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Characterization of Chemoautotrophic Bacterial Symbionts in a Gutless Marine Worm (Oligochaeta, Annelida) by Phylogenetic 16S rRNA Sequence Analysis and In Situ Hybridization

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The phylogenetic relationships of chemoautotrophic endosymbionts in the gutless marine oligochaete *Inanidrilus leukodermatus* to chemoautotrophic ecto- and endosymbionts from other host phyla and to free-living bacteria were determined by comparative 16S rRNA sequence analysis. Fluorescent in situ hybridizations confirmed that the 16S rRNA sequence obtained from these worms originated from the symbionts. The symbiont sequence is unique to *I. leukodermatus*. In phylogenetic trees inferred by both distance and parsimony methods, the oligochaete symbiont is peripherally associated with one of two clusters of chemoautotrophic symbionts that belong to the gamma subdivision of the *Proteobacteria*. The endosymbionts of this oligochaete form a monophyletic group with chemoautotrophic ectosymbionts of a marine nematode. The oligochaete and nematode symbionts are very closely related, although their hosts belong to separate, unrelated animal phyla. Thus, cospeciation between the nematode and oligochaete hosts and their symbionts could not have occurred. Instead, the similar geographic locations and habitats of the hosts may have influenced the establishment of these symbioses.

Symbioses between chemoautotrophic bacteria and marine invertebrates, first described nearly 15 years ago for the giant tube worm *Riftia pachyptila* from deep-sea hydrothermal vents (5, 12), are now known to occur in over 100 species from five host phyla. These symbioses are widespread in marine habitats, ranging from hydrothermal vents in the deep sea to sulfide-rich sediments in intertidal mudflats (reviewed in reference 15). The degree of integration within these partnerships also covers a broad range, from ecto- to endosymbiotic associations. In chemoautotrophic ectosymbioses, the bacterial partners occur externally on the host's surface; in endosymbioses, the bacteria exist internally as either extra- or intracellular symbionts. The bacterial symbionts are hypothesized to provide the animal host with a source of nutrition via chemosynthesis by using reduced inorganic sulfur compounds as an energy source for autotrophic fixation of carbon dioxide into organic compounds.

Chemoautotrophic symbionts have not been cultured from their hosts, nor has a free-living life stage of the symbionts been isolated from the environment. Comparisons of rRNA sequences have become particularly useful in characterizing symbionts, because these sequences can be obtained without isolating bacteria from their habitat or host (31). Comparisons of 16S rRNA bacterial sequences (7, 8, 26) show that the chemoautotrophic symbionts fall within the gamma subdivision of the *Proteobacteria*, one of the 11 major groups of the *Bacteria* (31).

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Within the animal phylum Annelida, bacterial symbioses appear to be widespread, ranging from ecto- to endosymbiotic associations in marine oligochaete worms (10, 18). Chemoautotrophic endosymbioses occur within a phylogenetically tightly related group of gutless oligochaetes of only two genera, *Inanidrilus* and *Olavius*, within the subfamily Phallodrilinae (11). *Inanidrilus leukodermatus* is one of the best studied gutless oligochaetes and is commonly found in inter- and subtidal calcareous sands of coral reefs around Bermuda and along the coast of Belize. Bacterial symbionts occur extracellularly in a thick layer just below the cuticle between extensions of the epidermal cells (Fig. 1). In *I. leukodermatus*, as in other gutless oligochaetes, two bacterial morphotypes, a smaller, rod-shaped form and a larger oval form with conspicuous polyhydroxybutyric acid granules in its cytoplasm (17) can be distinguished. The chemoautotrophic nature of these bacterial symbionts is indicated by the presence of enzymes that are characteristic of autotrophic organisms (13) and a stable carbon isotope composition in the same range as that of free-living chemoautotrophic bacteria (16). Symbiont transmission is assumed to occur vertically through genital pads that are packed with bacteria and transmit symbionts from the parental worm to the fertilized egg (17).

In this study, we determined the phylogenetic relationships of the symbiont from *I. leukodermatus* to chemoautotrophic symbionts from other host phyla, as well as to surface-attached and free-living bacteria, by comparative 16S rRNA analysis. In situ hybridization was used to confirm that the sequence obtained by PCR originated from the oligochaete symbiont. While the phylogenies of chemoautotrophic symbionts from three animal phyla (mollusks, vestimentiferans, and nematodes) have been well studied, symbionts from oligochaete hosts have not been previously characterized at the molecular level.

