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Phylogenetic Analysis of a Highly Specific Association between Ectosymbiotic, Sulfur-Oxidizing Bacteria and a Marine Nematode

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The phylogenetic relationship of chemoautotrophic, sulfur-oxidizing, ectosymbiotic bacteria growing on a marine nematode, a *Laxus* sp. (formerly a *Catanema* sp.), to known endosymbionts and free-living bacteria was determined. Comparative 16S rRNA sequencing was used to investigate the unculturable nematode epibionts, and rRNA-targeted oligonucleotide hybridization probes were used to identify the ectosymbionts in situ. Both analyses revealed a remarkably specific and stable symbiosis. Unique hybridization of a specific probe to the ectosymbionts indicated that only one species of bacteria was present and growing on the cuticle of the nematode. Distance and parsimony methods used to infer phylogenetic trees both placed the nematode ectosymbionts at the base of a branch containing chemoautotrophic, sulfur-oxidizing endosymbionts of three bivalve families and of the tube worm *Riftia pachyptila*. The most closely related free-living bacteria were chemoautotrophic sulfur oxidizers belonging to the genus *Thiomicrospira*. Furthermore, our results suggested that a second, only distantly related group of thioautotrophic endosymbionts has as its deepest branch surface-colonizing bacteria belonging to the genus *Thiothrix*, some of which are capable of sulfur-oxidizing chemoautotrophic growth.

The boundary zones between reducing and oxidizing marine environments harbor an extraordinary type of symbiosis. Sulfur-oxidizing, chemoautotrophic bacteria live in dense populations in or between cells of a variety of animals belonging to four different phyla (Annelida, Bivalvia, Pogonophora, and Vestimentifera) (5, 6, 14). The bacteria obtain energy by oxidizing reduced sulfur compounds and fix inorganic carbon through the Calvin-Benson pathway. Substantial amounts of the reduced carbon are diverted to the animal hosts, and in many cases this reduced carbon is the primary source of nutrition of the hosts (29). The hosts thus live autotrophically and are among the few animals on earth that are not fed directly by photosynthetically fixed carbon.

Recently, it was shown that a functionally similar symbiosis exists between members of the Stilbonematinae, a group of marine, sediment-inhabiting nematodes, and sulfur-oxidizing bacteria (23, 24). In this type of association the bacteria do not live within the host body but cover its surface with a dense coat. Although examples of bacterial epigrowth are known for other animals living in sulfidic environments (8, 21), the nematodes form by far the most intimate association (24). The bacterial epigrowth on the nematodes consists of morphologically uniform cells that grow in distinct patterns which are highly specific to the host species (24). The nematodes probably obtain most of their nutritional carbon by grazing on the epibionts (23). Behavioral adaptations of the worms appear to make their surfaces excellent substrates for sulfur-oxidizing symbionts. The nematodes migrate in the sediment with changes in the chemocline and repeatedly cross the boundary between oxidized and reduced sediment layers (22, 23). This ensures that there is a continuous supply of sulfide and oxygen and other electron acceptors necessary for the chemoautotrophic metabolism of the bacteria. Continuously high productivity may thus be possible under otherwise highly variable physicochemical conditions.

The evolutionary origin of the chemoautotrophic endosymbionts was recently elucidated by the results of 5S and 16S rRNA comparative sequence analysis (9–12, 30). On phylogenetic trees based on the 16S rRNA sequences, the bacteria formed two deeply divergent but loosely associated groups which cluster within the gamma subdivision of the *Proteobacteria*. Sulfur-oxidizing bacteria belonging to the genus *Thiomicrospira* were the only free-living strains that were loosely associated with the symbiont groups. The results of a comparative analysis indicated that the endosymbionts of members of at least two bivalve families formed uniquely associated monophyletic groups, suggesting that the symbiotic partners share a common ancestor (9). In the bivalves, symbiosis seems to have been an important source of evolutionary novelty and may have played a key role in the emergence of some families.

