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Phylogeny of 16S rRNA, Ribulose 1,5-Bisphosphate Carboxylase/Oxygenase, and Adenosine 5′-Phosphosulfate Reductase Genes from Gamma- and Alphaproteobacterial Symbionts in Gutless Marine Worms (Oligochaeta) from Bermuda and the Bahamas

Anna Blazejak,[1] Jan Kuever,[1]† Christer Erséus,[2] Rudolf Amann,[1] and Nicole Dubilier[1]*

Max Planck Institute of Marine Microbiology, Celsiusstrasse 1, D-28359 Bremen, Germany,[1] and Department of Zoology, Göteborg University, Box 463, SE-405 30 Göteborg, Sweden[2]

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Gutless oligochaetes are small marine worms that live in obligate associations with bacterial endosymbionts. While symbionts from several host species belonging to the genus *Olavius* have been described, little is known of the symbionts from the host genus *Inanidrilus*. In this study, the diversity of bacterial endosymbionts in *Inanidrilus leukodermatus* from Bermuda and *Inanidrilus makropetalos* from the Bahamas was investigated using comparative sequence analysis of the 16S rRNA gene and fluorescence in situ hybridization. As in all other gutless oligochaetes examined to date, *I. leukodermatus* and *I. makropetalos* harbor large, oval bacteria identified as Gamma 1 symbionts. The presence of genes coding for ribulose-1,5-bisphosphate carboxylase/oxygenase form I (*cbbL*) and adenosine 5′-phosphosulfate reductase (*aprA*) supports earlier studies indicating that these symbionts are chemoautotrophic sulfur oxidizers. Alphaproteobacteria, previously identified only in the gutless oligochaete *Olavius loisae* from the southwest Pacific Ocean, coexist with the Gamma 1 symbionts in both *I. leukodermatus* and *I. makropetalos*, with the former harboring four and the latter two alphaproteobacterial phylotypes. The presence of these symbionts in hosts from such geographically distant oceans as the Atlantic and Pacific suggests that symbioses with alphaproteobacterial symbionts may be widespread in gutless oligochaetes. The high phylogenetic diversity of bacterial endosymbionts in two species of the genus *Inanidrilus*, previously known only from members of the genus *Olavius*, shows that the stable coexistence of multiple symbionts is a common feature in gutless oligochaetes.

Gutless oligochaetes are small worms about 0.1 to 0.3 mm in diameter and 2 to 50 mm long that occur worldwide in marine sediments, with the highest diversity being found in tropical and subtropical coral reefs (11–15, 17, 20, 21). They form a monophyletic group that consists of only two genera, *Inanidrilus* and *Olavius*, within the subfamily Phallodrilinae (31). All worm species described so far, 25 *Inanidrilus* and 56 *Olavius* species (12), live in an obligate association with endosymbiotic bacteria. The lack of both a digestive and an excretory system led to the assumption that the symbiotic bacteria provide their hosts with a source of nutrition (19). Enzyme assays and uptake experiments with inorganic carbon indicated that at least some of the bacterial symbionts are thiotrophic; i.e., they use reduced sulfur compounds to fix CO_2 into organic carbon compounds (16). It is assumed that the transfer of these organic compounds to the host is the main mode of energy transfer, although digestion of the bacteria may also supply nutrients.

The bacterial symbionts occur in a thick layer just below the cuticle of the worm in an extracellular space above the epidermal cells of the worm (23). A phylogenetically diverse assemblage of bacteria co-occurs within the symbiotic layer, with up to six rRNA phylotypes being identified in a single host species (3). All gutless oligochaetes harbor a large (3- by 8-μm) thiotrophic symbiont with numerous sulfur and poly-beta-hydroxyalkanoate inclusions (4). These symbionts are found in all host species and form a closely related cluster of 16S rRNA sequences within the *Gammaproteobacteria* (8). Coexisting with these primary symbionts are smaller bacteria (0.7 by 1.9 μm) without any conspicuous inclusions that can belong to the *Gamma*-, *Alpha*-, or *Deltaproteobacteria* (3, 7, 10). While the metabolism of the gamma- and alphaproteobacterial symbionts is not clear, the deltaproteobacterial symbionts have been identified as sulfate reducers and are assumed to be engaged in a syntrophic sulfur cycle with the thiotrophic symbiont (3, 10). In some host species, spirochetes have also been identified as members of the oligochaete symbiont community (3, 7, 22).

The phylogeny of symbionts has been described for three host species from the genus *Olavius*—*Olavius loisae*, from Australia (7), *Olavius algarvensis*, from the Mediterranean Sea (10), and *Olavius crassitunicatus*, from Peru (3)—but for only a single species from the genus *Inanidrilus*, *Inanidrilus leukodermatus* (9). *I. leukodermatus* occurs in high abundance in calcareous sands of coral reefs around the island Bermuda in the northwest Atlantic Ocean, and the morphology, physiology, and ecology of this species have been studied intensively (9, 16, 19, 20, 23, 25). The symbionts of this species were the first to be characterized using molecular methods, and only a single thiotrophic gammaproteobacterial phylotype was identified despite the presence of multiple bacterial morphotypes (9). More recent studies on other gutless oligochaete species using im-

* Corresponding author. Mailing address: Max Planck Institute of Marine Microbiology, Celsiusstrasse 1, D-28359 Bremen, Germany. Phone: 49-421 2028-932. Fax: 49-421 2028-580. E-mail: ndubilie@mpi-bremen.de.

† Present address: Bremen Institute for Materials Testing, Paul-Feller-Str. 1, D-28199 Bremen, Germany.

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proved methods of 16S rRNA analysis and fluorescence in situ hybridization (FISH) have demonstrated that their multiple bacterial morphotypes correspond to multiple bacterial phylotypes (8). In the present study, we therefore reexamined the symbionts in *I. leukodermatus* and extended these analyses to include another *Inanidrilus* species from the northwest Atlantic Ocean, *Inanidrilus makropetalos*, that occurs in calcareous sands of coral reefs in the Bahamas (12).

