Phylogenetic Characterization and In Situ Localization of the Bacterial Symbiont of Shipworms (Teredinidae: Bivalvia) by Using 16S rRNA Sequence Analysis and Oligodeoxynucleotide Probe Hybridization†

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It has been proposed that a bacterium isolated from the gills of shipworms (teredinid mollusks) is, by virtue of its ability both to degrade cellulose and to fix dinitrogen, the symbiont that enables these mollusks to utilize wood as their principal food source. The phylogenetic affiliation of four of these bacteria isolated from wood-boring bivalve mollusks was determined by 16S rRNA sequence analysis by using the reverse transcriptase method with six oligodeoxynucleotide primers. The four bacterial strains tested had indistinguishable 16S rRNA sequences, supporting the previous conclusion, based on phenotypic characterization, that these isolates represent a single species. Evolutionary distance matrix analysis of the RNA sequence indicated that the bacterial symbiont falls within the gamma-3 subdivision of the *Proteobacteria* and is distinct from other known bacterial genera. In situ localization of the bacterial symbiont in tissue sections of the shipworm *Lyrodus pedicellatus* was determined by using a 16S rRNA-directed oligodeoxynucleotide hybridization probe specific for the bacterium isolated from shipworm gill tissue. Fluorescence microscopy showed that the specific probe bound to *L. pedicellatus* tissue at sites coincident with the location of symbiont cells and that it did not bind to other host tissues. This technique provided direct visual evidence that the cellulolytic, nitrogen-fixing bacterial isolates were the symbionts observed within the gill of *L. pedicellatus*.

The wood-boring mollusks of the family Teredinidae are host to a bacterial symbiosis which is of considerable economic and ecological importance. This symbiosis involves the presence of dense populations of gram-negative bacteria that reside within a specialized tissue, originally referred to as the gland of Deshayes, that is found in the gills of the molluscan host (19). These symbionts are purported to enable the teredinid bivalves to survive on a diet composed entirely of wood by providing the host with cellulolytic enzymes to digest wood and the nitrogenase complex to fix dinitrogen and help balance its nitrogen budget (8, 25).

The teredinid mollusks (commonly known as shipworms) are widely distributed throughout the world's oceans, with at least 14 genera currently recognized (24). They are highly efficient consumers of wood in the marine environment, where they play a major role in the destruction of wooden structures and in the recycling of organic carbon from cellulose. The latter may be of considerable consequence, since cellulose is among the most abundant biomolecules on earth and is a major organic sink for atmospheric carbon dioxide.

Waterbury et al. (25) obtained axenic cultures of a novel bacterium from the gills of six species of shipworms. These isolates are unique in that they represent the only marine bacteria currently known both to digest cellulose and to fix dinitrogen. Serial dilution experiments as well as the unique morphology and physiology of these isolates argue strongly

that these bacteria represent a free-living form of the same bacterium that is observed in symbiotic association within the gills of the teredinids. Characterization of these bacteria indicates that all belong to a single bacterial species and suggests that this bacterium will be found to occur in all shipworms.

As is the case in all studies based on isolates from natural populations, the validity of these conclusions depends on the assumption that the isolates are truly representative of the natural populations examined. In most cases, it is difficult to demonstrate that this is so. Several investigators have attempted to solve this problem by using comparative analysis of rRNA sequences found in nucleic acid extracts from natural samples to investigate the composition of populations in cases where the bacteria were not culturable (6, 9, 21, 22, 26). Still another approach is the use of oligodeoxynucleotide hybridization probes directed against 16S rRNA targets to detect and identify bacteria in natural samples. Hybridization probes directed against rRNA targets have been used successfully to distinguish and identify phylogenetic groups (1, 4, 10), physiological groups (23), and individual species (1, 4, 20) by in vitro hybridization with extracted nucleic acids immobilized on support membranes. 16S rRNA-directed oligodeoxynucleotide probes have also been used to identify phylogenetic groups and individual species by using intact cells from both cultured and natural bacterial populations (1).

