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Phylogenetic diversity of bacterial endosymbionts in the gutless marine oligochete *Olavius loisae* (Annelida)

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ABSTRACT: Endosymbiotic associations with more than 1 bacterial phylotype are rare among chemoautotrophic hosts. In gutless marine oligochetes 2 morphotypes of bacterial endosymbionts occur just below the cuticle between extensions of the epidermal cells. Using phylogenetic analysis, *in situ* hybridization, and denaturing gradient gel electrophoresis based on 16S ribosomal RNA genes, it is shown that in the gutless oligochete *Olavius loisae*, the 2 bacterial morphotypes correspond to 2 species of diverse phylogenetic origin. The larger symbiont belongs to the gamma subclass of the *Proteobacteria* and clusters with other previously described chemoautotrophic endosymbionts. The smaller symbiont represents a novel phylotype within the alpha subclass of the *Proteobacteria*. This is distinctly different from all other chemoautotrophic hosts with symbiotic bacteria which belong to either the gamma or epsilon *Proteobacteria*. In addition, a third bacterial morphotype as well as a third unique phylotype belonging to the spirochetes was discovered in these hosts. Such a phylogenetically diverse assemblage of endosymbiotic bacteria is not known from other marine invertebrates.

KEY WORDS: Symbiosis · Chemoautotrophic bacteria · Oligochaeta · 16S rRNA

INTRODUCTION

Symbioses with prokaryotic organisms have been one of the major driving forces in the evolution and diversification of eukaryotes (Margulis 1993). Eukaryotic cells carry the evolutionary history of endosymbiotic relationships in their mitochondria and plastids, organelles that appear to have evolved from prokaryotic ancestors of the alpha subclass of the *Proteobacteria* and cyanobacteria, respectively, through associations with ancestral eukaryotic hosts (Gray & Spencer 1996). More recent endosymbioses with prokaryotes have been established multiple times in many of the major metazoan groups (reviewed in Douglas 1994), and the diversity of these associations demonstrates their plasticity and evolutionary success.

Symbioses between chemoautotrophic bacteria and marine invertebrates were first discovered barely 2 decades ago during explorations of hydrothermal vents in the deep-sea. It is now known that these associations are geographically widespread, can be found in more common environments such as shallow-water sediments, and occur in more than 200 species representing at least 5 different animal host phyla (Fisher 1990, Cavanaugh 1994). Most chemoautotrophic hosts harbor a single endosymbiotic species of thioautotrophic metabolism (i.e. sulfur-oxidizing chemoautotroph). Only in a few host species has the presence of a second bacterial endosymbiont of methanotrophic metabolism

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(i.e. methane-utilizing) been detected (Distel 1998). Thus the stable coexistence of multiple endosymbiotic species is rare among these associations, perhaps because of competition among symbionts for resources and space, the increased evolutionary cost of recognizing, regulating, and transmitting more than 1 symbiont, or the dependence of the evolutionary unit on the survival of 3 or more separate organisms (Distel et al. 1995).

It is therefore intriguing that in 1 group of chemoautotrophic hosts, the gutless marine oligochetes from the animal phylum Annelida, all worms examined so far (at least 20 species) harbor 2 distinct bacterial morphotypes suggesting the presence of 2 distinct bacterial symbiont species. These hosts are an ideal group for studying the diversity and evolution of chemoautotrophic symbioses. The endosymbiotic associations occur within a monophyletic host group of 2 genera, *Olavius* and *Inanidrilus*, with 70 named species that are geographically widespread and easy to collect as they are commonly found in shallow-water sediments of tropical coral reefs (Erséus 1992). The associations are obligate for the hosts which lack mouth, gut, and anus, and symbiont transmission is assumed to occur directly from the parental generation to the fertilized egg through genital pads that are packed with the symbiotic bacteria (Giere & Langheld 1987).

The successful identification of multiple symbiont species can only be achieved using molecular methods, as chemoautotrophic symbionts have not yet been isolated from their hosts. The phylogenetic analysis of ribosomal RNA (rRNA) genes has provided a framework for characterizing uncultured organisms, because the gene sequences can be obtained without separating the bacteria from their habitat or host (Woese 1987, Pace 1997). Previously, molecular studies of gutless oligochetes were hampered by the small size of these meiofaunal worms (ca 200 µm × 10 mm) and the need to pool up to 100 individual worms for DNA isolation (Dubilier et al. 1995). Only recently have we developed methods that allow us to analyse single worms, enabling the characterization of symbionts in host species that do not occur in high abundances. This study uses phylogenetic analysis, *in situ* hybridization, and denaturing gradient gel electrophoresis (DGGE) based on 16S rRNA sequences to characterize the bacterial symbionts of the gutless oligochete *Olavius* (*Coralliodriloides*) *loisae* Erséus 1984.

MATERIALS AND METHODS

Specimen collection. *Olavius loisae* specimens were collected in April 1994 at about 3 m depth at Lizard Island in the Australian Great Barrier Reef. The worms were extracted from the sediment by decantation with seawater and identified alive under a light microscope. Only viable, active, and intact worms were used in this study.