Did the ectosymbionts arise from the same ancestral lineage of bacteria as the endosymbionts? Or did an independent evolutionary origin lead to a functionally similar symbiosis? Both are viable alternatives since sulfur-based chemoautotrophy and attachment to surfaces are found in bacteria belonging to very diverse phylogenetic groups (16). We investigated these questions by performing comparative 16S rRNA sequence analysis in which the nematode *Laxus* sp. (*Catanema* sp.) was used as a model organism. 16S rRNA-targeted in situ hybridization was used to identify the source of the sequence obtained. This technique also allowed us to estimate the specificity of the association which appears to be unique on the basis of morphological observations (24).

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MATERIALS AND METHODS

Organisms. Specimens were collected from a shallow, subtidal, coralline sandbar at Carrie Bow Cay, Belize Barrier Reef, in Belize, Central America (22). Worms were removed from the sediment by using a dissecting microscope, and batches of 20 to 50 individuals were frozen in liquid nitrogen for DNA extraction. Some live specimens were transported to a laboratory in natural sediment and were processed there for microscopic examination and in situ oligonucleotide hybridization.

Light and electron microscopy. Specimens were examined by light and electron microscopy to ensure proper identification of the microscopic worms and their symbionts. For light microscopic observations specimens were fixed in 4% formaldehyde and transferred into glycerin. For scanning electron microscopy, specimens were fixed in 4% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2), postfixed in 1% OsO_4 in 0.1 M sodium cacodylate buffer (pH 7.2), and dehydrated and critical point dried as described previously (24). For transmission electron microscopy animals were fixed in a mixture containing 3% glutaraldehyde, 2% formaldehyde, 0.1% acrolein, and 0.1% dimethyl sulfoxide in 0.1 M sodium cacodylate buffer (pH 7.2) made isotonic to seawater with $CaCl_2$, postfixed in 2% OsO_4 in 0.1 M sodium cacodylate buffer (pH 7.2), dehydrated, and embedded in Spurr's epoxy resin (19). The preparations were examined with a JEOL model JSM-35CF scanning microscope and a Zeiss model EM9-S2 transmission electron microscope.

DNA preparation. DNA was extracted from 20 to 50 whole specimens. The frozen worms were ground on ice with an Eppendorf tissue grinder in cold buffer (0.1 M NaCl, 1 mM EDTA, 1% sodium dodecyl sulfate [SDS], 20 mM Tris-HCl [pH 7.2]); this was followed by overnight proteinase K (50 μg/ml) digestion, phenol-chloroform extraction, and ethanol precipitation (4).

PCR amplification and sequencing. PCR amplification products were sequenced directly. Each 100 μl of PCR mixture contained 10 to 15 ng of total DNA and 100 pmol each of eubacterial 16S rRNA-specific primers 27f and 1492r (15). For direct PCR sequencing the product was purified with Geneclean (Bio 101, LaJolla, Calif.) according to the manufacturer's directions. Then it was denatured by boiling for 5 min and snap frozen in liquid nitrogen. The sequence was determined in both directions by using the Sequenase (U.S. Biochemicals) standard protocol. The sequencing primers used were primers 1492r, 1392r, 1101r, 690r, 519r, and 342r for the RNA-like strand and primers 1115f, 704f, 357f, and 27f for the opposite strand (15).

Probes and in situ hybridization. Specimens were extracted from sediment and processed immediately. Single worms were fixed in a cold, freshly prepared 4% paraformaldehyde solution in phosphate-buffered saline (PBS) (pH 7.4) (2), washed in cold PBS, and stored in PBS containing 30% ethanol until they were used.

A symbiont-specific probe was designed by using an alignment of all previously described chemoautotrophic endosymbiont sequences and choosing regions which maximized the number of mismatches between the *Laxus* sp. symbiont and the endosymbionts. The probe was then checked with sequences in the Ribosomal Database Project (18) by using the CHECK PROBE program and with GenBank sequences by using the BLAST algorithm (1). The positive control probe used was Eub338 (3), which hybridizes to (eu)bacterial 16S rRNAs, and the negative control probe used was Pce54 (26), which is targetted to a variable region of the 23S rRNA of *Pseudomonas cepacia*.