Here, we describe the use of 16S rRNA sequence information to phylogenetically characterize isolates from an unusual natural microbial population found within an animal tissue and to design oligodeoxynucleotide hybridization probes for examining the authenticity of these isolates as the

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FIG. 1. Alignments of 16S rRNA sequences in the target region of the *L. pedicellatus* isolate-specific probe (637r probe). Shaded regions represent nucleotides which mismatch with corresponding homologous nucleotides in the *L. pedicellatus* isolate sequence. The sequences compared are from the following organisms: *L. pedicellatus* isolate, *E. coli*, *Proteus vulgaris*, *P. aeruginosa*, *O. linum*, *Acinetobacter calcoaceticus*, *Rhodocyclus purpureus*, *Pseudomonas testosteroni*, *Agrobacterium tumefaciens*, and *Bacillus subtilis*. Sources of sequences are indicated in the legend to Fig. 2.

primary constituent of the symbiont population found in the gills of the tereidid mollusks.

MATERIALS AND METHODS

Organisms. The tereidid bacterial isolates chosen for sequence analysis were strain T7902 (ATCC 39867), isolated from *Lyrodus pedicellatus* (Quatrafages) collected at Long Beach, Calif.; strain T7901, isolated from *Bankia gouldi* (Bartsch) collected at Beaufort, N.C.; strain T8602, isolated from *Dicyanthifer manni* (Wright) collected at Ferguson Point, Australia; and strain T8402, isolated from *Teredora maleolus* (Turton) collected from floating wood 190 miles southeast of Woods Hole, Mass. Specimens of the host animals were collected and identified, and voucher specimens were deposited in the Mollusk Department of the Museum of Comparative Zoology, Harvard University, Cambridge, Mass., by R. D. Turner.

RNA purification and sequencing. RNA was purified from log-phase cells grown without aeration in liquid basal medium (25) supplemented with 0.5% (wt/vol) sucrose, 20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) buffer (pH 8.0), and 0.025% (wt/vol) NH_4Cl . Cells were harvested and resuspended in 7.5 ml of lysis buffer (5.0 M guanidinium thiocyanate, 50 mM Tris-HCl, 25 mM EDTA, 0.5% *N*-lauryl sarcosine, pH 7.5). After brief sonication, resuspended cells were passed through a French pressure cell at 20,000 lb/in². Following centrifugation, the clear lysate was then layered over 4 ml of 5.7 M CsCl–25 mM sodium acetate–10 mM EDTA, and the RNA was collected by ultracentrifugation in a Beckman SW41 rotor at 35,000 rpm for 20 h at 20°C. The RNA pellet was redissolved in 10 ml of extraction buffer (100 mM NaCl, 50 mM Tris-HCl [pH 7.4], 1 mM EDTA, 0.5% phenol), and residual protein was then removed by phenol-chloroform (4:1) extraction (15). The RNA was then ethanol precipitated, redissolved, and salt precipitated in 2 M NaCl overnight at 4°C (13). The high-molecular-weight rRNA was collected by centrifugation (5 min, 4°C, 13,000 × *g*), rinsed with 70% ethanol, and redissolved to a concentration of 2.0 mg ml⁻¹ in 10 mM Tris-HCl, pH 8.3.

Nucleotide sequences were determined by reverse transcription of rRNA templates (13) with six oligodeoxynucleotide primers specific to the small subunit rRNA (*Escherichia coli* positions 1407 to 1391, 1115 to 1100, 930 to 915, 704 to

690, 536 to 519, and 357 to 343 [14]). The resulting sequences were aligned with currently available 16S rRNA sequences. A distance matrix method (6, 12, 17) was employed to determine the optimal phylogenetic tree topology consistent with the pair-wise evolutionary distance estimates (7, 16–18).