In addition to the phylogenetic characterization of the *Inanidrilus* symbionts, we investigated genes coding for ribulose 1,5-bisphosphate carboxylase/oxygenase (RubisCO), the key enzyme for autotrophic CO_2 fixation in the Calvin-Benson-Bassham cycle, and adenosine 5′-phosphosulfate (APS) reductase, an enzyme involved in sulfur metabolism. Two distinct forms of RubisCO have been found in chemoautotrophic symbionts: form I, for which the *cbbL* gene is used as a functional marker, and form II, for which the *cbbM* gene is used (5, 25, 33, 38, 39). Form I RubisCO differs from form II structurally and is better adapted to CO_2 fixation under aerobic conditions (40, 41).

APS reductase is used in both the reductive and oxidative modes of sulfur metabolism and is therefore found in both sulfate reducers and sulfur oxidizers (18, 24). In sulfate reducers, APS reductase catalyzes the two-electron reduction of APS to sulfite and AMP, and in sulfur oxidizers, it catalyzes the reverse reaction (35). APS reductase consists of an alpha and beta subunit, encoded by the genes *aprA* and *aprB*, respectively, that form a $\alpha1\beta1$ heterodimer. The *aprA* gene has been proposed as a useful phylogenetic marker for bacteria involved in oxidative and reductive sulfur metabolism (24). The phylogeny of the *aprA* gene has been well studied in sulfate reducers (18), but nothing is known about the phylogeny of *aprA* from sulfur-oxidizing bacteria. Only a few sequences from free-living sulfur oxidizers are currently available in the GenBank database, and prior to this study, none from symbiotic sulfur oxidizers were known.

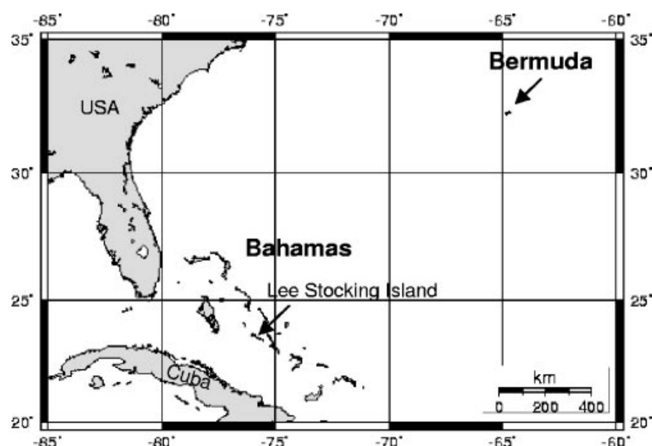

FIG. 1. Map of sampling sites in the northwest Atlantic Ocean. *I. leukodermatus* worms were collected in Bermuda, and *I. makropetalos* specimens were collected at Lee Stocking Island (the Bahamas).

MATERIALS AND METHODS

Specimen collection. *Inanidrilus leukodermatus* specimens were collected in June 1998 in Harrington Sound, Bermuda (Fig. 1). *Inanidrilus makropetalos* specimens were collected in April 1999 at Lee Stocking Island in the Bahamas (Fig. 1). The worms were extracted from shallow-water (<3 m) sediments by decantation with seawater and identified under a microscope. Specimens were fixed in 95% ethanol for DNA analyses and for FISH as described previously (9) and stored at 4°C.

DNA preparation. Six *I. leukodermatus* and three *I. makropetalos* individuals were prepared individually for PCR. Specimens were rinsed three times in MilliQ water, and DNA was isolated as described by Schizas et al. (36) in a protocol using proteinase K for digestion and the reagent GeneReleaser (BioVentures, Murfreesboro, Tenn.) for DNA purification.

PCR amplification. (i) 16S rRNA gene. Amplifications were performed with primers specific for the bacterial 16S rRNA gene (8F and 1492R [28]) using *Taq* DNA polymerase (Eppendorf, Hamburg, Germany). Template DNA (1 to 2 μl) was added after preheating the PCR mix (100 μl, total volume) to 80°C to avoid nonspecific annealing of the primers to nontarget DNA. The following thermocycling conditions were used: 1 cycle at 80°C for 5 min; 27 cycles at 95°C for 1 min, 40°C for 1 min, and 72°C for 3 min; and 1 cycle at 72°C for 10 min.

(ii) *cbbL* and *aprA* genes. The genes *cbbL*, coding for RubisCO form I, *cbbM*, for RubisCO form II, and *aprA*, for APS reductase, were amplified using *Taq* DNA polymerase (Eppendorf, Hamburg, Germany). For specific amplification of the genes coding for RubisCO forms I and II, the following primers were designed from available *cbbL* and *cbbM* sequences in the GenBank database: cbbLF (5′-CACCTGGACCACVGTBTGG-3′) and cbbLR (5′-CGGTGYATGTGCAGCAGCAT5CCG-3′) for the *cbbL* gene and cbbMF (5′-ATCATCAARCCSAARCTSGGYCTGCG-3′) and cbbM1R (5′-GAGGTSACSGCRCCRTGRCCRGCMCGRTG-3′) or cbbM2R (5′-GAGGTSACSGCRCCRTGRCC-3′) for the *cbbM* gene. For specific amplification of the *aprA* gene, the primers aps1F (5′-TGGCAGATCATGATYMAYGG-3′) and aps4R (5′-GCGCCAACYGGRCCRTA-3′) (J. Kuever, unpublished data) were used. Template DNA (0.5 μl) was added to the PCR mix (50 μl, total volume). The following thermocycling conditions were applied: 1 cycle at 95°C for 3 min; 27 cycles at 95°C for 1 min, 48°C (for *cbbL* and *cbbM* genes) or 54°C (for *aprA* genes) for 1 min, and 72°C for 3 min; and 1 cycle at 72°C for 5 min. The PCR products from each individual were purified using a QIAquick PCR purification kit (QIAGEN, Hilden, Germany) and directly sequenced in both directions (~696 bp for *cbbL* genes and ~396 bp for *aprA* genes).