For hybridization experiments in which digoxigenin (DIG)-labeled probes and an alkaline phosphatase-conjugated anti-DIG Fab fragment detection system (32) were used, permeabilization of the cells with lysozyme (1 mg/ml, 20 min, 25°C) was necessary. This was followed by a short rinse in hybridization buffer (0.9 M NaCl, 0.01% SDS, 10 mM Tris [pH 7.2]). Hybridization experiments were performed at 46°C for 2 h in microtiter dishes containing 16 μl of hybridization buffer to which 2 μl of a DIG-labeled oligonucleotide solution (50 μg/μl) was added. Then 10 μl of a 1:4 dilution of the alkaline phosphatase-antibody conjugate (Boehringer) was added to each well, and the preparation was incubated for 1 h at 27°C. Subsequently, the nematodes were transferred to a well containing wash buffer (150 mM NaCl, 100 mM Tris-HCl [pH 7.5]), washed for 15 min at 29°C, and then rinsed in 100 mM Tris-HCl (pH 9.5)–100 mM NaCl–50 mM $MgCl_2$. Alkaline phosphatase activity was detected by the formation of a dark blue, water-insoluble precipitate after incubation in a substrate solution containing nitroblue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate in 100 mM Tris-HCl (pH 9.5)–100 mM NaCl–50 mM $MgCl_2$ at room temperature for 2 h (32). Each nematode was then mounted on a microscope slide and, after air drying for a short time, embedded in Eukitt (O. Kindler, Freiburg, Germany). Micrographs were taken with a Zeiss Axioplan microscope by using Kodak type Tmax 400 film.

Sequence analysis. The nematode ectobacterium sequence was compared with the 16S rRNA gene sequences of chemoautotrophic endosymbionts (10, 11) and representative free-living and symbiotic bacteria obtained from the Ribosomal Database Project (18) (Table 1). The sequence was checked by referring to predicted bacterial secondary structures available from the Ribosomal Database Project, and sequences were aligned manually by using the Genetic Data Environment sequence editor (28). All nucleotide positions that could be unambiguously aligned for all taxa were included in the analysis. The final data set comprised 895 nucleotide positions. Phylogenetic analyses were performed by using programs contained in the PHYLIP version 3.4 package (13) and were implemented through the Genetic Data Environment on a Sun SPARCstation IPC. The distance programs used were DNADIST, SEQBOOT, and FITCH (13). A parsimony analysis was performed by using the DNAPARS and DNABOOT programs (13). Bootstrap values are given if they were greater than 50 of 100 trees; values greater than 75 of 100 trees are assumed to be significant support for the grouping of organisms in an associated node (33).

Nucleotide sequence accession number of the *Laxus* sp. symbiont sequence. The *Laxus* sp. symbiont nucleotide sequence has been deposited in the GenBank database under accession number U14727.

RESULTS

Short description of *Laxus* sp. When a preparation was examined with a light microscope, the bacterial epigrowth on *Laxus* sp. (*Catanema* sp.) was immediately apparent. The symbionts are morphologically uniform rods and stand very tightly together in an upright position (Fig. 1). They form a complete coat which has occasional bare spots that may result from the grazing activity of the worm (24) (Fig. 1A). Only the anteriormost end of the animal is always completely free of bacteria, and the covering starts with a distinct border (Fig. 2A).

TABLE 1. Organisms whose 16S rRNA sequences were used in the phylogenetic analysis and levels of sequence identity between the 16S rRNA of the *Laxus* sp. symbiont and representative 16S rRNA sequences of members of the domain *Bacteria*[a]

Mode of living	Organism or host	Proteobacterial subdivision	% Identity[b]
Surface attached	*Thiothrix nivea*	γ	86.4
Endosymbiotic[c]	*Agrobacterium tumefaciens*	α	81.0
	Rickettsia rickettsii	α	77.8
	Neisseria gonorrhoeae	β	80.9
	Calyptogena magnifica[d]	γ	85.6
	Lucinoma aequizonata[d]	γ	90.5
	Riftia pachyptila[d]	γ	92.7
	Solemya reidi[d]	γ	91.9
	Solemya velum[d]	γ	90.2
	Thyasira flexuosa[d]	γ	90.3
	Vesicomya cordata[d]	γ	85.2
Free living	*Pseudomonas testosteroni*	β	79.5
	Chromatium vinosum	γ	90.2
	Escherichia coli	γ	82.0
	Oceanospirillum linum	γ	87.1
	Pseudomonas aeruginosa	γ	89.5
	Pseudomonas mendocina	γ	88.4
	Thiobacillus hydrothermalis	γ	86.6
	Thiomicrospira sp. strain L12	γ	87.1
	Thiomicrospira thyasirae	γ	86.4
	Vibrio harveyi	γ	84.0

[a] All of the sequences used were obtained from the alignment published by the Ribosomal Database Project (18).
[b] Identity values were calculated by using an alignment containing 895 sequence positions.
[c] Bacteria are listed as symbiotic if they have the potential to spend at least portions of their lives as symbionts.
[d] For chemoautotrophic symbionts the names of the hosts are given.