Preparation of tissues and hybridization of fluorescent probes. *L. pedicellatus*, originally collected from Long Beach, Calif., has been maintained in culture at the Woods Hole Oceanographic Institution since 1975. Live specimens (3 to 5 mm in length) were extracted from their burrows in wooden boards and placed immediately in Bouin's fixative (0.9% picric acid, 9% formalin, 5% acetic acid in filtered seawater). The specimens were fixed for 48 h and then transferred to 50% ethanol. After 2 h, the specimens were placed in 70% ethanol. Changes of 70% ethanol were made twice daily until the yellow color of the Bouin's fixative was removed from the specimens. Samples were then embedded in paraffin and serially sectioned (5 μm). Sections were mounted on gelatin-coated slides and stored at room temperature.

Sections for hybridization with fluorescent probes were placed on slides and baked at 55 to 58°C for 30 min to promote binding of the sections. After 5 min of cooling at room temperature, the sections were deparaffinized by im-

FIG. 2. Evolutionary distance tree (based on 16S rRNA sequences) comparing the nitrogen-fixing, cellulolytic bacterium isolated from the shipworm *L. pedicellatus* with selected reference species from the class *Proteobacteria* (with the gram-positive eubacterium *B. subtilis* as an out-group). The tree was constructed from evolutionary distance values by using a least-squares, distance matrix method (17). The horizontal scale bar corresponds to two nucleotide substitutions per 100 sequence positions. Sequence sources are as follows, with similarity values to *L. pedicellatus* given in brackets: (alpha subdivision) *R. purpureus* [0.850] (26a), GenBank accession number M34132; *P. testosteroni* [0.813] (27); (gamma subdivision) *Calypotgena magnifica* symbiont [0.867]; *Lucinoma annulata* symbiont [0.886]; *Riftia pachyptila* symbiont [0.874] (6); *P. aeruginosa* [0.896] (26a), GenBank accession number M34133; *O. linum* [0.896] (26a), GenBank accession number M22365; *A. calcoaceticus* [0.871] (26a), GenBank accession number M34139; *V. harveyi* [0.874] (20a), GenBank accession number M58172; *P. vulgaris* [0.852] (3); *E. coli* [0.863] (2); *L. pedicellatus* symbiont, this work; (beta subdivision) *A. tumefaciens* [0.835] (27); (gram-positive eubacteria) *B. subtilis* [0.815] (11).

FIG. 3. Dot blot hybridization demonstrating the specificity of the teredinid isolate-specific probe. Bulk RNA from the indicated organisms was immobilized on nylon membranes and incubated with 32 P-labeled positive control (universal probe, 519r), negative control (515f), and teredinid isolate-specific probe (637r). Target nucleic acids included bulk RNA from *O. linum*, *V. harveyi*, and *P. aeruginosa*; from bacterial isolates of the shipworms *T. malleolus*, *L. pedicellatus*, *D. manni*, and *B. gouldi*; and from the gill of the shipworm *L. pedicellatus*. Reprinted from *Endocytobiology IV* (4th International Colloquium on Endocytobiology and Symbiosis) with permission of the publisher.

mersion in xylenes for 15 min and in fresh xylenes for an additional 15 min. The sections were rehydrated through a graded ethanol series by 5-min incubations in 95, 80, and 70% ethanol. Sections were incubated for 15 min in 0.2 M HCl, rinsed briefly with 0.2 M Tris–50 mM EDTA (pH 7.8), and then treated with proteinase K (Sigma) in 0.2 M Tris–50 mM EDTA (pH 7.8) for 3 min at 37°C. Sections were then postfixed in 3.7% formalin in phosphate-buffered saline (145 mM NaCl, 100 mM Na_2HPO_4 , pH 7.5) for 20 min and rinsed in 0.2 M Tris–50 mM EDTA (pH 7.8). To reduce background fluorescence, the sections were incubated in 50 mM sodium borohydride–50 mM Tris (pH 7.8) for 30 min in the dark. The slides were rinsed in distilled water and dehydrated through a graded ethanol series by 5-min incubations in 70, 80, 95, and 100% ethanol. Sections were stored in the dark at room temperature.