Transmission electron microscopy (TEM). Six *Olavius loisae* individuals were fixed in Trump's fixative (McDowell & Trump 1976) and prepared for electron microscopical examination as described previously (Dubilier et al. 1995).

DNA preparation and polymerase chain reaction (PCR) amplification. *Olavius loisae* individuals were rinsed in 0.2 µm filtered seawater, placed singly in 20 µl of the cell lysis reagent GeneReleaser (BioVentures), and stored in GeneReleaser at +4°C for up to 2 wk. In the home laboratory the samples were vortexed for 30 s, microwaved for 5 min at 900 W, heated at 80 to 85°C for 5 min, and stored at –20°C.

For PCR amplifications of the 16S rRNA gene, 20 µl of ultrapure water was added to the GeneReleaser samples and each diluted sample was then split equally between 2 tubes. Samples were overlayed with oil, heated at 94°C for 5 min, and placed on ice for 5 min. PCR reagents were added directly to the mix to a total volume of 100 µl. Amplifications were performed with primers specific for the *Bacteria* 16S rRNA gene (27f and 1492r, Lane 1991).

Cloning and sequencing. PCR products from 4 different host individuals were cloned separately and sequenced. In 1 worm PCR products were also directly sequenced to determine if a single sequence predominated. Cloning was performed with the TA cloning kit (Vector Laboratories) or the pGEM-T Easy Vector System (Promega). Plasmid DNA was prepared using the QIAprep Plasmid Kit (Qiagen). Clones were screened and grouped into families by single nucleotide track sequencing (Coulter-Mackie 1994) using the 519r universal sequencing primer (Lane 1991). This screening method was verified by partially sequencing at least 4 clones per clone family. Sequencing was performed manually using Sequenase V.2 (United States Biochemical), or with an automated ABI 373S DNA sequencer using the *Taq* Dyedeoxy Terminator Cycle Sequencing Kit (Applied Biosystems). For each host individual a representative clone from each of the numerically dominant clone families was almost fully sequenced (1450 to 1500 bp) in both directions. The symbiont sequences from *Olavius loisae* were submitted to GenBank under Accession Numbers AF104472 (γ-*Proteobacteria* symbiont), AF104473 and AF104474 (α-*Proteobacteria* symbionts), and AF104475 (spirochete symbiont).

Phylogenetic analysis. Sequences were automatically inserted into an alignment of ~6000 small subunit rRNA sequences using the ARB program package (Strunk et al. 1998). Alignments were verified by comparison with manual alignments based on the pre-

dicted secondary structure of *Escherichia coli* (Gutell et al. 1994). Sequence similarities were calculated with ARB using the Olsen correction. Phylogenetic analyses were performed with ARB using maximum parsimony, distance matrix (neighbor joining), and maximum likelihood methods. Only sequences that were at least 90% complete were used in these analyses. Bootstrap analyses (1000 replicates) were performed with the Phylip program (Ver. 3.5) (Felsenstein 1989) for parsimony methods and with ARB for distance analyses.

Fluorescence *in situ* hybridization. Approximately 20 *Olavius loisae* individuals were fixed, embedded, and pretreated as described previously (Dubilier et al. 1995). Hybridizations were carried out for 4 h at 46°C as described by Manz et al. (1992). Hybridizations were carried out at the formamide concentration determined to be specific (see below) for each probe: 55% formamide for the Oloi-alpha and 0% formamide for the Oloi-gamma probe (see below for probe definition). Slides were mounted in a non-fluorescent medium (Vectashield, Vector), and viewed with a Zeiss Axioskop microscope.

Oligodeoxynucleotide probes were designed for 16S rRNA sequences of each of the 3 numerically dominant clone families and designated Oloi-alpha (sequence TTCCGCTACCCTCTCCCGGAC; *Escherichia coli* positions 656 to 676), Oloi-gamma (sequence CCTTTCCCCCATAGGACGT; *E. coli* positions 181 to 199), Oloi-spiro 1 (sequence ATGAGCTATCCCCAACCAAA; *E. coli* positions 138 to 157) and Oloi-spiro 2 (sequence CCCTTTCAACACTCCGCCTA; *E. coli* positions 188 to 200). The probes were checked against sequences in GenBank using BLAST (Altschul et al. 1990) and against small-subunit rRNA sequences in the Ribosomal Database Project using CHECK-PROBE (Olsen et al. 1992) and contained at least 1 mismatch to all entered sequences and at least 2 mismatches to all known symbionts from marine invertebrates. The *Bacteria* probe EuB338 and the γ-*Proteobacteria* probe GAM42a were used as positive controls (Amann et al. 1995). As a further positive control, sections were stained with DAPI (10 min in 1 μg ml^{-1}) after hybridizations with the Oloi probes, to check for the presence of all 3 bacterial morphotypes. As negative controls the β-*Proteobacteria* probe BET42a (Amann et al. 1995) as well as a probe with 2 mismatches to the targeted sequence of the Oloi-alpha probe (sequence TTCCGCTAGCCTCTGCCGGAC; *E. coli* positions 656 to 676) were used. The oligonucleotide probes were synthesized and labeled fluorescently with Cy3 by Biometra (Göttingen, Germany).