Cloning and sequencing. 16S rRNA PCR products from each individual were cloned separately using a TA cloning kit (Invitrogen, Breda, The Netherlands) according to the manufacturer's protocol. For screening of 16S rRNA genes, clones were randomly picked and selected for the correct insert size (~1,500 bp) by PCR with the vector primers M13F and M13R. For template DNA, a small amount of cells from each clone colony was picked with a sterile toothpick and resuspended in 10 μl sterile water. After preheating of this suspension to 95°C for 5 min, template DNA was amplified by PCR as described above using a total volume of 30 μl. Clones with PCR products of the correct size were prepared using the MontagePlasmid Miniprep$_{96}$ kit (Millipore, Bedford, Mass.) and screened by partial sequencing of 300 to 500 bp using the 16S rRNA primer GM1F. Sequencing reactions were run using ABI BigDye on an ABI Prism 3100 genetic analyzer (Applied Biosystems, Foster City, Calif.). Sequences were aligned and compared using the Bioedit program (www.mbio.ncsu.edu/BioEdit/bioedit.html). Sequences were grouped together in a clone family if they shared ≥ 99% sequence identity (percent identical nucleotides). For each host individual, a representative clone from each clone family was fully sequenced in both directions.

Phylogenetic analyses. The 16S rRNA, *cbbL*, and *aprA* sequences were checked against sequences in GenBank using BLAST (1) for similarity searches. Chimeras were identified using CHIMERA_CHECK 2.7 from the Ribosomal Database Project (6) and by eye in sequence alignments and excluded from further analysis. The sequence data were analyzed using the ARB software package (www.arb-home.de).

Sequence similarity of the nucleotide sequences was calculated by distance analysis with the Jukes-Cantor correction. Phylogenetic trees for 16S rRNA sequences were estimated using parsimony, distance, and maximum-likelihood analyses with different filter sets, considering only sequences with a length of at least 1,255 bp.

Phylogenetic trees for the *cbbL* gene were generated from amino acid sequences using maximum-likelihood analyses with a 25% amino acid frequency filter. The leading and tailing amino acid stretches of full-length sequences were excluded from the analyses, eliminating those regions found in some but not all RubisCO sequences. The partial RubisCO form I sequences (*cbbL*, 211 amino acids) from this study were added to the maximum-likelihood tree using maximum parsimony. The phylogeny of the *aprA* gene was calculated from partial

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TABLE 1. Oligonucleotide probes used in this study

Probe	Specificity	Sequence (5′–3′)	Position[a]	[FA][b] in CARD FISH	Reference
EUB338	Bacteria	GCTGCCTCCCGTAGGAGT	338–355	55	2
GAM42a	Gammaproteobacteria	GCCTTCCCACATCGTTT	1027–1043[b]	55	27
NON338	Negative control	ACTCCTACGGGAGGCAGC	338–355	30	43
ALF968	Alphaproteobacteria, several members of *Deltaproteobacteria*	GGTAAGGTTCTGCGCGTT	968–985	40	29
InaGAM1	Gamma 1 symbiont in *I. leukodermatus* and *I. makropetalos*	CGCTCCCGAAGGCACCTA	1020–1038	55	This study
IleuALF1a	Alpha 1a symbiont in *I. leukodermatus*	CTCGGGTCCCCGCGACCG	999–1016	45	This study
IleuALF1b	Alpha 1b symbiont in *I. leukodermatus*	CTCCCGGACTCGAGCACA	646–663	45	This study
IleuALF3	Alpha 3 symbiont in *I. leukodermatus*	GAATGTCTCCACTCTCCG	1004–1021	45	This study
ImakALF1b	Alpha 1b symbiont in *I. makropetalos*	TCCGGTCTCCGCGACCCC	998–1015	55	This study
ImakALF2	Alpha 2 symbiont in *I. makropetalos*	ATCGCCACCCATTGTCAC	1244–1261	55	This study

[a] Position in the 16S rRNA of *Escherichia coli*, except for GAM42a, for which the position in the 23S rRNA of *E. coli* is given.
[b] Formamide concentration in the hybridization buffer, in percent (vol/vol).

sequences (131 amino acids) using maximum-likelihood analyses with a 25% amino acid frequency filter.

FISH. Six *I. leukodermatus* and three *I. makropetalos* specimens were prepared for FISH analyses of bacterial endosymbionts as described previously (3). Symbionts were detected by CARD (catalyzed reporter deposition) FISH with horseradish peroxidase (HRP)-labeled probes and tyramide signal amplification as described by Schönhuber et al. (37). Tissue sections were hybridized with the HRP-labeled probe for 3 h at 35°C. After washing for 15 min at 35°C in washing buffer (37), the sections were equilibrated for 15 min at room temperature in phosphate-buffered saline (PBS) buffer (pH 7.3). The moist tissue sections were incubated with amplification solution (1× phosphate-buffered saline, pH 7.3; 0.0015% [vol/vol] H_2O_2; and 1% Alexa Fluor 488, 546, or 633 dye [Molecular Probes, Leiden, The Netherlands]) for 10 min at 37°C in the dark and rinsed in PBS buffer for 15 min at room temperature. After air drying, tissue sections were embedded in the mounting fluid Vecta Shield (Vecta Laboratories, Burlingame, CA) and stored for microscopic evaluation at −20°C for <1 to 2 days. For dual and triple hybridizations, the CARD FISH protocol was repeated two or three times on the same sections using different probes and Alexa dyes. For this purpose, after the last washing step the tissue sections were covered with 0.01 M HCl for 10 min at room temperature to inactivate the HRP. After washing for 3 min in sterile water, tissue sections were hybridized with another probe as just described.

The oligonucleotide probes designed in this study target 16S rRNA sequences isolated from *I. leukodermatus* and *I. makropetalos* (Table 1). The probes were checked against sequences in GenBank using BLAST (1) and against small-subunit rRNA sequences in the Ribosomal Database Project using PROBE MATCH (32) and contained at least one mismatch relative to all entered sequences. The general bacterial probe EUB338, the general gammaproteobacterial probe GAM42a, and the general alphaproteobacterial probe ALF968 were used as positive controls, and the antisense probe NON338 was used as a negative control. All hybridizations were performed at formamide concentrations ensuring high specificity (Table 1).

Nucleotide sequence accession numbers. The 16S rRNA sequences from *Inanidrilus leukodermatus* were submitted to GenBank under accession numbers AJ890100 (Gamma 1 symbiont), AJ890099 (Alpha 1a symbiont), AJ890098 (Alpha 1b symbiont), AJ890093 (Alpha 1c clone associated with *I. leukodermatus*), and AJ890097 (Alpha 3 symbiont), and those from *Inanidrilus makropetalos* were submitted under accession numbers AJ890094 (Gamma 1 symbiont), AJ890095 (Alpha 1b symbiont), and AJ890096 (Alpha 2 symbiont). Sequences of genes coding for RubisCO and APS reductase from *Inanidrilus leukodermatus* symbionts were submitted to GenBank under accession numbers AM228902 (*aprA* gene) and AM228899 (*cbbL* gene), and those from *Inanidrilus makropetalos* symbionts were submitted under accession numbers AM228901 (*aprA* gene) and AM228900 (*cbbL* gene).