Sequence identity and specificity of the association. PCR amplification performed with the bacterial 16S rRNA primers produced a single band of the expected size (about 1,470 bp) on an agarose gel. Direct sequencing of this PCR product yielded a single unambiguous sequence. This suggests that a single bacterial sequence was amplified from the batches of 20 to 50 worms from which DNA was extracted and that only one bacterial species was associated with the animals.

The source of the sequence obtained was further tested by oligonucleotide in situ hybridization. We designed a specific probe (Lax659, 5′-TGGTAGAGGGGGGTGGAA-3′) that was complementary to the variable region from position 659 to position 676 (*Escherichia coli* numbering) of the symbiont sequence which exhibited three or more mismatches with all previously published sequences for sulfur oxidizers and endosymbionts. Initially, fluorescent labeling of the probes was used (3, 7). However, the hydrophobic fluors appeared to bind to the cuticle, which gave a strong nonspecific signal that overshadowed the specific labeling of the bacteria. Therefore, DIG-labeled oligonucleotide probes, in combination with anti-DIG–alkaline phosphatase detection, were used to circumvent problems of nonspecific fluorescence. With this method hybridization of the probe with the target sequence can be detected by the formation of a darkly colored precipitate (32). Bacterial cells on the worm's surface hybridized with the specific probe Lax659 (Fig. 2C) and with universal bacterial probe Eub338 (Fig. 2A and B). Probe Pce54, which was targeted to *P. cepacia* and was used as a negative control, revealed only low levels of nonspecific binding of DIG-labeled oligonucleotides to *Laxus* sp. ectobacteria (Fig. 2D). These data corroborate the hypothesis that the sequence amplified was the sequence of the epibiont and that the epigrowth consisted only of one species of bacteria.

Phylogeny of the *Laxus* sp. symbionts. A phylogenetic analysis in which we used distance (Fig. 3) and parsimony methods (data not shown) unambiguously placed the *Laxus* sp. symbiont in the gamma subdivision of the *Proteobacteria* (Fig. 3). The two methods gave essentially identical trees that consistently associated the ectosymbiont with the chemoautotrophic endosymbionts. The *Laxus* sp. ectosymbiont fell at the base of the branch that contains only intracellular symbionts of members of the bivalve families Thyasiridae, Lucinidae, and Solemyidae and of the deepsea tube worm *Riftia pachyptila* (Fig. 3). The most closely related free-living organisms are sulfur-oxidizing bacteria belonging to the genus *Thiomicrospira*. The symbionts of members of the bivalve families Vesicomyidae and Mytilidae form their own branch with *Thiothrix nivea* as their closest free-living relative. However, addition of the nematode symbiont sequence consistently split the previously described endosymbiont cluster and moved free-living, nonsulfur bacteria between the two previously described groups (Fig. 3). This is inconsistent with the results of previous analyses which suggested that there is a much closer relationship between the two symbiont groups. However, in these analyses the relationship between the deep branches could not be determined with a high degree of