Computer-assisted rRNA sequence analysis revealed a contiguous sequence segment unique to the teredinid bacterial isolates. The 26-nucleotide sequence (positions 662 to 637 by the *E. coli* numbering system) was identical in all four of the teredinid isolates, but it had at least six mismatches with the homologous sequences of other organisms examined (Fig. 1). A complementary oligodeoxynucleotide was synthesized and was fluor labeled by conjugation with an ethyl amino group (Aminolink I; Applied Biosystems) incorporated into the 5' end of the oligodeoxynucleotide, following the manufacturer's instructions. The sequence of the teredinid isolate-specific probe (637r) was GCT GTA CTC AAG TTA CCC AGT TCT AA. Fluorescently labeled probes were prepared as described by DeLong et al. (4), except that the fluor used was Texas Red (Molecular Probes, Eugene, Oreg.).

The specificity of the oligodeoxynucleotide probe binding was tested by dot hybridization with 32 P-end-labeled probes. Target nucleic acids (500 ng per dot) were immobilized on nylon support membranes (GeneScreen Plus) and were hybridized with the specific probe (637r), a probe complementary to a universally conserved region of the 16S rRNA (positive control), or the complement of the positive control probe (negative control) (10). Hybridizations were performed with shaking for 24 h at 37°C in 5.0 ml of hybridization buffer (1.0 M NaCl, 10% dextran sulfate, 1.0% sodium dodecyl sulfate, 10 mM Tris [pH 8.0], 4 mM EDTA) containing 1.0 μCi of 32 P-end-labeled probe per incubation. Each strip was washed three times (10 min per wash) in $0.2\times$ SET ($1\times$ SET is 150 mM NaCl, 20 mM Tris-HCl [pH 7.8], 1.0 mM EDTA) at 37°C.

In situ hybridizations were performed in a hybridization buffer which contained $5\times$ SET, 0.2% bovine serum albumin (fraction V; Sigma), 10% dextran sulfate (average molecular weight, 5,000; Sigma), and 0.1% polyadenylic acid (Sigma). Fluor-labeled oligodeoxynucleotides were added to the hybridization buffer to a final concentration of 1.7 ng/ μl , and 15 μl was applied to each section. Coverslips were placed over

FIG. 4. (A) Stylized representation of *L. pedicellatus*, with the left side of the mantle (except the mantle collar, which surrounds the siphons) removed to show the relation of the gill to the shell, visceral mass, pallets, and siphons. The anterior-posterior axis is left to right and the dorsal-ventral axis is top to bottom. F, foot; VM, visceral mass; G, gill; MC, mantle collar; S, siphons. Dotted lines indicate the section of the animal (posterior to the visceral mass) depicted in panel B. (B) Cross-section demonstrating the structure of the gill and associated blood vessels. EF, efferent branchial vein; AF, afferent branchial vein; IJ, interlamellar junction; GF, gill filament; M, mantle. The box indicates the orientation of the tangential section of the gill depicted in panel C. (C) Tangential section showing two adjacent gill filaments and their associated interlamellar junctions and symbiont-containing vesicles. The tangential sections shown in Fig. 5 and 6 are taken from a region of the gill tissue equivalent to that shown here.