RESULTS

Clone library analysis. Bacterial 16S rRNA sequences from six *I. leukodermatus* specimens were grouped into five distinct clone families, with a total of 510 clones analyzed (Table 2). Within each clone family, sequence similarity was never less than 99.6%. Phylogenetic analyses (see below) revealed that in *I. leukodermatus* one clone family belongs to the *Gammaproteobacteria* (Gamma 1) and four to the *Alphaproteobacteria* (Alpha 1a, 1b, 1c, and 3). Sequences belonging to the Gamma 1 clone family were found in all individuals. For the Alpha 1a, Alpha 1b, and Alpha 3 clone families, at least three out of six

TABLE 2. 16S rRNA clone libraries from six *I. leukodermatus* individuals and three *I. makropetalos* individuals

Worm	No. of clones analyzed	No. (%) classified as:					
		Gamma 1	Alpha 1a	Alpha 1b	Alpha 1c	Alpha 2	Alpha 3
I. leukodermatus							
1	78	54 (69)	16 (21)	2 (3)	6 (8)		0 (0)
2	99	57 (58)	29 (29)	0 (0)	0 (0)		13 (13)
3	93	33 (36)	3 (3)	31 (33)	0 (0)		26 (28)
4	93	78 (84)	0 (0)	5 (5)	0 (0)		10 (11)
5	53	48 (91)	0 (0)	3 (6)	0 (0)		2 (4)
6	94	79 (84)	0 (0)	15 (16)	0 (0)		0 (0)
I. makropetalos							
1	101	55 (56)		2 (2)		41 (42)	
2	114	95 (83)		0 (0)		19 (17)	
3	103	92 (89)		3 (3)		8 (8)	

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I. leukodermatus individuals harbored sequences belonging to these groups. In contrast, sequences belonging to the Alpha 1c clone family were present in only a single individual.

Bacterial 16S rRNA sequences from the three *I. makropetalos* specimens (318 clones analyzed) were grouped into three distinct clone families, with at least 99.6% sequence similarity within each clone family (Table 2). Phylogenetic analyses (see below) confirmed that in *I. makropetalos* one clone family belongs to the *Gammaproteobacteria* (Gamma 1) and two to the *Alphaproteobacteria* (Alpha 1b and 2). Sequences belonging to the Gamma 1 and Alpha 2 clone families were found in all three *I. makropetalos* individuals and from the Alpha 1b clone family in two out of the three specimens examined.

Phylogenetic analyses 16S rRNA gene. Parsimony, distance, and maximum-likelihood analyses of the 16S rRNA sequences from the five *I. leukodermatus* and three *I. makropetalos* clone families confirmed that these belong to one bacterial group of the *Gammaproteobacteria* and five phylogenetically distinct bacterial groups of the *Alphaproteobacteria*. The five *I. leukodermatus* and three *I. makropetalos* sequences are unique to these hosts and differ from those of symbionts from other host species or free-living bacteria.

Gammaproteobacterial symbionts. The 16S rRNA sequence of the *I. leukodermatus* Gamma 1 symbiont from this study differed from the *I. leukodermatus* Gamma sequence described in an earlier study (8) by three nucleotides (positions 82, 861, and 862 based on *E. coli* numbering). All three nucleotide discrepancies are in stem regions, and these led to mismatched stem structures in our earlier sequence (8), while the stems are conserved in the sequence from this study. Furthermore, these sites are conserved in all Gamma 1 symbionts and the nucleotides in this study are in consensus with all other Gamma 1 symbionts, suggesting that these three substitutions were caused by PCR or sequencing error in the earlier study (8).

In all phylogenetic analyses, the *I. makropetalos* and the *I. leukodermatus* Gamma 1 sequences consistently group together (98.5% sequence similarity) (Fig. 2a). The *Inanidrilus* Gamma 1 sequences group in the same cluster as the sequences of Gamma 1 symbionts from *Olavius* hosts and the ectosymbiont of the marine nematode *Laxus* sp. (≥95.7% sequence similarity) (Fig. 2a). These symbiotic sequences, together with a clone sequence isolated from the Kazan mud volcano in the eastern Mediterranean Sea, are most closely related to a clade of free-living, phototrophic, sulfur-oxidizing bacteria from the family *Chromatiaceae*.

Alphaproteobacterial symbionts. The alphaproteobacterial sequences from *I. leukodermatus* and *I. makropetalos* belong to five phylogenetically distinct bacterial groups: Alpha 1a, 1b, and 1c, Alpha 2, and Alpha 3 lineages (Fig. 2b). The Alpha 1a to 1c sequences from both species are clustered (≥92% sequence similarity), whereas the *I. makropetalos* Alpha 2 and *I. leukodermatus* Alpha 3 sequences are phylogenetically separate from the Alpha 1 sequences (≥88.3% sequence similarity) and from each other.

Within the Alpha 1 group, *I. leukodermatus* contained sequences belonging to all three subgroups (Alpha 1a, 1b, and 1c), while in *I. makropetalos* only sequences belonging to the Alpha 1b group were found. The Alpha 1a sequence from *I. leukodermatus* is most closely related to the Alpha 1a endosymbionts of the gutless oligochaete *Olavius loisae* from the Australian Great Barrier Reef in all treeing methods (≥97.8% sequence similarity). The closest relatives of these sequences are the Alpha 1c sequence from *I. leukodermatus* (≥92% sequence similarity) and a cluster of clone sequences isolated from Atlantic Ocean and Arctic Ocean bacterioplankton communities and from Pacific Ocean deep-sea sediments (≥97.2% sequence similarity). The Alpha 1b *I. leukodermatus* and *I. makropetalos* sequences consistently grouped together in all three phylogenetic analyses (95.5% sequence similarity). These two sequences are distantly related to the Alpha 1a clade.