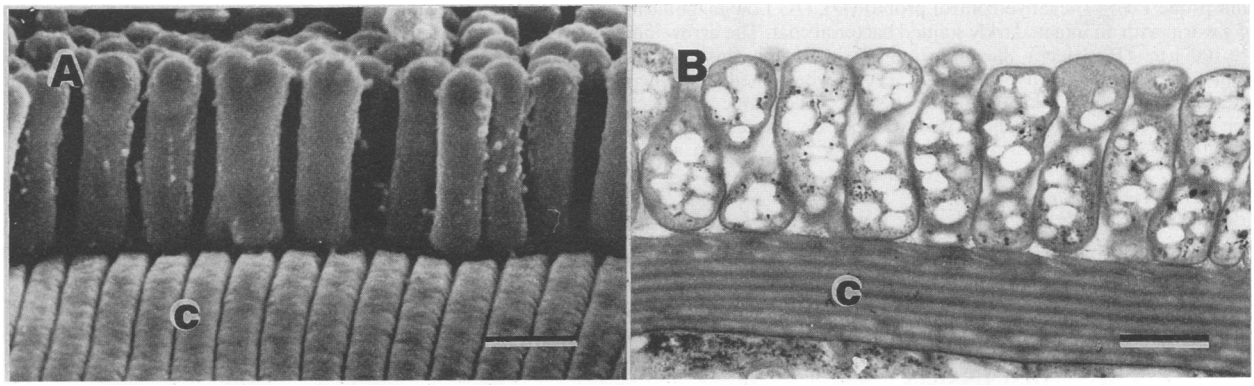

FIG. 1. Morphology of the *Laxus* sp. symbionts and their arrangement on the nematode cuticle. (A) Scanning electron micrograph of ectosymbionts viewed from the side in a spot where the bacterial coat was ruptured. The annulated structure is the worm cuticle. c, cuticle. Bar = 1 μm. (B) Transmission electron micrograph showing the highly ordered array of the bacteria. c, cuticle. Bar = 1 μm.