The specificity of the Oloi-probes was checked by hybridizations with bacteria that have 16S rRNA target sites with only 1 or 2 mismatches to the Oloi-alpha and -gamma probes, respectively. These bacteria were *Geobacter metallireducens* DSM 7210 (target sequence GUACGGGAGAGGGUAGUGGAA; *Escherichia coli* positions 656 to 676) for the Oloi-alpha probe, and *Alcaligenes faecalis* DSM 30030 (target sequence ACGCCCUACGGGGGAAAGG, *E. coli* positions 181 to 199) for the Oloi-gamma probe. Bacterial cells were harvested during log phase growth, and fixed and hybridized as described by Amann (1995).

DGGE analysis. The 16S rRNA genes from 2 *Olavius loisae* specimens and *O. loisae* clones were amplified by PCR with the primers 341f-GC clamp and 907r using a 'touchdown' PCR as described by Muyzer et al. (1998). Denaturant gradient gels (20 to 80%) of 1 mm thickness and 6% (w/v) polyacrylamide were run for 18 h at a constant voltage of 100 V in 1× TAE electrophoresis buffer, pH 8.3 (40 mM Tris, 20 mM sodium acetate, 1 mM EDTA) using the D-Code system (Bio-Rad). After electrophoresis, gels were stained for 20 min in ethidium bromide (0.5 mg ml^{-1}) and photographed on a UV transillumination table (302 nm) with a Polaroid camera.

DGGE bands were sequenced according to Muyzer et al. (1998) by excising the bands from the gel with a sterile razor blade, and beating the gel slices in 0.5 ml sterile glass beads with the same volume of sterile water for 3 × 1 min at highest speed in a bead beater. After incubation overnight at 4°C, the supernatant was used as template DNA for reamplification with the same primers as described above. After purification of the PCR products with the Qiaquick Purification Kit (Qiagen), these were sequenced automatically as described above.

RESULTS

TEM

As in all other gutless oligochetes, 2 bacterial morphotypes were found in *Olavius loisae* that occurred extracellularly in a layer between the cuticle and the epidermis (Fig. 1). The larger morphotype (~1.4 × 2.7 μm) contained numerous cytoplasmic globules, some of which were membrane-bound, while the smaller morphotype (~0.3 × 1.2 μm) had no cytoplasmic inclusions except for a conspicuous net of DNA threads. Two of the 6 worms examined harbored a third bacterial morphotype of intermediate width (~0.7 μm) but greater length (~3 μm) than the 2 other morphotypes. In contrast to the even distribution of the large and small morphotypes throughout the bacteria-containing region, the third intermediate morphotype only occurred peripherally just below the cuticle (Fig. 1). Electron-dense bodies at the apical ends of this third morphotype as well as invaginations of the cell wall were regularly observed.

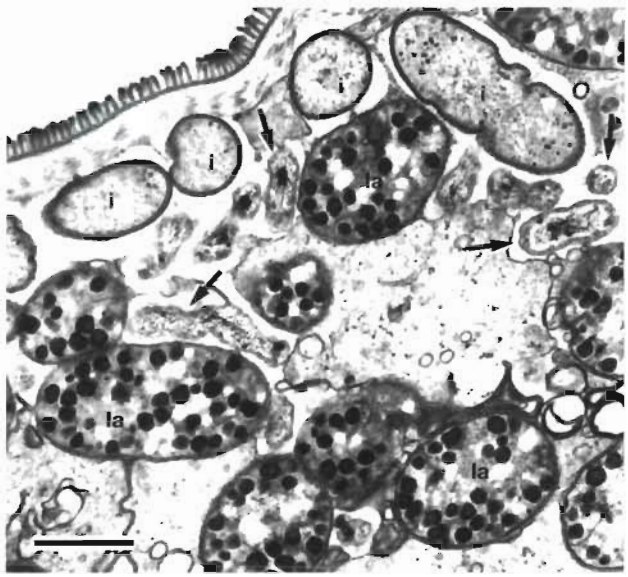

Fig. 1. Transmission electron micrograph of bacterial symbionts in *Olavius loisae* showing symbiont-containing region just below the cuticle of the worm. Note 3 distinct bacterial morphotypes: large bacteria (la) with cytoplasmic globules and small bacteria (arrows) interspersed among the large bacteria which both occur in all 6 specimens examined while intermediate sized bacteria (i) (also referred to as the third morphotype) occurred in 2 out of 6 worms. Bar = 1 µm

Clone families

Bacterial 16S rRNA amplification products from 4 *Olavius loisae* specimens were cloned individually after direct sequencing of PCR products from 1 specimen revealed a heterogeneous pattern. Three distinct clone families were identified with 63 out of a total of 66 analysed clones belonging to 1 of these 3 families (Table 1). Partial 16S rRNA sequence comparisons revealed that within each clone family members shared nearly identical sequences. BLAST similarity searches (Altschul et al. 1990) and phylogenetic analyses (see below) identified the 3 clone families as members of the (1) α-*Proteobacteria*, (2) γ-*Proteobacteria*, and (3) spirochetes.