In addition to the Alpha 1 sequences, both host species contained another alphaproteobacterial sequence, called Alpha 2 in *I. makropetalos* and Alpha 3 in *I. leukodermatus* (Fig. 2b). The phylogeny of the Alpha 2 sequence from *I. makropetalos* was similar in maximum-likelihood and parsimony analyses, with a clone sequence from an Aegean Sea bacterioplankton community being the closest relative (91.1% sequence similarity). These two sequences are most closely related to the free-living halophilic bacteria *Rhodovibrio salinarum* and *Rhodovibrio sodomensis* (≥89.3% sequence similarity) and bacteria isolated from a dinoflagellate and coral mucus (≥90.2% sequence similarity). In distance analyses the *I. makropetalos* Alpha 2 sequence does not cluster with the Aegean Sea clone sequence, but otherwise the relationships to the next relatives are similar to those shown in Fig. 2b.

In *I. leukodermatus* the relationship of the Alpha 3 sequence to other 16S rRNA sequences varied with the phylogeny method used. In maximum-likelihood analyses the closest relatives were the nitrogen-fixing symbionts *Sinorhizobium meliloti* and *Sinorhizobium fredii* (≥95.7% sequence similarity), while parsimony and distance analyses placed the Alpha 3 sequence within a clade of bacteria belonging to the genera *Mesorhizobium*, *Aminobacter*, *Hoeflea*, and *Agrobacterium* and symbionts from the marine brittle star *Ophiactis balli* and the ant *Tetraponera binghami*.

***cbbL* and *aprA* genes.** The *cbbL* gene of RubisCO form I was amplified from all six *I. leukodermatus* and three *I. makropetalos* individuals. The sequences from each host species were identical between individuals. The *cbbM* gene of form II RubisCO was not detectable in either species despite multiple PCR assays under various conditions. Comparative phylogenetic analyses showed that the *I. leukodermatus* and *I. makropetalos cbbL* sequences are closely related to each other (99.5% amino acid sequence identity) (Fig. 3a). The closest relatives of these sequences are RubisCO form IA sequences from gammaproteobacterial sulfur-oxidizing endosymbionts of the snail *Alvinoconcha hessleri*, the clam *Solemya velum*, and the free-living sulfur-oxidizing bacterium *Allochromatium vinosum* (*cbbL*-2) (≥89% amino acid sequence identity). *A. vinosum* has two *cbbL* gene copies, of which only *cbbL*-2 is expressed (42).

The *aprA* gene, coding for the alpha subunit of APS reductase, was found in all six *I. leukodermatus* and three *I. makropetalos* specimens and showed no sequence variation within each host species. The *I. leukodermatus* and *I. makropetalos aprA* sequences are closely related to each other (99.2% amino acid sequence identity) (Fig. 3b). In the absence of *aprA* sequences from other symbiotic sulfur-oxidizing bacteria, the closest relatives to the two *Inanidrilus* sequences are *aprA* sequences from free-living sulfur-oxidizing bacteria, such as

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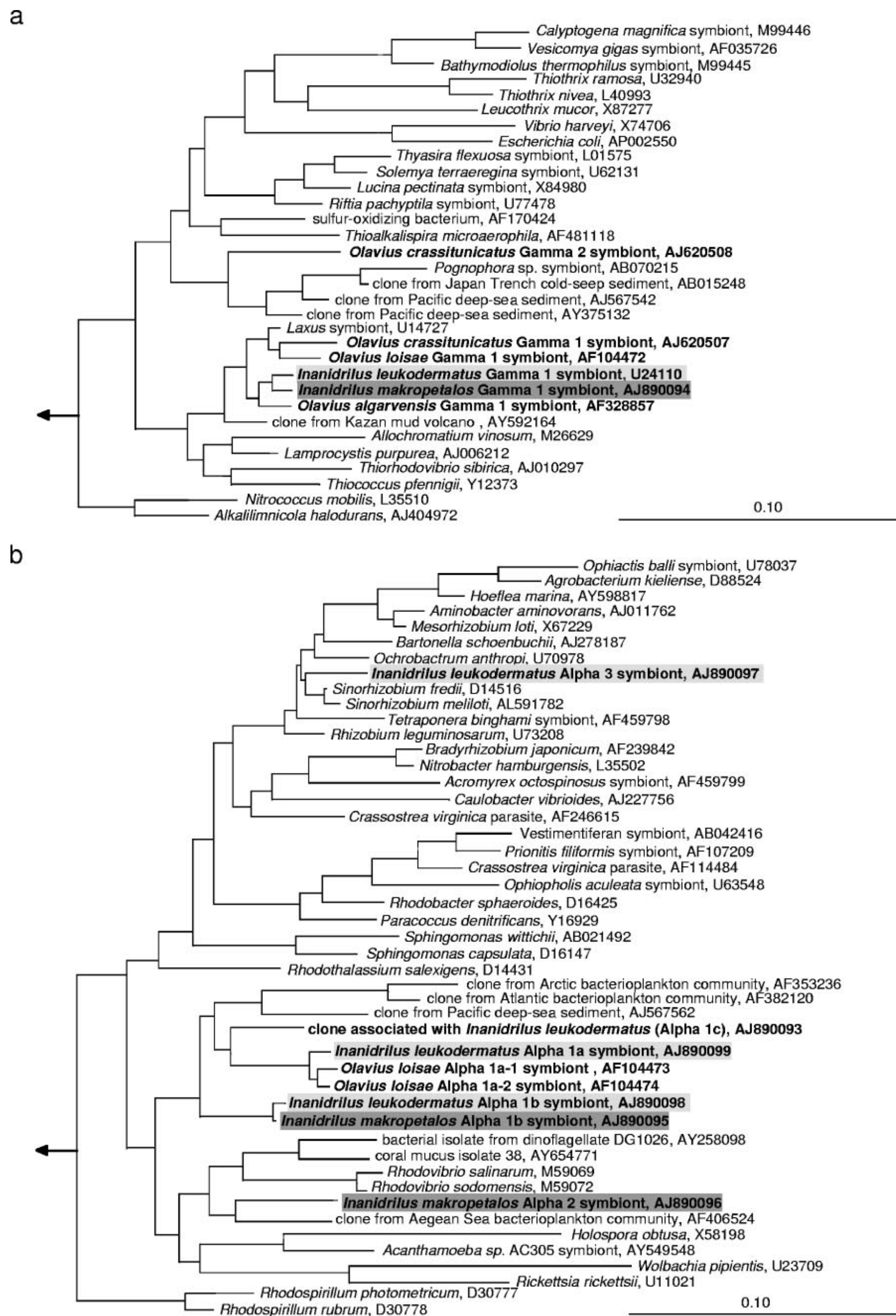

FIG. 2. Phylogenetic placement of bacterial symbionts in *I. leukodermatus* and *I. makropetalos* based on 16S rRNA sequences. Maximum-likelihood trees of members of the (a) *Gammaproteobacteria* and (b) *Alphaproteobacteria* are shown. Symbionts of gutless oligochaetes are listed in bold type, with the *I. leukodermatus* symbionts boxed in light gray and *I. makropetalos* symbionts in dark gray. The bars represent 10% estimated sequence divergence.