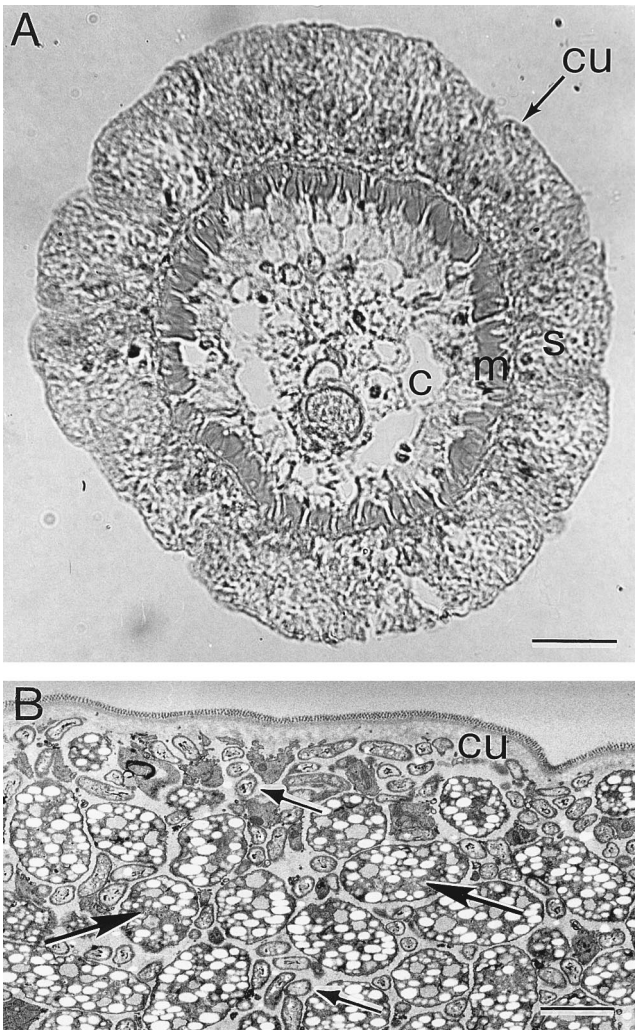

FIG. 1. Gutless oligochaete *I. leukodermatus*. (A) Light micrograph of cross section of a worm. Bar = 20 μm. (B) Transmission electron micrograph of symbiont-containing region just below the cuticle. Note smaller and larger symbiont morphotypes (smaller and larger arrows, respectively). Bar = 2 μm. c, coelomic cavity; m, muscle tissue; s, symbiont-containing region between cuticle and epidermis. cu, cuticle.

MATERIALS AND METHODS

Specimen collection. *I. leukodermatus* worms were collected in November 1992 by scuba divers at a depth of approximately 5 m at Flatts Inlet, Bermuda. Worms were extracted from sediment by decantation with seawater and identified under a microscope.

Light microscopy and transmission electron microscopy. *I. leukodermatus* worms were fixed immediately after collection in Trump's fixative (21). After dehydration in an acetone series, specimens were embedded in Spurr resin (30) and sectioned on an ultramicrotome. For light microscopy, thin sections (0.5 to 1.0 μm) were stained with toluidine blue. For electron microscopy, ultrathin sections were stained with uranyl acetate and lead citrate and examined with a Zeiss EM 902A.

DNA preparation. Approximately 100 *I. leukodermatus* individuals were pooled after identification and stored for 2 days at −20°C. Pooled worms were homogenized in a glass tissue grinder in 500 μl of lysozyme buffer (50 mM Tris, 0.1 M EDTA, 0.08% Triton X [pH 8.0]). The homogenate was incubated with lysozyme (final concentration, 12.5 mg ml^{-1}) for 3 h at 4°C and then with proteinase K (final concentration, 1 mg ml^{-1}) for 6 h at 22°C. Cell debris was precipitated by centrifugation for 10 min (14,000 × *g*, 25°C), and DNA was extracted from the supernatant by phenol-chloroform extraction and ethanol precipitation (27).