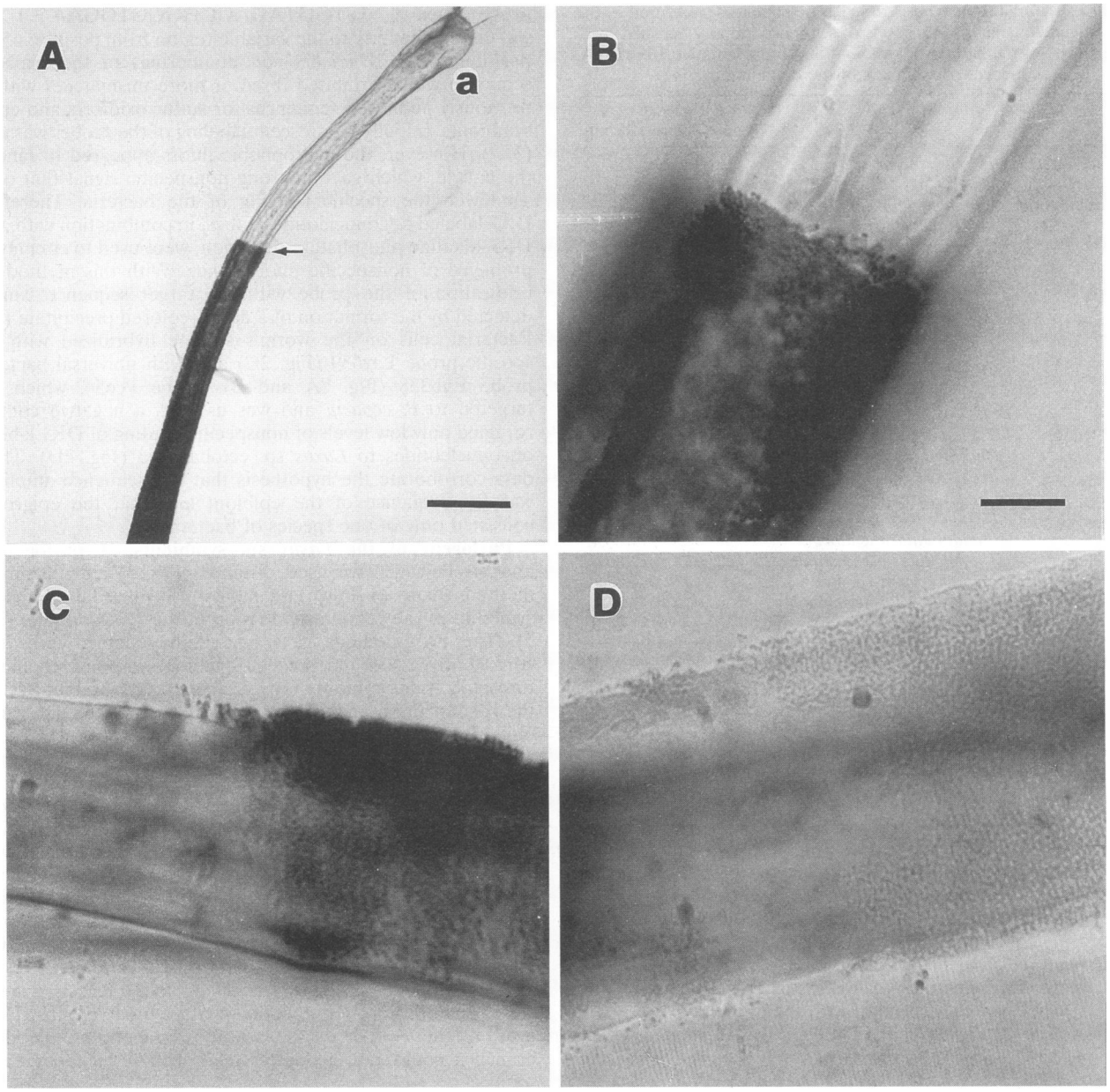

FIG. 2. In situ hybridization of whole worms with probe Eub338 (universal bacterial probe) (A and B), probe Lax659 (symbiont-specific probe) (C), and probe Pce54 (negative control probe) (D). (A) Low-magnification micrograph of in situ hybridization preparation showing the anterior end of a worm with an intact, darkly stained bacterial coat. The arrow indicates the beginning of the bacterial coat. a, anterior end of the nematode. Bar = 100 μm. (B) Higher magnification of the region shown in panel A, showing the sharp border between the anterior end and the symbiont-covered region. Bar = 10 μm. (C) Area where the bacterial coat has been ruptured and some of the cuticle lies free. Note the absence of bacteria that do not hybridize with the specific probe. Same magnification as panel B. (D) Worm prepared with negative control probe Pce54, showing low background staining. The dark spot on the left is inside the worm and is not due to a stained structure on the surface. Same magnification as panel B.

confidence since the levels of identity between the *Laxus* sp. symbiont sequence and the sequences of even its closest relatives are relatively low (Table 1) and bootstrap values remained below our assumed level of significance value (75 of 100 trees) (Fig. 3).

DISCUSSION

One of the most remarkable features of the symbiosis between the nematode *Laxus* sp. (*Catanema* sp.) and its epibacteria is the extremely high specificity of the association. This is shown by (i) the striking morphological uniformity among the ectosymbionts (Fig. 1) (24), (ii) shared features rarely observed among bacteria, like longitudinal division (24), and (iii) the phylogenetic homogeneity observed. PCR with primers capable of amplifying the 16S rRNA gene of any eubacterial species resulted in an unambiguous sequence for the symbiont. This indicates that the PCR products were dominated by a single 16S rRNA sequence since a level of