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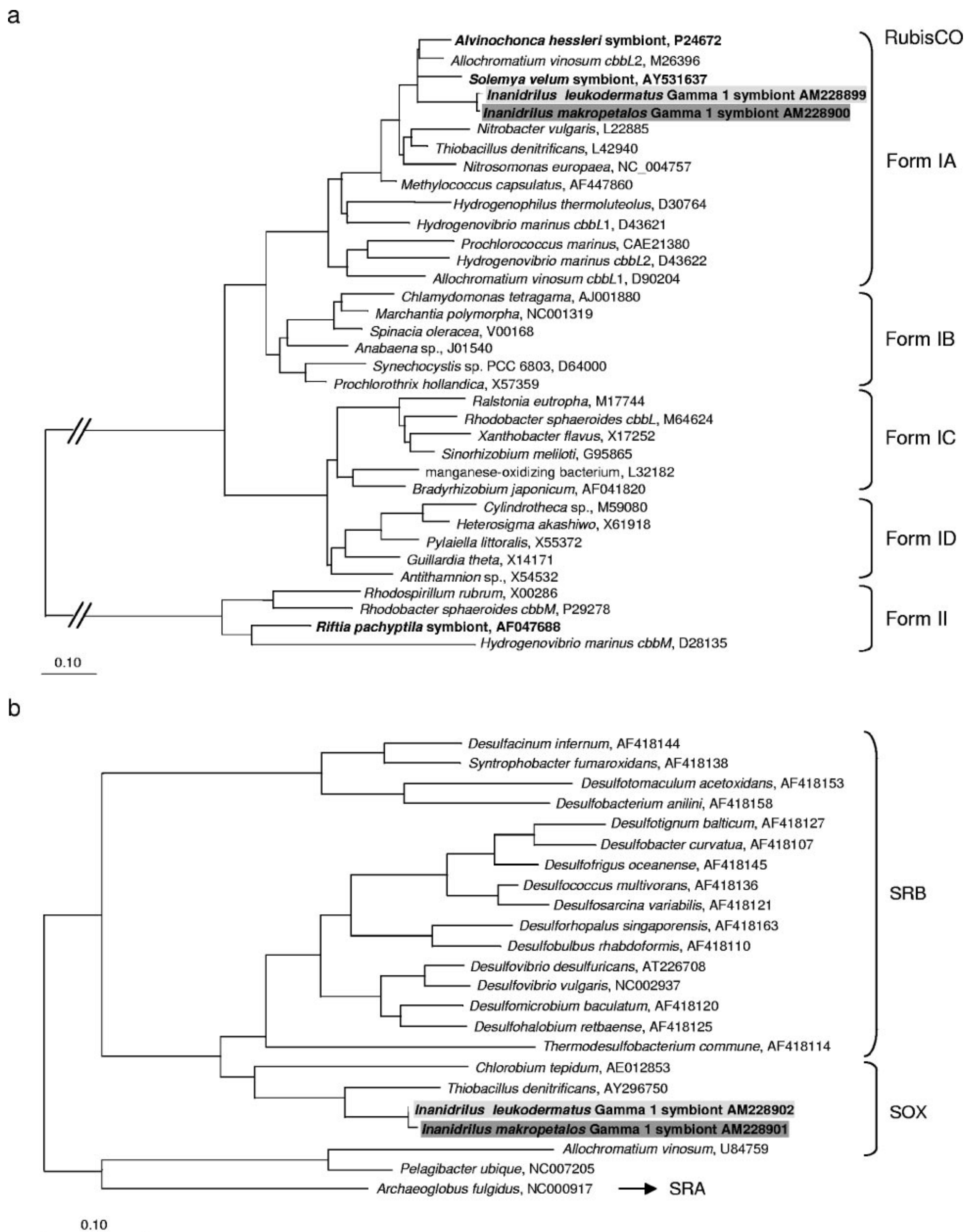

FIG. 3. Phylogenetic placement of symbiotic RubisCO large-subunit (a) and APS reductase alpha-subunit (b) sequences from *I. leukodermatus* and *I. makropetalos* based on maximum-likelihood analyses. The bars represent 10% estimated sequence divergence, except for the estimated divergence between RubisCO form I and II sequences, which corresponds to 124%. Abbreviations: SRB, sulfate-reducing bacteria; SOX, sulfur-oxidizing bacteria; SRA, sulfate-reducing archaeon. Symbionts of chemosynthetic hosts are listed in bold type.