PCR amplification and sequencing. DNA sequences were determined directly from PCR amplification products, as previously described (7), with biotinylated primers (27f or 1492r) specific for amplification of *Bacteria* 16S rRNA genes. Single-stranded DNA, prepared from amplification products with streptavidin-coated magnetic beads (Dynal), was sequenced directly by using Sequenase V.2 (United States Biochemical) according to the recommended protocol.

Sequence analysis. Sequence data management and phylogenetic analyses were performed with the Genetic Data Environment sequence editor (28) and a SPARC station IPC (Sun Microsystems). The *I. leukodermatus* symbiont sequence was compared with the 16S rRNA sequences of symbiotic, pathogenic, and free-living bacteria that were available from the Ribosomal Database Project (RDP) (20) (see Table 1). A smaller data set of 798 nucleotide positions was used to include two taxa (*Thiothrix nivea* and *Thiobacillus ferrooxidans* m1) for which less sequence information was available. A larger data set without these taxa included 1,035 nucleotide positions in order to maximize the number of characters used. Sequence alignment, phylogenetic distance and parsimony methods, and bootstrap analyses were performed as described previously (7), by using the distance correction algorithms of Jukes and Cantor and the tree-fitting method of DeSoete (6) for distance analyses and the Phylip 3.5 program (14) for parsimony and bootstrap analyses. For each data set, 100 bootstrap replicates were used. Bootstrap values greater than 50% (50 of 100 trees) are given but are considered to support the grouping of organisms in an associated node only at values greater than 75% (33).

Fluorescent in situ hybridizations. Fluorescent in situ hybridizations were performed to confirm that the 16S rRNA sequence from *I. leukodermatus* originated from endosymbionts and not from bacteria on the surfaces of worms or from contaminants. An oligodeoxynucleotide probe specific to the *I. leukodermatus* symbiont was designed from a highly variable region of the 16S rRNA gene and designated *I. leu.* (sequence, TCTGACTTATTCGGCCGCCTAC; *Escherichia coli* positions 581 to 602). This region differed by at least 3 nucleotides from all other known chemoautotrophic symbiont sequences. The probe was checked against sequences entered in the GenBank database by using BLAST (1) and against small-subunit rRNA sequences entered in the RDP database by using CHECK-PROBE (20); it contained at least two mismatches to all other entered sequences. A negative control probe was designed from the same region as the *I. leu.* probe; it contained four mismatches to the targeted *I. leukodermatus* sequence. This probe was complementary to the symbiont sequence of another gutless oligochaete species (*Olavius tantalus* [10a]) and designated *O. tan.* (sequence, TCTGACTGGTCCGACCACCTAC; *E. coli* positions 581 to 602). A (eu)bacterial universal probe (sequence, GCTGCCTCCC GTAGGAGT; *E. coli* positions 338 to 356) (2) was used as a positive control. Probes were biotinylated as described previously (7) and purified after synthesis by polyacrylamide gel electrophoresis (27).

I. leukodermatus specimens were prepared for in situ hybridizations by fixation in phosphate-buffered seawater (pH 7.4) with 4% formaldehyde, then dehydrated in an ethanol series, and embedded in paraffin. Samples were sectioned serially (6 μm) onto slides coated with 3-aminopropyltriethyloxysilane (Sigma), deparaffinized in xylene, and rehydrated in an ethanol series. Sections were incubated for 10 to 15 min in 0.2 M HCl, rinsed for 10 min in prehybridization buffer (20 mM Tris [pH 8.0]), incubated for 3 min at 37°C in 0.5 μg of proteinase K (Sigma) per ml of prehybridization buffer, rinsed as described before, and postfixed for 5 min in 3.7% formaldehyde in prehybridization buffer. Hybridizations were carried out for 4 h at 42°C in hybridization buffer consisting of 5× SET (0.75 M NaCl, 5 mM EDTA, 0.1 M Tris buffer [pH 7.8]), 0.2% bovine serum albumin, and 0.025% sodium dodecyl sulfate, with 50 ng of biotinylated probe per section. At the end of hybridization, sections were washed three times for 5 min each in 0.2× SET at 42°C and fluorescently labeled on ice with 20 μg of avidin-conjugated fluorescein (Vector) ml^{-1} in binding buffer (0.1 M $NaHCO_3$, 1.45 M NaCl), and excess label was washed off three times for 10 min each in 0.1× binding buffer on ice. In an attempt to enhance the fluorescent signal, a sandwich method (as modified for oligonucleotides by Distel et al. [9] from the method of Pinkel et al. [25]) was used on some sections by incubation in successive rounds of biotinylated antiavidin (Vector) and avidin-fluorescein. Control sections were hybridized without a probe to check background autofluorescence, as well as fluorescence caused by nonspecific binding of avidin-conjugated fluorescein. Sections were mounted in a nonfluorescent medium (Vectashield; Vector) and viewed with a Zeiss Axioskop microscope and a Bio-Rad MRC600 confocal microscope.