In 3 out of 4 host individuals, the α-*Proteobacteria* clone family was numerically dominant (Table 1). One representative clone from each of these 3 hosts was nearly fully sequenced (~1450 bp). Two of these hosts shared nearly complete sequence identity (0.07% difference; GenBank Accession Number AF104473), while a third host differed by 1.4% from the 2 other specimens (GenBank Accession Number AF104474). The spirochete clone family was the second most dominant group, occurring in 3 of the 4 host worms (Table 1). One representative clone from each of these 3 hosts was nearly fully sequenced (~1500 bp), and sequence differences between host individuals of at most 0.2% were observed (GenBank Accession Number AF104475). The γ-*Proteobacteria* clone family was found in only 2 of the 4 host individuals (Table 1), and their sequences differed by only 0.5% (~1500 bp of a representative clone from each of the 2 hosts was sequenced; GenBank Accession Number AF104472).

Phylogenetic analyses

Parsimony, distance, and maximum likelihood analyses of the 16S rRNA sequences from each of the 3 *Olavius loisae* clone families confirmed that these belong to 3 phylogenetically distinct bacterial groups: (1) the γ-*Proteobacteria*, (2) the α-*Proteobacteria*, and (3) the spirochetes (Fig. 2). All 3 *O. loisae* sequences are unique to this host and differ from those of symbionts from other host species or of free-living bacteria.

In all 3 phylogenetic analyses the *Olavius loisae* γ-*Proteobacteria* sequence consistently falls in a clade with 2 other chemoautotrophic symbionts (Fig. 2A): the endosymbiont from another gutless oligochete *Inanidrilus leukodermatus* and the ectosymbiont from the marine nematode *Laxus oneistus* (96% similarity to both symbionts). Bootstrap values of 100% for both parsimony and distance methods demonstrate that the oligochete-nematode symbiont clade is monophyletic but the relationships within this clade cannot be resolved.

The *Olavius loisae* α-*Proteobacteria* sequence forms an isolated branch within the α-subclass and is not closely related to other symbiotic, parasitic, or free-living α-*Proteobacteria* (Fig. 2B). The free-living bacteria *Rhodospirillum rubrum*, *R. photometricum*, *R. sodomense*, and *R. salinarum* are consistently placed on neighboring branches of the *O. loisae* alpha sequence (ca 85% similarity) in all 3 treeing methods, but bootstrap analyses do not support a close relationship.

Table 1 16S rRNA clone libraries from *Olavius loisae* individuals (No. of clones: number of clones analysed, Gamma: γ-*Proteobacteria*, Alpha: α-*Proteobacteria*, Spiro: spirochete)

Worm	No. of clones	Gamma	Alpha	Spiro	Others
O. loisae 1	17	3 (18%)	10 (59%)	3 (18%)	1 (6%)
O. loisae 2	18	1 (6%)	0 (0%)	16 (89%)	1 (5%)
O. loisae 3	10	0 (0%)	9 (90%)	0 (0%)	1 (10%)
O. loisae 4	21	0 (0%)	17 (81%)	4 (19%)	0 (0%)