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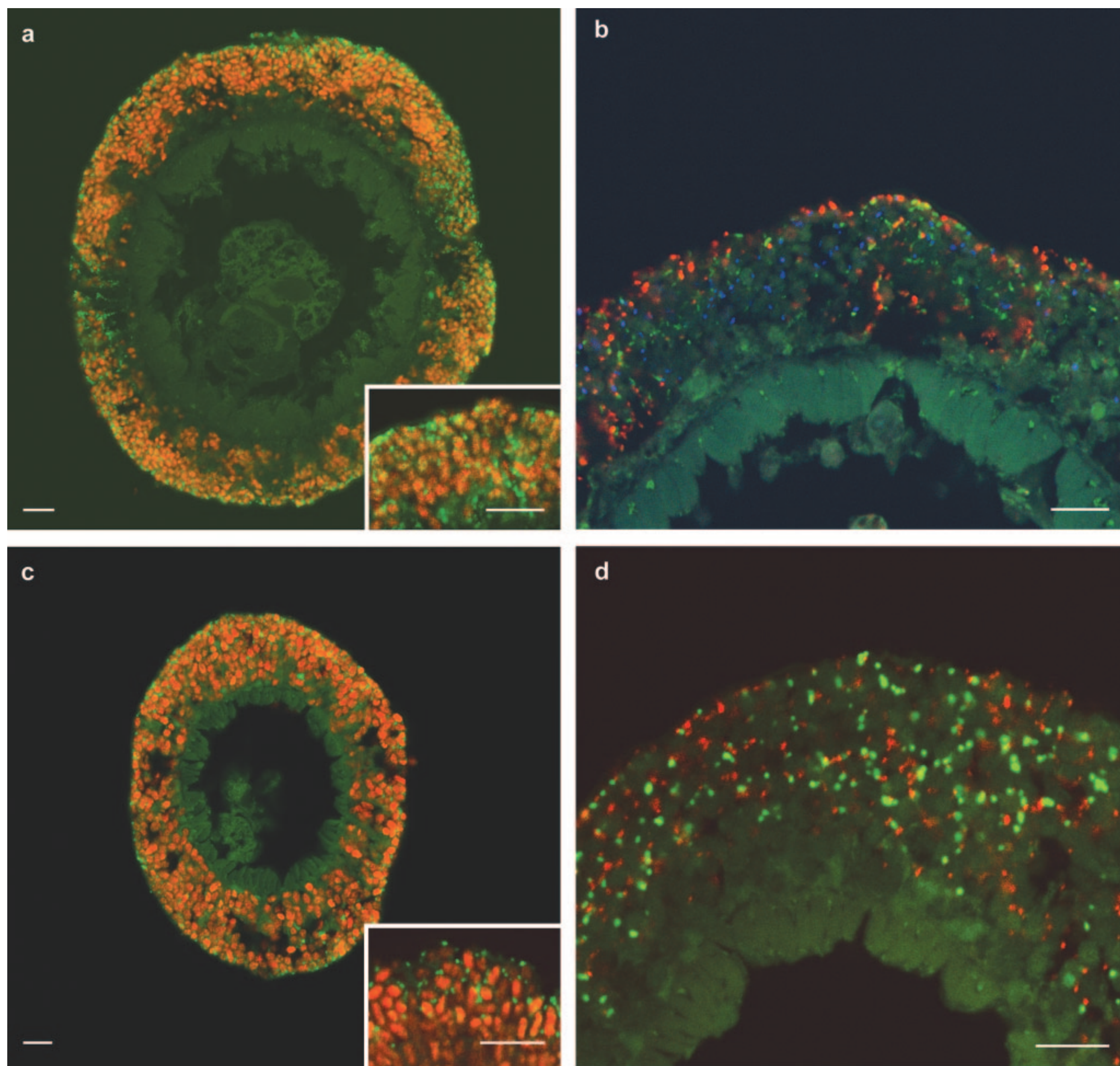

FIG. 4. In situ identification of bacterial symbionts in *I. leukodermatus* (a and b) and *I. makropetalos* (c and d). Epifluorescence images show a cross section through the entire worm and the symbiont-containing region of the worm's body wall (all scale bars, 10 μm). (a) Dual hybridization with InaGAM and IleuAlpha1a-IleuAlpha1b-IleuAlpha3 probes, showing gammaproteobacterial symbionts in red and alphaproteobacterial symbionts in green. (Inset) Symbionts at a higher magnification. (b) Triple hybridization with Alpha 1a, Alpha 1b, and Alpha3 probes, showing the Alpha 1a symbionts in green, Alpha 1b symbionts in red, and Alpha 3 symbionts in blue. (c) Dual hybridization with InaGAM and ImakAlpha 1b-ImakAlpha2 probes, showing gammaproteobacterial symbionts in red and alphaproteobacterial symbionts in green. (inset) Symbionts at a higher magnification. (d) Dual hybridization with ImakAlpha1b and ImakAlpha2 probes, showing the Alpha 1b symbionts in green and Alpha 2 symbionts in red.

the betaproteobacterium *Thiobacillus denitrificans* (86.6% amino acid sequence identity) and *Chlorobium tepidum* of the green sulfur bacteria phylum (60% amino acid sequence identity).

In situ identification. FISH with oligonucleotide probes confirmed that four of the five 16S rRNA phylotypes isolated from *I. leukodermatus* and all three phylotypes isolated from *I. makropetalos* originated from bacteria in the symbiont-containing region between the cuticle and the epidermis of the worm (Fig. 4a and c). In *I. leukodermatus*, the gammaproteobacterial symbiont (Gamma 1) and three alphaproteobacterial symbionts (Alpha 1a, Alpha 1b, and Alpha 3) co-occurred in all six specimens. The Alpha 1c 16S rRNA sequence, found in a single *I. leukodermatus* specimen, was not observed in any of the six worms examined despite the design of six specific probes and multiple in situ hybridizations under different con-

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ditions. This suggests that the Alpha 1c sequence originated from a contaminant or from a symbiont that is very rare. In *I. makropetalos*, all three bacterial phylotypes, the gammaproteobacterial symbiont (Gamma 1) and the two alphaproteobacterial symbionts (Alpha 1b and Alpha 2), were observed to coexist in all examined host specimens.

The hybridization patterns of the Gamma 1 symbionts in the symbiont-containing regions of *I. leukodermatus* and *I. makropetalos* were similar (Fig. 4a and c). In both species, the general probe for *Gammaproteobacteria*, GAM42a, and the specific probe InaGAM for the Gamma 1 sequences from these worms hybridized to large, oval bacteria throughout the symbiont-containing region (Fig. 4a and c), described as the large morphotype in *I. leukodermatus* (9, 23). This morphotype also hybridized with the specific probe IleuGAM for the Gamma 1 symbionts in *I. leukodermatus* (9, 23).

The hybridization signal of the general probe for *Alphaproteobacteria*, ALF968, was limited to small bacterial cells distributed throughout the entire symbiont region of *I. leukodermatus* and *I. makropetalos*. The hybridization patterns of the probes specific to the Alpha symbionts of *I. leukodermatus*, Alpha 1a, 1b, and 3 (Fig. 4b), and of *I. makropetalos*, Alpha 1b and 2 (Fig. 4d), corresponded to those from the general alphaproteobacterial probe ALF968, indicating that all alphaproteobacterial symbionts in the worms were identified with the specific probes.