Nucleotide sequence accession number. The symbiont sequence from *I. leukodermatus* was submitted to GenBank under accession number U24110.

RESULTS

PCR amplifications with *Bacteria* 16S rRNA primers produced a single DNA band of ~1.5 kb, as detected by agarose gel electrophoresis. Although a pooled sample of 100 *I. leukodermatus* worms was used, only one unambiguous sequence was detectable after direct sequencing of PCR products. This result implies that one bacterial species is dominant within these worms. However, differential lysis of the smaller and larger symbiont morphotypes during DNA preparation cannot be excluded.

In situ hybridizations confirmed that the 16S rRNA sequence originated from the *I. leukodermatus* symbiont, not from a bacterial contaminant. The species-specific *I. leu.* probe hybridized specifically to the region between the cuticle and the epidermis, as determined by the bright fluorescence visible in the subcuticular region of the worm's body (Fig. 2B). Light microscopy and transmission electron microscopy show that this location corresponds to the site of bacterial symbionts (Fig. 1). A negative control, the *O. tan.* probe, did not hybridize to the subcuticular region of *I. leukodermatus* or to any other region of the worm (Fig. 2C). The fluorescent signal from the *I. leu.* probe was similar to that of the positive control, a *Bacteria* universal probe (Fig. 2A). While the larger symbiont morphotype can be readily identified with the *I. leu.* and *Bacteria* probes on the basis of hybridization patterns that are consistent with the size, shape, and distribution of these larger bacteria, the smaller symbiont morphotype was not unambiguously identified by either epifluorescence or confocal microscopy. Attempts to detect the smaller morphotype through enhancing the fluorescent signal by a sandwich method (9) (using avidin-fluorescein and biotinylated antiavidin in successive labeling rounds) caused background fluorescence to increase to such high levels that a specific signal was no longer detected. The difficulty in detecting a hybridization signal from the smaller symbiont morphotype may have been due to differential preservation or low concentrations of the probe target, rRNA. The latter alternative is supported by the fact that division stages have not been observed for the smaller morphotype but are regularly visible for the larger morphotype.

The phylogenetic relationships of the *I. leukodermatus* symbiont to other chemoautotrophic symbionts and free-living bacteria were determined by comparing their 16S rRNA sequences by distance and parsimony methods on data sets of 798 and 1,035 nucleotide positions. The smaller data set was used to include two taxa for which less sequence data were available (*Thiothrix nivea* and *Thiobacillus ferrooxidans* m1). The resulting trees from these two data sets were essentially the same by distance and parsimony methods, and significant bootstrap values (>75%) were not influenced by the size of the data set, so only the distance tree with 798 characters is shown here (Fig. 3).

Phylogenetic analysis of the *I. leukodermatus* symbiont sequence indicates that it falls within the gamma subdivision of the *Proteobacteria* and clusters with other chemoautotrophic symbionts (Fig. 3). The oligochaete symbiont forms a monophyletic group with the ectosymbiont of the marine nematode *Laxus* sp. (subfamily Stilbonematinae) on the basis of bootstrap values of 100% for both distance and parsimony trees. The two sequences differ by only 1.5% (Table 1). The oligochaete and nematode symbionts are peripherally associated (distance and parsimony bootstrap values, below 50%) with a group of chemoautotrophic symbionts from other marine invertebrates, the bivalves *Solemya velum* and *S. reidi*, the vestimentiferan tube worm *R. pachyptila*, and bivalves from the superfamily Lucinacea (left grey box in Fig. 3). A second group of chemoautotrophic symbionts from bivalve hosts of the families Mytilidae and Vesicomyidae (right grey box in Fig. 3) forms a monophyletic group (bootstrap values, 100% for both distance and parsimony trees) that is distinct from the other cluster of chemoautotrophic symbionts. Phylogenetic distance and parsimony analyses, as well as the phylogenetic tree from the RDP database (20), show that the following free-living, sulfur- and/or iron-oxidizing bacteria consistently fall in the vicinity of the chemoautotrophic symbionts: *Thiobacillus ferroxidans*, *Ectothiorhodospira shaposhnikovii*, *Thiothrix nivea*, a *Thiomicrospira* sp., and *Thiomicrospira thyasirae*. However,