FIG. 5. Morphology of the symbiont-containing region within the gill tissue of the shipworm *L. pedicellatus*. (A) Light micrograph of a hematoxylin-eosin-stained 5- μ m tangential section of paraffin-embedded gill tissue. Individual gill filaments (GF) and the underlying darkly staining interfilamentar junctions are seen with their dorsoventral axes extending from the upper right to lower left corner of the micrograph. Scale bar, 100 μ m. (B) Photomicrograph taken with differential interference phase microscopy showing the SCVs within the interlamellar junctions. The box in panel A indicates the orientation here. The region within the SCVs appears either striated or dimpled depending on the orientation of the rod-shaped symbiotic bacteria with respect to the plane of the section. Host cell nuclei (BN) are also visible. Scale bar, 10 μ m. (C) Transmission electron micrograph of the symbiotic bacteria within an SCV. Scale bar, 1 μ m.

the sections and sealed with rubber cement, and the slides were placed in a humid chamber at 37°C for 5 to 16 h. Coverslips were removed by immersion in 5 $\times$ SET, and the sections were washed three times (10 min per wash) in 0.2 $\times$ SET at 37°C. Slides were then air dried and stored in the dark at 4°C before they were viewed.

Specimens for phase-contrast and electron microscopy

were fixed in 3% glutaraldehyde in Sorrenson's phosphate buffer (0.4 M sucrose, 0.1 M Na₂HPO₄, pH 7.3) and were postfixed, dehydrated, embedded, sectioned, and stained as described by Distel and Felbeck (5).

Nucleotide sequence accession number. The sequence data reported here have been deposited in GenBank under accession numbers M64338, M64339, and M64340.

FIG 6. UV fluorescence micrograph showing three serial 5- μ m tangential sections of paraffin-embedded tissue from the symbiont-containing region of the gill of *L. pedicellatus*. Portions of two adjacent interlamellar junctions are visible, each containing one SCV surrounded by host tissue (H). The dorsal-ventral axis is right to left. Sections were incubated with oligodeoxynucleotide probes labeled with the fluorescent marker Texas Red. Section A was incubated with the negative control probe (515f), section B was incubated with the teredinid bacterial isolate-specific probe (637r), and section C was incubated with the positive control (universal) probe (519r). Reprinted from *Endocytobiology IV (4th International Colloquium on Endocytobiology and Symbiosis)* with permission of the publisher.

RESULTS AND DISCUSSION

Partial sequence analysis of the 16S rRNAs of four bacterial strains isolated from the gills of teredinid mollusks showed them to be identical in primary sequence at all 891 nucleotide positions determined for each of the isolates. This result was in agreement with the phenotypic analysis of Waterbury et al. (25) that indicated that these isolates represent a single bacterial species. The results of the distance matrix analysis using a single summary sequence (Fig. 2) showed that the teredinid isolate falls within the gamma-3 subdivision of the *Proteobacteria*. Of known 16S rRNA full and partial sequences (177 examined), the teredinid bacterial sequence was most similar to that of *Oceanospirillum linum* (similarity = 0.896) and *Pseudomonas aeruginosa* (similarity = 0.896).

An oligodeoxynucleotide hybridization probe (637r) was designed that was specific for the shipworm bacterial symbiont (Fig. 1). Dot blot hybridizations, performed to test the specificity of probe 637r, showed that the 32 P-labeled probe hybridized with the bulk RNA extracted from the four teredinid isolates tested (Fig. 3). This probe did not bind to bulk RNA from *Vibrio harveyi*, *P. aeruginosa*, or *O. linum*, close relatives of the teredinid isolates on the basis of 16S rRNA sequence analysis (Fig. 2). The teredinid isolate-specific probe did, however, bind to bulk nucleic acid purified from the symbiont-containing gill tissue of *L. pedicellatus*.

These results indicated that the teredinid symbiont rRNA was present in the bulk rRNA isolated from the gill tissue of *L. pedicellatus*. The quantity of probe binding to bulk RNA from *L. pedicellatus* gill in the dot blot hybridization was considerably less than that binding to bulk RNA from the teredinid isolates. This was to be expected, since the *L.*

pedicellatus sample contained both host and symbiont RNA and, therefore, a smaller proportion of the total gill RNA was composed of bacterial 16S rRNA. These results were consistent with the hypothesis that the teredinid isolates were representative of the symbiont population observed in the gill tissue of the shipworms by light and electron microscopy. However, the observed signal could have been due to the presence of these bacterial cells as an abundant superficial contaminant associated with gill preparations.