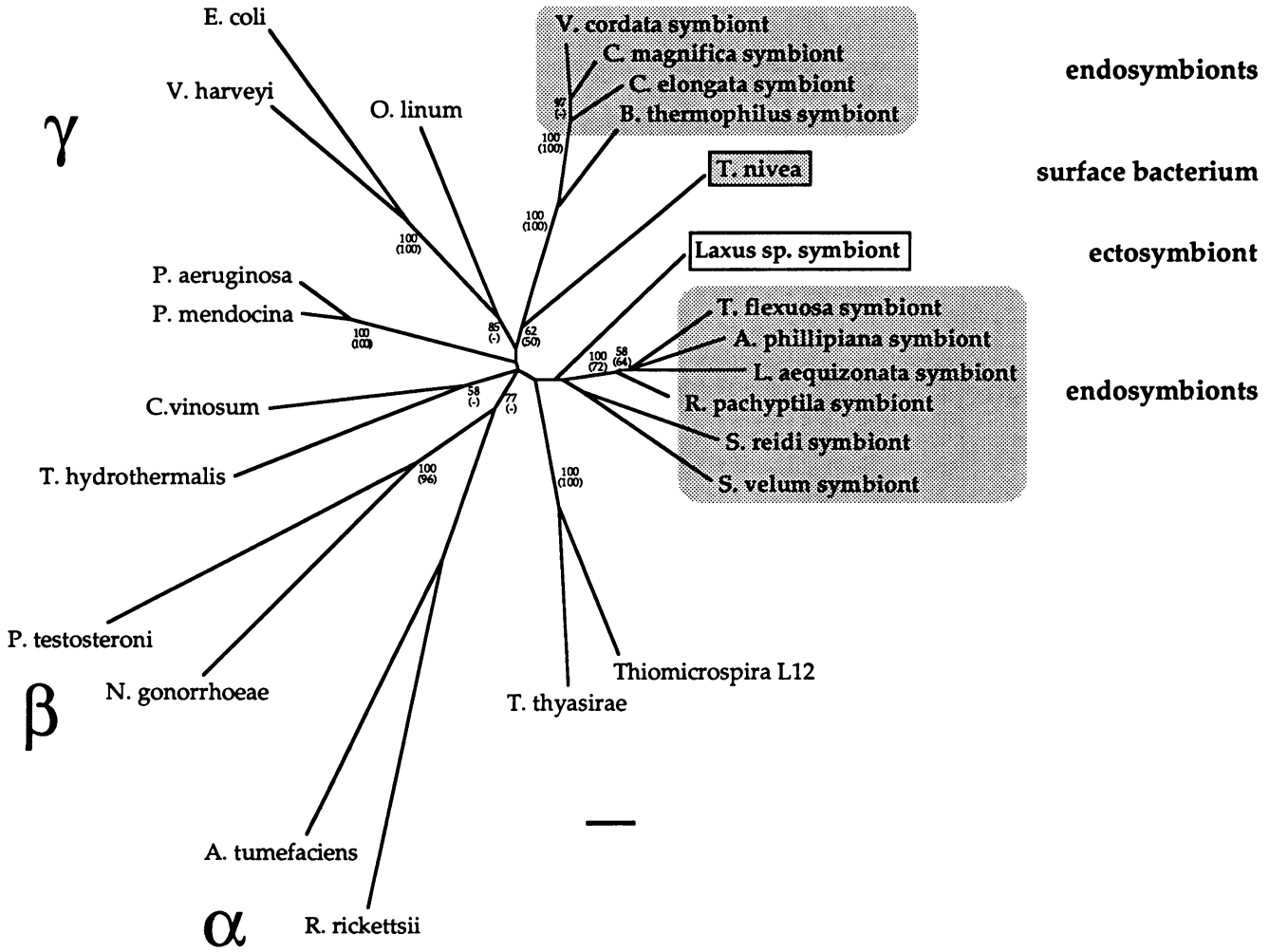

FIG. 3. Evolutionary distance tree based on the 16S rRNA sequences of the *Laxus* sp. symbiont and representative symbiotic and free-living members of the *Proteobacteria*. Two species belong to the α and β subdivisions of the *Proteobacteria*; all other species are members of the γ subdivision of the *Proteobacteria*. Bootstrap values that are greater than 50 of 100 trees are shown at the nodes. Bootstrap values from both distance (upper) and parsimony (lower) analyses refer to species distal to the associated node. For species names see Table 1. Bar = 1% nucleotide difference per sequence position.

heterogeneity of at least ≥5% is readily detectable on sequencing autoradiographs (8a) and implies that a single bacterial species is associated with the animals. The results of in situ hybridization with a symbiont-specific oligonucleotide probe independently confirmed these conclusions. We detected no bacterium attached to the cuticle that hybridized with the universal bacterial probe but did not hybridize with the specific probe. Even if bare spots occurred on the nematode cuticle, no bacteria other than the symbionts colonized those areas (Fig. 2) (24). This indicates that the association is extremely specific and that a bacterial monoculture exists on the animal.

The association of the *Laxus* sp. ectosymbionts with one of the chemoautotrophic endosymbiont groups was surprising and was not expected a priori. Not only do the host organisms belong to separate phyla, but they also live in different geographic locations and environments, and the two types of associations are physically and mechanistically very different. Furthermore, chemoautotrophic sulfur oxidation is extremely widespread in bacteria (16). In the *Proteobacteria* alone it occurs in at least four of the five subdivisions (the alpha, beta, and gamma subdivisions and the *Thiovolum-Campylobacter* group) (16). However, all of the symbionts examined to date unambiguously belong to the gamma subdivision. Although the relationship between the deep branches cannot be stated with certainty, it seems likely that the most recent common ancestors of the symbionts were sulfur oxidizers. Within this putative group some representatives may have evolved mechanisms to attach to animals living in sulfidic environments. Such specialization could have constituted an early step in the chain of events which led to the establishment of some of these modern symbioses.