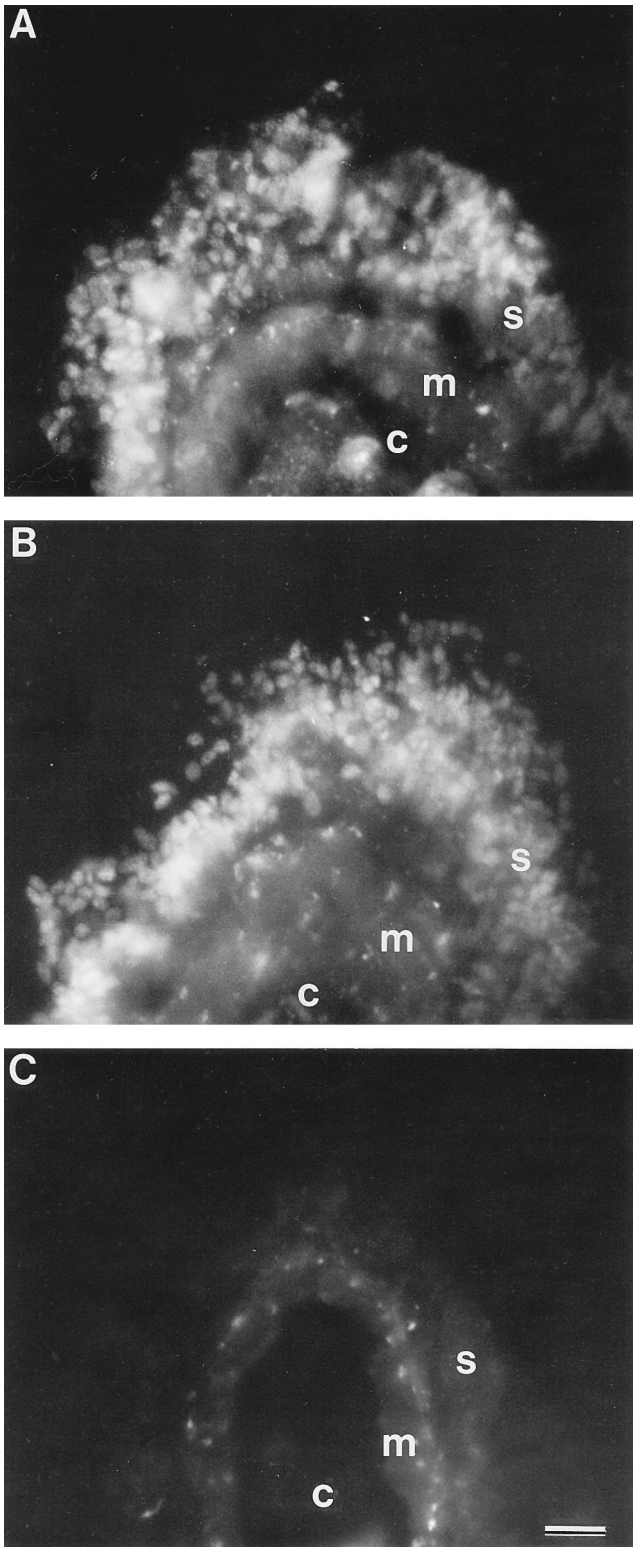

FIG. 2. Epifluorescence micrographs from in situ hybridizations. The body wall of the worm with the symbiont-containing region is shown. Probes: universal *Bacteria* (A), species-specific *I. leu.* (B), and negative control, *O. tan.* (C). c, coelomic cavity; m, muscle tissue; s, symbiont-containing region. Bar = 10 μm.