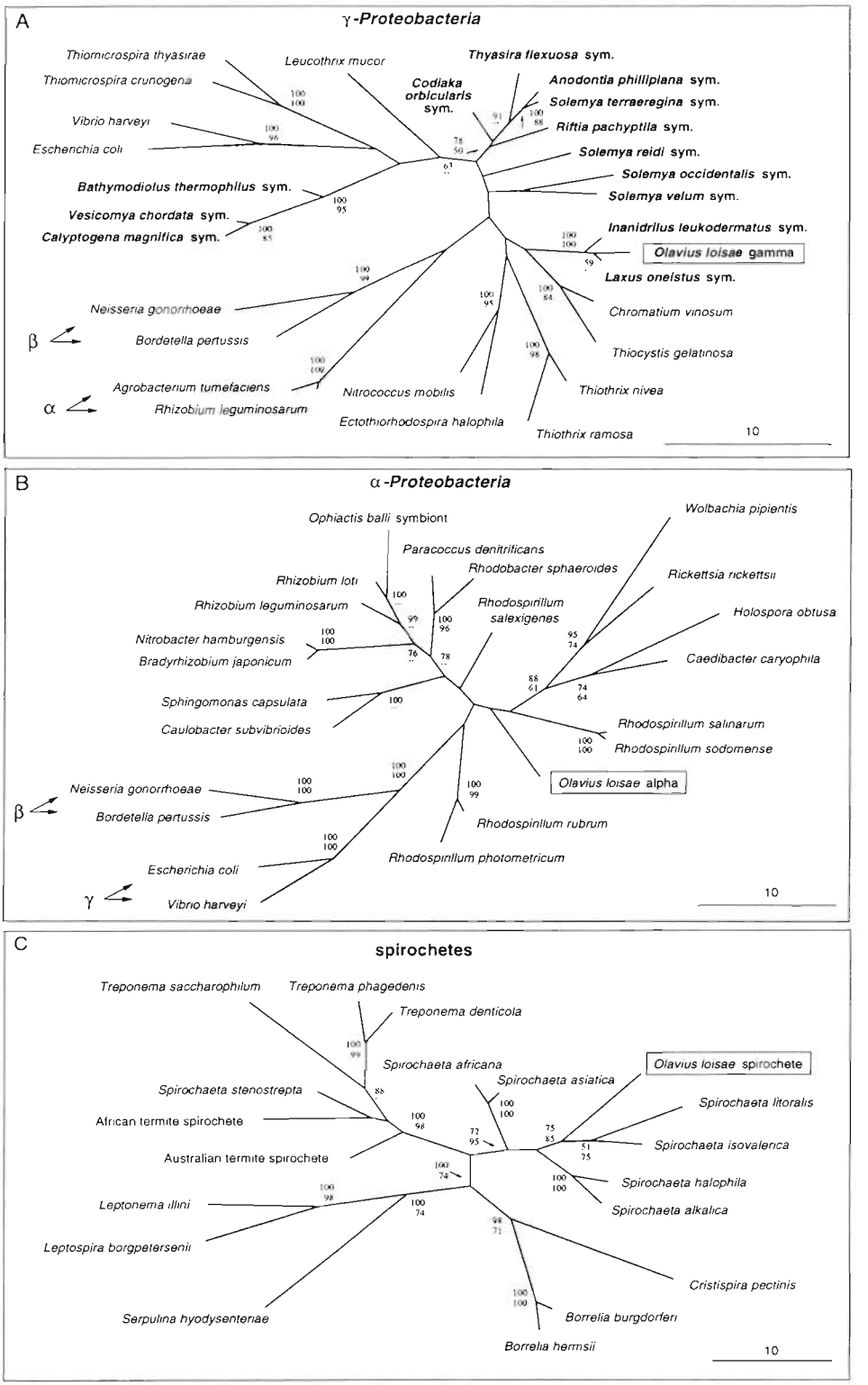

Fig. 2. Phylogenetic placement of bacterial symbionts from *Olavius loisae* based on 16S rRNA sequences. Maximum likelihood trees of members of the (A) γ-*Proteobacteria* (α- and β-*Proteobacteria*, marked with arrows; chemoautotrophic symbionts are listed in bold type [sym: symbiont]), (B) α-*Proteobacteria* (β- and γ-*Proteobacteria*, marked with arrows), and (C) spirochetes. Bootstrap values greater than 50% are shown, with upper and lower values representing those from parsimony and distance analyses, respectively. Sequences determined in this study are framed; the α-*Proteobacteria* symbiont sequence in (B) represents the consensus sequence between AF104473 and AF104474. Bar = 10 nucleotide substitutions per 100 nucleotide positions

The *Olavius loisae* spirochete sequence is consistently placed within the *Spirochaeta* group of the spirochetes by all 3 phylogenetic analyses with a distance bootstrap value of 95% strongly supporting the grouping of these organisms (Fig. 2C). The closest relatives to the *O. loisae* spirochete (ca 85% sequence similarity) are the free-living marine spirochetes *Spirochaeta isovalerica* and *S. litoralis*, as supported by all 3 treeing methods (distance and parsimony bootstrap values 85 and 75%, respectively).

In situ identification

In situ hybridizations with oligonucleotide probes confirmed that the α- and γ-*Proteobacteria* 16S rRNA sequences originated from the symbiotic bacteria of *Olavius loisae*. The species-specific probes Oloi-alpha and Oloi-gamma hybridized specifically to the symbiont-containing region of the host between the cuticle and the epidermis (Fig. 3). The hybridization patterns of the 2 probes were distinctly different. The signal from the Oloi-gamma probe was consistent with the size, shape, and distribution of the large bacterial morphotype (Fig. 3A) and was identical to the signal from the probe for all γ-*Proteobacteria*. In negative controls, the probe for all β-*Proteobacteria* did not hybridize to the symbiont region or any other part of the worm. The signal from the Oloi-alpha probe was consistent with the appearance and distribution of the small bacterial morphotype (Fig. 3B), while negative control probes with a 2 bp mismatch did not hybridize. The signals from both the Oloi-alpha and Oloi-gamma probe were distributed evenly throughout the symbiont-containing region, paralleling the regular distribution of the large and small bacterial morphotype between the cuticle and epidermis. This shows that these signals could not have originated from the third intermediate bacterial morphotype, which occurred only in a thin layer just below the cuticle.

The specificity of the Oloi-alpha and Oloi-gamma probes was demonstrated in hybridizations with reference bacteria that had nearly identical sequences at the targeted 16S rRNA sites (data not shown). The signal of the Oloi-alpha probe disappeared at 55% formamide in hybridizations with *Geobacter metallireducens* (1 mismatch) while the probe signal in hybridizations with the *Olavius loisae* α-*Proteobacteria* symbionts was still strong at the same formamide concentration (Fig. 3B). The Oloi-gamma probe failed to hybridize with *Alcaligenes faecalis* (2 mismatches) at even 0% formamide, proving the specificity of this probe at low stringencies.