While the distribution of the Gamma 1 symbionts in the symbiont-containing region was similar in the two host species, the distribution of the Alpha symbionts was different. The two Alpha symbionts of *I. makropetalos*, Alpha 1b and 2, were observed in all cross sections of the three individuals. In contrast, the distribution of three Alpha symbionts of *I. leukodermatus*, Alpha 1a, 1b, and 3, varied between individuals and within an individual, with some cross sections showing the coexistence of all three symbionts and some showing only one or two of the Alpha symbionts. These differences appeared to be random, with no obvious patterns of coexistence between the three Alpha symbionts.

DISCUSSION

Like all other gutless oligochaetes examined to date, *I. leukodermatus* and *I. makropetalos* harbor large, oval bacteria identified as Gamma 1 symbionts (Fig. 2a and 4). These symbionts are more closely related to each other than to the Gamma 1 symbionts from hosts belonging to the genus *Olavius*. The close phylogenetic relationship of the *Inanidrilus* Gamma 1 symbionts is congruent with the close relationship of host species belonging to the genus *Inanidrilus* (10) and could therefore have been caused by cospeciation. Alternatively, their biogeography may have played a role, as the two host species are geographically much closer to each other (both from the northwest Atlantic Ocean) than to *Olavius* species from the Mediterranean Sea and the Pacific Ocean. Additional studies on Gamma 1 symbionts from both *Inanidrilus* and *Olavius* host species to better understand the roles of biogeography and cospeciation in these associations are in progress.

The Gamma 1 symbionts of gutless oligochaetes have been identified as chemoautotrophic sulfur oxidizers based on their close phylogenetic relationship to free-living sulfur oxidizers, the presence of sulfur in their cells, and immunohistochemical studies showing the presence of form I RubisCO in these symbionts (10, 25). This study provides further evidence that these symbionts are thiotrophic, based on the presence of the *cbbL* gene of form I RubisCO and the *aprA* gene of APS reductase. The close relationship of the *I. leukodermatus* and *I. makropetalos cbbL* and *aprA* genes to those of free-living and symbiotic sulfur oxidizers indicates that the oligochaete sequences originated from their sulfur-oxidizing Gamma 1 symbionts. An alternative explanation, that some or all of the alphaproteobacterial symbionts of these hosts are sulfur-oxidizing chemoautotrophs and that they are the source of these genes, is unlikely. The oligochaete Alpha symbionts are not related to known alphaproteobacterial sulfur oxidizers, and it has been shown that these small bacteria in *I. leukodermatus* do not contain sulfur and are not labeled by a form I RubisCO antiserum (25).

Prior to this study, alphaproteobacterial symbionts had been found in only a single gutless oligochaete species: *O. loisae*, from the Australian Great Barrier Reef (7). The presence of these symbionts in two species of the host genus *Inanidrilus* that are geographically very distant from *O. loisae* indicates that symbioses with alphaproteobacterial symbionts may be more widespread in gutless oligochaetes than previously assumed. The Alpha 1 symbionts occur in all three host species with alphaproteobacterial symbionts and are relatively closely related to each other (≥92.6% sequence similarity). Within this clade, different phylotypes can co-occur in the same species, such as Alpha 1a and 1b symbionts in *O. loisae* and *I. leukodermatus*. In addition to the Alpha 1 lineage of symbionts, *I. leukodermatus* and *I. makropetalos* harbor additional alphaproteobacterial symbionts, the former Alpha 2 and the latter Alpha 3 symbionts, that are phylogenetically distinct from each other and the Alpha 1 symbionts.

The metabolism of the alphaproteobacterial symbionts is not currently known. The closest free-living relatives of the Alpha 1 symbionts from both host species and the Alpha 2 symbionts from *I. makropetalos* are the halophilic bacteria *Rhodovibrio salinarum* and *R. sodomensis* (≥89.2% sequence similarity). Both species are photoheterotrophic under anoxic conditions, while only *R. salinarum* can also grow in the dark under aerobic conditions as a chemoheterotroph (26, 30). Tests for photoautotrophic growth with reduced sulfur compounds were negative for both species (26, 30). The common feature of these bacteria is their use of fermentation products such as lactate, acetate, succinate, malate, and pyruvate as electron and carbon sources during anaerobic growth (26, 30). These metabolites are produced by marine invertebrates when oxygen concentrations become limiting and are excreted in animals without symbionts. It was suggested that in gutless oligochaetes with sulfate-reducing deltaproteobacterial symbionts, these might take up the anaerobic waste products of their hosts, thus recycling these valuable carbon compounds (10, 34). It is intriguing that in all gutless oligochaetes, either alpha- or deltaproteobacterial symbionts coexist with the Gamma 1 symbionts, and it is tempting to speculate that the alphaproteobacterial symbionts might play a role similar to that of the deltaproteobacterial symbionts by recycling the anaerobic waste products of the worms.

The Alpha 3 symbiont from *I. leukodermatus* is most closely

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related to nitrogen-fixing *Sinorhizobium* symbionts of leguminous plants (≥95.7% sequence similarity), suggesting that the Alpha 3 symbionts might also fix N_2. However, numerous attempts to amplify the *nifH* gene, involved in bacterial N_2 fixation, were unsuccessful (N. Dubilier and J. Zehr, unpublished data). Since this symbiont occurs only in *I. leukodermatus*, it does not appear to be essential for nitrogen uptake, as other oligochaete hosts are clearly able to acquire nitrogen without this Alpha 3 symbiont.

The association between the multiple symbiotic bacteria and the gutless oligochaetes *I. leukodermatus* and *I. makropetalos* is highly specific and stable within each host species. FISH analyses showed that the symbionts occur regularly in all examined individuals. In addition, the 16S rRNA sequences of the Gamma 1 symbiont from *I. leukodermatus* worms collected in 1992 (9) and in 1998 (this study) are identical (with the exception of three nucleotide substitutions caused by sequencing error), indicating a high specificity and evolutionary stability of this symbiont over this period of time. A high phylogenetic diversity of symbiotic bacteria was first observed in the gutless oligochaete *O. crassitunicatus* that harbors up to six bacterial phylotypes (3). This study shows that the stable coexistence of multiple endosymbionts is not limited to a single species but rather appears to be a common feature in oligochaete symbioses.

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