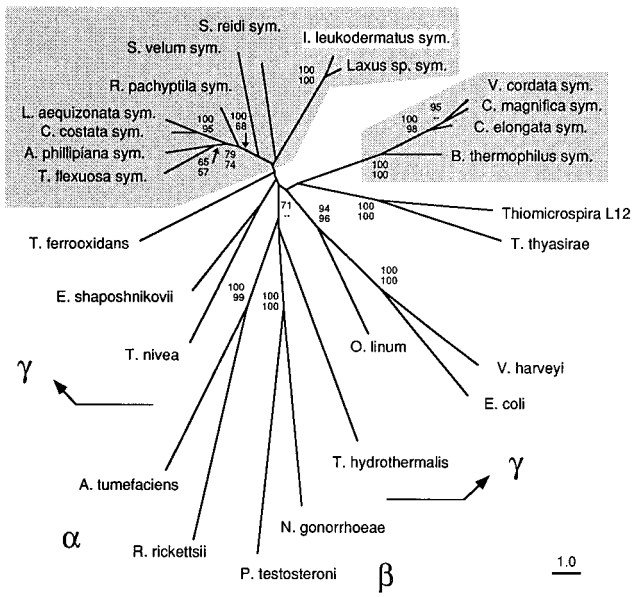

FIG. 3. Evolutionary distance tree (based on 16S rRNA sequences) comparing symbionts (sym.) from the gutless oligochaete *I. leukodermatus* with free-living bacteria and other chemoautotrophic symbionts. Bootstrap values greater than 50% are shown, with upper and lower values representing those from distance and parsimony analyses, respectively. A total of 798 nucleotide positions were compared. Shaded boxes represent sequences from chemoautotrophic symbionts. *Proteobacteria* subdivisions (α, β, and γ) are also indicated. Bar = 1 nucleotide substitution per 100 nucleotide positions. Full species names are listed in Table 1. All sequences were obtained from the RDP database (20).

bootstrap values between these deep-branching, free-living bacteria and the chemoautotrophic symbionts are below 50% in all nodes; thus, their relationships cannot be resolved.

DISCUSSION

The 16S rRNA sequence determined in this study is concluded to originate from the *I. leukodermatus* symbiont on the basis of PCR and in situ hybridization results. Direct sequencing of PCR products obtained from a homogenate of 100 pooled worms resulted in one unambiguous sequence, even in regions of the 16S rRNA gene that are known to be highly variable. This result suggests that the two bacterial morphotypes represent structural dimorphism of a single bacterial symbiont species in *I. leukodermatus*. The two bacterial morphotypes occur in approximately equal amounts, as observed by transmission electron microscopy (Fig. 1B). Therefore, the relative numbers of these two morphotypes alone do not explain the failure to detect a second genotype. Structural polymorphism is not unusual in bacteria despite molecular evidence of only one monospecific population and has been observed for the chemoautotrophic symbionts of bivalves and tube worms (8, 15), the chemoautotrophic bacterium *Thiomicrospira thyasirae* (32), and certain pathogens, e.g., *Coxiella burnetii* and members of the genus *Chlamydia* (3). While the results from direct sequencing of PCR products suggest the presence of only one symbiont genotype in *I. leukodermatus*, the possibility that a second symbiont species occurs in these worms cannot be ruled out, since the smaller morphotype was not resolved by in situ hybridization with the symbiont-specific probe.

The single 16S rRNA symbiont sequence obtained from these worms is unique to *I. leukodermatus* and differs from those found in symbionts of other host species and free-living

TABLE 1. Sequence identities and evolutionary distances between the *I. leukodermatus* symbiont and representative *Proteobacteria* species used in analysis of 16S rRNA sequences[a]

Organism	% Identity	% Evolutionary distance
Gamma subdivision		
Chemoautotrophic symbionts[b]		
Laxus sp.	98.5	1.5
Solemya reidi	91.7	8.8
Solemya velum	91.1	9.5
Riftia pachyptila	92.7	7.6
Lucinoma aequizonata	91.0	9.6
Codakia costata	91.5	9.0
Anodontia phillipiana	90.7	9.9
Thyasira flexuosa	90.4	10.3
Vesicomya cordata	86.7	14.6
Calyptogena magnifica	87.2	14.0
Calyptogena elongata	87.7	13.4
Bathymodiolus thermophilus	87.0	14.3
Free-living bacteria		
Thiobacillus ferrooxidans m1	89.3	11.5
Thiobacillus hydrothermalis r3	87.6	13.6
Ectothiorhodospira shaposhnikovii	90.6	10.0
Thiothrix nivea JP2	88.2	12.8
Thiomicrospira sp. strain L12	88.0	13.1
Thiomicrospira thyasirae TG-2	86.8	14.5
Vibrio harveyi	85.8	15.7
Escherichia coli	85.5	16.2
Oceanospirillum linum	88.2	12.8
Beta subdivision		
Pseudomonas testosteroni	82.0	20.6
Neisseria gonorrhoeae B5025	84.7	17.1
Alpha subdivision		
Rickettsia rickettsii R	82.3	20.1
Agrobacterium tumefaciens	85.2	16.5