The localization of the spirochete 16S rRNA sequence in *Olavius loisae* was not successful despite the design of 2 specific probes, Oloi-spiro 1 and 2, and multiple *in situ* hybridizations under varying conditions. The expected identification of the third intermediate bacterial morphotype as the spirochete was thus not possible. In an attempt to establish whether the third morphotype represented a morphological variant of the α- or γ-*Proteobacteria* symbiont, simultaneous hybridizations with DAPI staining and the symbiont specific probes were examined. The intermediate bac-

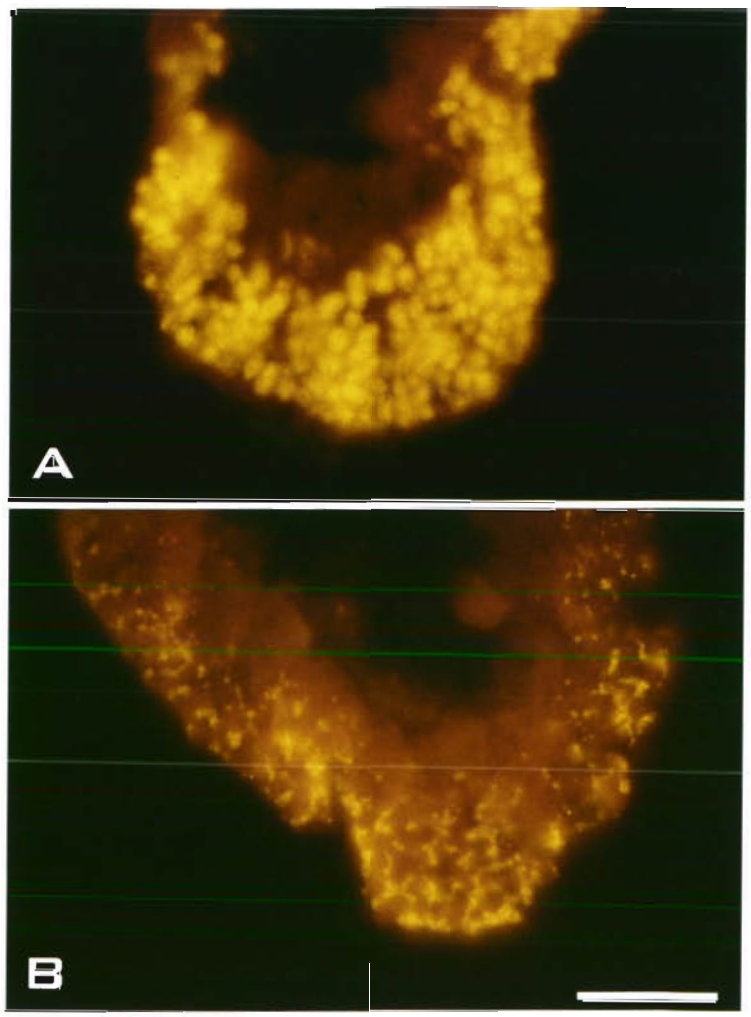

Fig. 3. *In situ* identification of bacterial symbionts in *Olavius loisae*. Epifluorescence micrographs show the body wall of the host with the symbiont-containing region. Probes: (A) Oloi-gamma probe and (B) Oloi-alpha probe. Bar = 20 µm, valid for both micrographs

teria were clearly labeled by DAPI but not by the Oloi-alpha probe, indicating that they are not a morphological variant of the α-*Proteobacteria* symbiont. Similar experiments with the Oloi-gamma probe could not be resolved because the signal from the large bacterial morphotype was so strong that background fluorescence did not allow the distinction of the third bacterial morphotype (nor of the small bacterial morphotype). The same problem was encountered with the *Bacteria* universal probe EuB338 so that it remained unclear whether the third bacterial morphotype could at all be successfully labeled.

DGGE analysis

To further address the origin of the 3 bacterial 16S rRNA sequences isolated from *Olavius loisae*, PCR products from 2 worm individuals and clones isolated from the worms were compared using DGGE. In the *O. loisae* hosts, 3 distinct bands were present indicating that *O. loisae* harbors 3 bacterial phylotypes (representative data from 1 specimen shown in Fig. 4). Each band corresponded in its migration pattern to the respective band from the α-*Proteobacteria*, γ-*Proteobacteria*, and spirochete clone isolated from *O. loisae* (Fig. 4). The congruence in migration patterns between the *O. loisae* host bands and the 3 bacterial clone bands strongly suggests that these are identical. This assumption was verified by sequencing the 3 DGGE bands present in *O. loisae*. Each DGGE band sequence was identical (γ-*Proteobacteria* and spirochete sequence) or nearly identical (2 bp difference for the α-*Proteobacteria* sequence) to its corresponding clone library sequence over 420 to 500 nucleotides.