To determine whether the teredinid isolates were representative of the authentic gill symbiont population in *L. pedicellatus*, in situ hybridizations were performed by using the previously described probes applied directly to 5- μ m-thick serial sections of paraffin-embedded shipworms. These oligodeoxynucleotide probes were covalently coupled to Texas Red, allowing detection by fluorescence microscopy. Light and electron microscopy were used to determine whether probe binding coincided with the location of the symbionts in the host tissue.

The symbionts of *L. pedicellatus* are found within symbiont-containing vesicles (SCVs) that line the interlamellar junctions and efferent branchial vein within the gills (19, 24, 25) (Fig. 4). Ultrastructural evidence to date is insufficient to determine whether the bacterial cells are located intracellularly or are present within invaginated pockets in host cells (19, 24a, 25). No organelles or other cytoplasmic constituents are seen within the SCVs; however, nuclei, mitochondria, and endoplasmic reticula are present in the cytoplasm surrounding the SCVs. The SCVs were readily identifiable structures that often appeared as irregular ringlike structures in cross-section, with the rod-shaped bacteria, aligned along their longitudinal axes, radiating from a central nonstaining region. In photomicrographs taken with differential interfer-

ence phase microscopy, the bacterium-containing structures have a striate or dimpled appearance depending on whether the bacteria were sectioned longitudinally or on end (Fig. 5B). Transmission electron micrographs (Fig. 5C) revealed that these patterns were indeed due to the presence of densely packed, rod-shaped gram-negative bacteria.

When hybridizations were performed on sections of paraffin-embedded specimens of *L. pedicellatus*, the teredinid isolate-specific probe (637r) bound specifically to the symbiont-containing vesicles in the gill and to no other structures in the host animal. Figure 6 shows three serial sections containing SCVs in two adjacent interlamellar junctions. When the teredinid isolate-specific probe was applied, only the SCVs bound the fluorescent probe (Fig. 6B). The negative control probe (515f) hybridized with neither the host tissue nor the symbiont cells (Fig. 6A). Finally, the universal probe (519r) hybridized with both the symbiont-containing vesicles and the surrounding host tissue within the interlamellar junctions (Fig. 6C).

The binding of the universal probe to both the bacterium-containing regions of the gills and the host tissue cytoplasm indicated that the ribosomes of both the host and the symbionts are accessible to that probe. Therefore, the binding selectivity of the teredinid isolate-specific probe (637r) was clearly a function of the sequence specificity of the probe and was not due to an artifact such as the differential permeability of these cells. The results with the negative control probe demonstrated that background fluorescence (fluorescence not due to sequence-specific probe binding) was negligible. These results strongly support the conclusion that the teredinid bacterial isolates grown in pure culture by Waterbury et al. (25) are representative of the true gill symbiont of the shipworm *L. pedicellatus*.

The teredinid isolates examined in this investigation were chosen from four teredinid genera that were collected from widely dispersed geographic locations. The observation that all four were indistinguishable with respect to 16S rRNA sequence further strengthens the argument that all of the isolates represent a single bacterial species. To date, isolates have been obtained from 23 species of shipworm representing 9 of the 14 currently recognized teredinid genera (25a). The results that we present provide strong evidence that this single bacterial species has worldwide distribution in many, if not all, teredinid species. The homogeneity in the symbiont populations of the teredinid bivalves suggests relatively recent common ancestry or recent exchange among these populations. In contrast, the sulfur-oxidizing symbionts found in closely related species of lucinid clams show distinct differences in their 16S rRNA sequences (6). This dissimilarity must certainly reflect fundamental differences in the ways in which these symbiont systems are maintained, function, and evolve.

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