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 (Oligochaeta, Annelida) have been 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, coexistence 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).

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 Phalldrilinae (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.
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 (20) and a SPPC II workstation 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) (Table 1). A smaller data set of 706 nucleotide positions was used to include two taxa (Thiodoxis nivea and Thiothrix ferruginosa) 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. Seed parsimony, 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 DeSoto (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, TCTGACCTATTGCGGCGCTTAC; Escherichia coli positions 581 to 602). This region differed by at least 3 nucleotides from all other known chemosymbiotic 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 (Olivius tanaitus [10a]) and designated O. tan. (sequence, TCTGACTGCTGGACCACTAC; E. coli positions 581 to 602). (A) 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. (B) Confocal micrograph of cross section of a worm. Bar = 20 μm. (C) Transmission electron micrograph of symbiont-containing region and free-living bacterium that were available from the Ribosomal Database Project (RDP) (20)(see Table 1). A smaller dataset of 706 nucleotide positions was used to include two taxa (Thiodoxis nivea and Thiothrix ferruginosa) for which

**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 EM902A.

**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) for 3 h at 4°C and then with proteinase K (final concentration, 1 mg/ml) 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.

**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 superfamilies 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 ferrooxidans*, *Ectothiorhodospira shaposhnikovii*, *Thiobrix nivea*, a *Thiomicrospira* sp., and *Thiomicrospira thyasirae*. However,
The presence of only one symbiont genotype in results from direct sequencing of PCR products suggest the those found in symbionts of other host species and free-living these worms is unique to those worms.

Probe.

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 bacteria. (Here we define free-living bacteria as cultivable, nonsymbiotic bacteria. It is unknown whether chemosynthetic symbionts also exist as free-living forms in plankton or sediments.) The I. leukodermatus symbiont shares 98.5% sequence identity with the closest symbiotic relative 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 chemosynthetic 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 vestimentiferans (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 divergence 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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