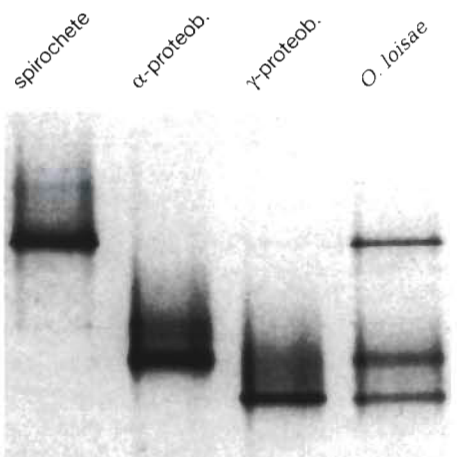

Fig. 4. DGGE profiles of 16S rRNA PCR fragments from an *Olavius loisae* specimen (right lane) and spirochete, α-*Proteobacteria*, and γ-*Proteobacteria* clones isolated from *O. loisae* (3 left lanes)

DISCUSSION

The symbiont morphology of *Olavius loisae* is highly unusual because of the presence of 3 distinct bacterial morphotypes. In all gutless oligochetes studied so far, only 2 morphotypes of bacterial endosymbionts have been described (Giere et al. 1995, Giere 1996). In the first host species studied with molecular methods, *Inanidrilus leukodermatus*, only a single 16S rRNA phylotype was identified, and it was not possible to distinguish whether the 2 bacterial morphotypes represent structural dimorphism of a single symbiont species or correspond to 2 distinct phylotypes (Dubilier et al. 1995). The results presented here prove that in *O. loisae* the large and small bacterial morphotype represent 2 distinct phylotypes belonging to the γ- and α-subclasses of the *Proteobacteria*. The phylogeny of the third bacterial morphotype remains inconclusive, but its identification as a spirochete is consistent with the data presented here (see discussion below).

The *Olavius loisae* association is distinctly different from other chemoautotrophic symbioses. It is the only known association with a symbiont belonging to the α-*Proteobacteria*, as all previously analyzed symbionts of chemoautotrophic hosts belong to either the γ- or the ε-subclasses of the *Proteobacteria*. The coexistence of 2 such phylogenetically diverse endosymbionts as the γ- and α-*Proteobacteria* symbionts of *O. loisae* has not been previously described in a chemoautotrophic association. Of the few hosts known to harbor 2 coexisting phylotypes, such as some bivalves with thioautotrophic and methanotrophic bacteria, both symbiont species belong to the γ-*Proteobacteria* (Distel 1998). Furthermore, the presence in *O. loisae* of a third bacterial phylotype belonging to the spirochetes appears unique among chemoautotrophic symbioses.

The large bacterial morphotype of *Olavius loisae* was clearly identified as a γ-*Proteobacteria* symbiont in this study on the basis of 16S rRNA analysis, DGGE, and *in situ* hybridizations. Although inferring metabolic capabilities from 16S rRNA phylogeny alone can be misleading, the thioautotrophic nature of the *O. loisae* gamma symbiont is suggested by its position in the oligochete-nematode clade which is composed exclusively of symbionts already characterized as thioautotrophic (Polz et al. 1994, Dubilier et al. 1995), and by the very close evolutionary relationships among these symbionts. Furthermore, electron spectroscopy imaging analyses of the *O. loisae* gamma symbiont indicate the presence of sulfur in membrane-bound cytoplasmic globules (Krieger & Giere unpubl. results), a common morphological feature in thioautotrophic bacteria (Steudel 1989).

The monophyly of the oligochete-nematode clade, first determined between the symbionts of the oligochete *Inanidrilus leukodermatus* and the nematode

Laxus oneistus (Dubilier et al. 1995), is reconfirmed in this study with the addition of the *Olavius loisae* gamma sequence. Such a close evolutionary relationship between the symbionts of this clade is surprising given the distinct differences in morphology between the endosymbionts of the oligochetes and the ectosymbionts of the nematode and the very distant relationship between these 2 host groups. The overall topology of the chemoautotrophic symbiont tree was altered by the addition of the *O. loisae* gamma sequence: the oligochete-nematode symbiont clade was consistently separated from the chemoautotrophic symbionts of the *Solemya-Riftia-Thyasira* clade, in contrast to results from previous analyses which suggested a closer evolutionary relationship between these 2 groups of symbionts (Polz et al. 1994, Dubilier et al. 1995, Distel 1998). While no free-living bacteria were previously known to fall within a cluster of chemoautotrophic symbionts, it now appears as if the oligochete-nematode clade is separated from other chemoautotrophic symbionts by free-living bacteria such as *Chromatium vinosum* and *Thiocystis gelatinosa*.

Results from *in situ* hybridizations, DGGE, and 16S rRNA analysis demonstrated that the small bacterial morphotype of *Olavius loisae* is an α-*Proteobacteria* symbiont. These bacteria represent a unique symbiotic lineage in marine invertebrates. While α-*Proteobacteria* sequences have been isolated from an echinoderm (Burnett & McKenzie 1997) and a sponge (Althoff et al. 1998), *in situ* hybridization data is lacking that would demonstrate that these sequences originated from the bacteria harbored by these hosts. The presence of a novel phylotype in *O. loisae* suggests the acquisition of new traits in this symbiotic association. Furthermore, competition with the γ-*Proteobacteria* symbiont for host or environmental resources would be reduced if the α-*Proteobacteria* symbiont inhabited a different symbiotic niche. At this point, the physiological nature of the α-*Proteobacteria* symbiont remains open as it is too distantly related to other free-living or symbiotic bacteria to infer its metabolism from 16S rRNA phylogeny. It is tempting to speculate that the α-*Proteobacteria* symbiont is responsible for nitrogen recycling in *O. loisae*, because this host as well as all other gutless oligochetes are unique and remarkable in that they appear to lack nephridia, excretory organs used for the disposal of nitrogenous wastes (Giere 1996). To our knowledge, all other chemoautotrophic metazoan hosts, even those without a gut, bear nephridia or other excretory organs, and their absence in gutless oligochetes suggests that their role has, at least in part, been taken over by the symbionts. However, the γ-*Proteobacteria* symbiont and the spirochete must also be considered, as the assimilation of inorganic nitrogen is common among bacteria.

