*) illustrates the major phylogenetic groupings so far defined within the eubacteria.

type (*8*). These symbionts are among the group III (*9*) purple photosynthetic bacteria, which also includes *Escherichia coli* and the *Pseudomonas* spp., *aeruginosa* and *fluorescens*. The sulfur-oxidizing symbionts straddle the division between the enterics and the typical pseudomonads, average 5*S* rRNA sequence homologies being 85 percent in representatives of both genera. This is approximately the extent of the homology that spans the Enterobacteriaceae. The closest relatives of the symbionts among the free-living, sulfur-oxidizing chemoautotrophs in our 5*S* sequence collection are *Thiomicrospira pelophila* and L-12 (*10*) (a *Thiomicrospira*-like hydrothermal vent isolate).

Because of the phylogenetic affinities of the symbionts, their fundamental biochemical properties must be similar to those of the enterics and pseudomonads. On the other hand, our and other analyses indicate that many organisms classified as *Pseudomonas* spp. on the basis of the usual microbiological tests are phylogenetically disparate (*11*); those considered in Fig. 3 are scattered throughout the purple photosynthetic line assemblage. Equally notable is the incidence of sulfur-based energy metabolism in the array shown and the close evolutionary association of well-known heterotrophs with chemoautotrophic phenotypes. Taxonomic hierarchies based on autotrophy and heterotrophy therefore appear to correlate poorly with phylogenetic associations.

The 5*S* rRNA sequence homologies of the host animals with other invertebrates (*12*) also are of interest. The *Riftia* 5*S* rRNA is as close in primary structure to that of bivalves [*Riftia:Calyptogena*, 0.98 ± 0.01 homology; *Riftia:Mytilus edulis*, 0.96 ± 0.02 homology with one standard deviation of the population (*13*)] as the bivalves are to each other (*Calyptogena:Mytilus*, 0.98 ± 0.01 homology). The *Riftia* 5*S* rRNA homology to that of the bivalves is higher than the 5*S* rRNA homology of the bivalves to that of the snail *Arion rufus*, which is also usually considered a member of the phylum Mollusca (*14*). The *Arion* 5*S* rRNA homologies with the *Riftia, Calyptogena*, and *Mytilus* 5*S* rRNA's are, respectively, 0.87 ± 0.03, 0.89 ± 0.03, and 0.90 ± 0.03. These relations call into question the evolutionary relevance of assigning *Riftia* (and other pogonophorans, if *Riftia* is representative of that group) to a singular phylum (*14*). The close evolutionary positioning of *Riftia* and *Calyptogena* might seem heretical, given the morphological differences. However, the rRNA's evolve slowly: a

single nucleotide change in 5*S* rRNA represents millions of years of evolution. Moreover, there is evidence in the fossil record for substantial morphologic discontinuity occurring in the molluscs, within relatively short geologic time spans (*15*).

Although microscopic examinations have indicated that the invertebrates we considered might harbor more than one type of symbiont (*16*), we recovered only one end-labeled, prokaryotic 5*S* rRNA from each. However, different 5*S* rRNA's vary in their efficiencies of end-labeling because of varying secondary structures at their termini, so it remained possible that other 5*S* rRNA's present in low abundance were not detected. This uncertainty was overcome by first digesting the mixed, unlabeled 5*S* rRNA population with ribonuclease T_1 or A, end-labeling the resultant oligonucleotides, and resolving the products by two-dimensional electrophoresis (Fig. 1D). Oligonucleotides are labeled uniformly with 5′-^{32}P, so that the relative radioactivities of species-specific oligonucleotides (defined by the RNA sequences) establish the 5*S* rRNA stoichiometries and are a test for the presence of unsuspected RNA's. Such analyses revealed no novel 5*S* rRNA's, and therefore we conclude that the symbiont populations in these animals are at least 90 percent homogeneous. In the case of the *Riftia* trophosome, we inspected material from two regions of the worm—the anterior one-third and the extreme posterior. The 5*S* rRNA's isolated from both segments were identical. By the criterion of 5*S* rRNA oligonucleotide content, presuming equivalent extraction efficiencies, the prokaryotic symbionts contribute more than 75 percent of the *Riftia* trophosomal ribosomes. The symbiont contributions to the *Calyptogena* and *Solemya* gill tissues are about 75 and 20 percent, respectively. In the one case examined (*Solemya*), no bacterial 5*S* rRNA could be extracted from nongill tissue.

The techniques used to characterize these two-component symbioses are applicable to more complex microbial populations as well. Isolation and analysis of rRNA's (or their genes) from diverse environments could be a powerful and general tool for the microbial ecologist.

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30. In evaluating the agreement of phylogenies with sequence data, it is assumed that all of the sequence positions are subject to independent base variation. Base-paired nucleotides are the most obvious exceptions to this assumption; if one nucleotide is known, its pairing partner can be predicted reliably. We have accounted for this redundancy by decreasing the weighting of paired positions to half that of unpaired positions. This data treatment results in more self-consistent phylogenies; that is, a better overall agreement between the observed pairwise distances and those implicit in the "optimal" trees.
31. We thank H. Jannasch, P. Comita, and C. Wirsen (Woods Hole Oceanographic Institution) for samples of *R. pachyptila* and *C. magnifica*; C. Cavanaugh (Harvard University) for assistance with *S. velum*; T. Schmidt (Ohio State University) and D. Nelson (Woods Hole Oceanographic Institution) for assistance in growing some reference organisms; and C. Woese (University of Illinois) and G. Fox (University of Houston) for supplying some unpublished 5*S* rRNA reference sequences. Supported by NIH research grant GM20147 (N.R.P.).
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Chromosome Organization and Heterochromatin Elimination in *Parascaris*

Abstract. *A cytological analysis by modern banding techniques of gonial metaphases in two* Parascaris *forms that have been considered varieties but now seem to be two species* [P. univalens *(karyotype* 2n = *2) and* P. equorum *(karyotype* 2n = *4)] reveals a different chromosome organization in each.* Parascaris univalens *chromosomes contain only terminal heterochromatin, while* P. equorum *chromosomes also contain intercalary heterochromatin. In the somatic cells of both species during early embryogenesis, chromatin diminution occurs in and consists of the elimination of all heterochromatin independent of its localization in the chromosomes.*

Past studies on the nematode *Parascaris equorum* revealed the existence of two morphologically indistinguishable varieties, one with a single chromosome pair (univalens variety) and the other with two chromosome pairs (bivalens variety) (*1, 2*). The results of a series of electrophoresis studies have revealed a degree of isolation between the univalens and bivalens varieties, suggesting that they belong to two different diploid species for which the names *P. equorum* (karyotype $2n = 4$) and *P. univalens* (karyotype $2n = 2$) were proposed (*3*). The chromosomes of these two species, which appear similar in germ-cell metaphases when conventional staining techniques are used, are polycentric or holocentric (*4, 5*) and undergo chromatin diminution during development (*1*).

Diminution consists of chromosome fragmentation and the elimination of heterochromatin in presomatic cells. The result is the generation of a difference in chromosome number and quality between germ and somatic cells. Because it has been shown that the maintenance of germ line–limited chromatin is necessary for the germ line quality of blastomeres, diminution seems to be a good example of cell differentiation associated with genomic change (*6, 7*).

Attempts to characterize germ line–limited DNA in various species of *Ascaris* have produced conflicting results. Some studies suggest that this DNA is