Highly specific recognition mechanisms in two partners may play a crucial role in the maintenance of a stable association over time. The hosts must be recolonized by their symbionts several times, since nematodes typically shed their cuticles up to four times during their lives. We have found no evidence that there is direct lateral or horizontal transmission of the symbionts. Thus, there are probably times in a worm's life when its uncolonized cuticle is exposed to the environment and susceptible to attachment of other bacteria, such as surface

parasites. However, *Laxus* sp. specimens have never been found to be colonized by bacteria other than their characteristic symbionts (24). Only when the animals appear weakened do many morphologically different bacteria start to settle on the surface, and the worms usually die soon thereafter. Defense against surface settlement appears to be a common feature of nematodes since in the vast majority of species the cuticle is free of epigrowth (23, 24). Therefore, in healthy *Laxus* sp. specimens powerful exclusion and highly specific recognition mechanisms must be active.

Historically, the first step in the association may have occurred when certain bacteria were able to overcome the nematode's normal defense mechanisms. If these bacteria were sulfur-oxidizing chemoautotrophs, benefits for both partners may have promoted the continuation of the association. Surface colonizers may have represented an easily accessible food source for the worms, and the reproductive rate may have been rapid enough so that the worms could be fed and a stable surface population could be sustained. The migratory behavior of the worms in the chemocline may provide a predictable supply of reduced sulfur compounds and electron acceptors like oxygen or nitrate for continuous growth of the chemoautotrophs (23, 25). However, the key to the success of the early association was probably the development of high specificity and strong attachment mechanisms that may have provided the necessary stability and continuity for the two partners to cospeciate.

It has been proposed (27) that specific surface association could have been the first step toward endosymbiosis. In our analyses both endosymbiont groups appeared to be associated with ectosymbionts as their deepest branch. The *Laxus* sp. epibiont is found at the base of the first endosymbiont group, which contains the bacterial partners of the tubeworm *R. pachyptila* and members of the bivalve families Solemyidae, Thyasiridae, and Lucinidae. *Thiothrix nivea*, a surface-attaching species, is placed at the base of the second group, which contains symbionts of members of two additional bivalve families. Representatives of the genus *Thiothrix* are capable of chemoautotrophic metabolism (20, 23a) and have been found in association with animals, living either on their body surfaces (17, 21) or in their guts (31). Thus, the ability to grow on animal surfaces may be an ancient trait in these two bacterial groups, and monopolization of the outside may be an important precondition for entry into the host body.

While it is difficult to estimate a time frame for the evolution of the ectosymbiosis, many traits of the nematode-epibacterium association suggest that there was a long period of coevolution. Both the host and the symbiont appear to be very highly adapted to the presence of the other partner. Not only does a single species colonize the surface, but the bacteria also always grow in a characteristic upright position (24). Each cell multiplies by fission along its longitudinal axis, and division appears to be suppressed if free space on the cuticle becomes limiting (24). Like all of the chemoautotrophic endosymbionts (6), the ectosymbionts have not been successfully cultured, which may be an indication of the complexity of their physiological niche. The worms also exhibit morphological and behavioral adaptations in addition to their unusual reliance on epibionts as food. Individuals rarely lack the epigrowth (23, 24). The bacterial coat always begins with a sharp line behind the head region of the worm (19, 24), where the diameter of the worm decreases, so that the overall circumference does not increase (19). Recently, Distel et al. (9) presented evidence which suggests that some current chemoautotrophic symbioses may have become established more than 250 million years ago on the basis of a comparison of symbiont phylogeny and fossil records for the symbiotic bivalve families Lucinidae and Thyasiridae. Although nematode fossils are not available, the specificity and specialization of the ectosymbiosis described in this paper suggest that it is also ancient. Further examination of host and symbiont phylogeny will be needed to estimate a minimum age of the symbiosis and to determine the extent of coevolution.

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