[a] Values are for a data set with 798 nucleotide positions. All sequences were drawn from the RDP database (20). Sequence identities and evolutionary distances (with the Jukes and Cantor correction) were calculated by using the Genetic Data Environment program.

[b] For each symbiont, the name of the host is given.

bacteria. (Here we define free-living bacteria as cultivable, nonsymbiotic bacteria. It is unknown whether chemoautotrophic symbionts also exist as free-living forms in plankton or sediments.) The *I. leukodermatus* symbiont shares 98.5% sequence identity with the ectosymbiont of the marine nematode *Laxus* sp. (26), and both parsimony and distance analyses place the oligochaete and nematode symbionts in a monophyletic group (Fig. 3). Such a close relationship between these two symbionts is surprising because in most cases in which chemoautotrophic symbionts form monophyletic groups, their hosts also form a taxonomic unit, suggesting that cospeciation between symbiont and host occurred (7). In contrast to the extremely close evolutionary relationship between the oligochaete and nematode symbionts, their hosts fall into two animal phyla. Morphological and molecular analyses show that the closest symbiotic relatives of the oligochaete annelids are vestimentiferan (e.g., *R. pachyptila*) and pogonophoran tube worms (19, 29), whereas the nematodes are often placed in a group of phyla, termed Nemathelminthes, that is considered to be very distant from annelids, vestimentiferans, and pogonophores (23). Thus, if cospeciation had occurred, the oligochaete symbiont would be expected to be more closely related to the symbiont of *R. pachyptila* than to the nematode symbiont.

Given the close evolutionary relationship of the oligochaete and nematode symbionts in such distantly related hosts, it is

tempting to speculate on the time frame involved in the establishment of these symbioses. Separation of these two symbionts from a common free-living ancestor could have occurred prior to establishment of these symbioses, i.e., separate but closely related free-living bacteria could have associated with hosts very distantly related from one another. In such a case, the node between the oligochaete and nematode ancestors would represent a time point prior to establishment of these symbioses. Although it is difficult to calibrate the molecular clocks of bacteria, substitution rates of 0.01 to 0.02 nucleotides per site per 50 million years have been estimated (4, 22, 24). Thus, the establishment of an extracellular endosymbiosis with full reduction of the oligochaete gut may have taken only 20 to 40 million years (based on a difference of ~0.008 nucleotides per site between the oligochaete symbiont and its ancestral node with the nematode symbiont [Fig. 3]). Alternatively, speciation of the oligochaete and nematode symbionts could have occurred after establishment of these symbioses. According to this scenario, an ancestral, free-living bacterial species was able to form an association with very distantly related animals but consequently diverged into the nematode ectosymbiont and oligochaete endosymbiont. In such a case, the oligochaete and nematode symbioses could have become established anywhere along their long common branch (Fig. 3) and so may be considerably older than the 20 to 40 million years suggested by the divergence of their symbionts. After this long shared period, in which the hosts must have recruited their symbionts from a common free-living bacterial population, the divergence of the oligochaete symbiont from the nematode symbiont may be explained by the introduction of vertical transmission of symbionts via oligochaete eggs (17). Unfortunately, there is no fossil record for either oligochaetes or nematodes, so calibration of host evolution and symbiont evolution is not possible. Regardless of the time frame involved in establishment of these symbioses, the similar geographic locations and/or environments of the hosts must have been essential in the evolution of these associations, as their symbionts clearly had a common ancestor until relatively recently. Indeed, modern populations of *I. leukodermatus* and this *Laxus* sp. often co-occur in sulfide-rich sediments of Bermuda and Belize, suggesting that these symbioses were established in one of the ways discussed above.

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