The degree of 16S rRNA sequence heterogeneity among different *Olavius loisae* individuals was unusually high in the α-*Proteobacteria* symbionts when compared with the divergence among the γ-*Proteobacteria* symbionts (0.5%) and the spirochete phylotypes (0.2%). The α-*Proteobacteria* sequences of 2 *O. loisae* specimens differed by only 0.07%, but the difference between these 2 hosts and a third was 1.4%. Most of these differences (85%) involved compensatory base pair changes or A-G changes which do not disrupt rRNA secondary structure (Gutell et al. 1994). Such an overwhelming majority of these types of changes can not be explained by PCR or sequencing error. Multiple 16S rRNA copies are also unlikely because these would have been detected by DGGE, as described previously in the free-living bacterial species *Paenibacillus polymyxa* (Nübel et al. 1996). The most likely explanation for the observed heterogeneity is intraspecific variation, that is, individual hosts harbor different strains of the same symbiont. In other chemoautotrophic associations in which this question has been addressed, the symbionts of the same host population share identical 16S rRNA sequences (Durand et al. 1996, Distel 1998).

The presence in *Olavius loisae* of a third bacterial phylotype belonging to the spirochetes was demonstrated using 16S rRNA analysis and DGGE. This phylotype was found in 3 out of 4 host worms, was the second most dominant clone, and was identified as 1 of the 3 bacterial phylotypes of *O. loisae* by DGGE. The consistency of these results argues against the possibility that the spirochete is a contaminant carried over from the seawater. In addition, the worms were rinsed carefully in 0.2 µm filtered seawater to help eliminate contaminating bacteria. It is also unlikely that the spirochete is associated with the surface of *O. loisae* as light microscopical examinations of live worms freshly extracted from the sediment, as well as transmission and scanning electron microscopical studies of fixed specimens, always revealed a clean and bacteria-free outer surface. Furthermore, bacteria were never found in host tissues other than the symbiont-containing region below the cuticle, despite examinations of numerous cross and longitudinal sections from different body regions. Thus, the most likely explanation is that the third bacterial morphotype corresponds to the third spirochete phylotype. This is consistent with the fact that 3 distinct phylotypes and 3 distinct morphotypes were regularly found in *O. loisae*, and 2 phylotypes (α- and γ-*Proteobacteria* sequences) were unambiguously designated to 2 morphotypes (small and large symbionts). We can only speculate on why the 2 *in situ* probes specific to the spirochete phylotype of *O. loisae* did not hybridize to the third morphotype. Perhaps this morphotype could not be labeled due to

low rRNA concentrations in the bacteria. An alternative explanation that cannot be currently excluded is that the third morphotype represents a morphological variant of the α- or γ-*Proteobacteria* symbiont.

The morphology of the third morphotype lacked characteristics that are typical of spirochetes such as periplasmic flagella, organelles used in locomotion (Canale-Parola 1992). However, their absence would not be surprising considering the loss of motility that the endosymbiotic association would cause. Furthermore, it is well known that an endosymbiotic existence can cause extensive structural changes. For example, the symbiotic form of rhizobial bacteria of leguminous plants has a completely different morphology, including the absence of flagella, than the free-living form (Smith & Douglas 1987). The association of a spirochete with a chemoautotrophic host is not ecologically improbable considering their regular occurrence in sulfide-rich muds and occasional isolation from hydrothermal vents (Canale-Parola 1992). Their possible role in the association, if any, remains unclear, just as the role of spirochete symbionts in the digestive system of termites, cockroaches, and bivalve mollusks is not understood (Margulis & Hinkle 1992). A parasitic association seems highly unlikely as the worms with the spirochete phylotype appeared as viable and active as the worms without this phylotype.

The order in which the bacteria associated with *Olavius loisae* became established is not yet known but it seems reasonable to assume that the obligate symbionts must have been acquired at an early point in the evolution of these associations. The obligacy of the large, thioautotrophic symbionts for gutless oligochetes is clear as they provide the worms with nutrition via chemosynthesis. It appears as if the small bacterial symbionts are equally important for the association, as all gutless oligochetes harbor these 'secondary' endosymbionts, their distribution is non-random and similar in all hosts (Giere et al. 1995, Giere 1996), and they are inherited through vertical transmission in those species examined (Giere & Langheld 1987). The putative spirochete symbiont of *O. loisae* is not obligate for these hosts, being present in only a part of the population. Perhaps these bacteria are 'recently acquired guests which are still in need of adaptation' (Buchner 1965) and the association can be considered as a symbiosis in the making.

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