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Brief report

Multiple bacterial symbionts in two species of co-occurring gutless oligochaete worms from Mediterranean sea grass sediments

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Summary

Gutless oligochaete worms are found worldwide in the pore waters of marine sediments and live in symbiosis with chemoautotrophic sulfur-oxidizing bacteria. In the Mediterranean, two species of gutless oligochaete worms, *Olavius algarvensis* and *O. ilvae*, co-occur in sediments around sea grass beds. These sediments have extremely low sulfide concentrations (< 1 μM), raising the question if *O. ilvae*, as shown previously for *O. algarvensis*, also harbours sulfate-reducing symbionts that provide its sulfur-oxidizing symbionts with reduced sulfur compounds. In this study, we used fluorescence *in situ* hybridization (FISH) and comparative sequence analysis of genes for 16S rRNA, sulfur metabolism (*aprA* and *dsrAB*), and autotrophic carbon fixation (*cbbL*) to examine the microbial community of *O. ilvae* and re-examine the *O. algarvensis* symbiosis. In addition to the four previously described symbionts of *O. algarvensis*, in this study a fifth symbiont belonging to the *Spirochaetes* was found in these hosts. The symbiotic community of *O. ilvae* was similar to that of *O. algarvensis* and also included two gammaproteobacterial sulfur oxidizers and two deltaproteobacterial sulfate reducers, but not a spirochete. The phylogenetic and metabolic similarity of the symbiotic communities in these two co-occurring host species that are not closely related to each other indicates that syntrophic sulfur cycling provides a strong selective advantage to these worms in their sulfide-poor environment.

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Introduction

Gutless oligochaetes are small worms of less than 0.1–0.3 mm diameter and 2–50 mm length that are found worldwide in marine sediments (Bright and Giere, 2005; Dubilier *et al*., 2006). With no mouth or gut, these worms are dependent on their symbiotic bacteria for nutrition. The endosymbionts are extracellular and occur in a thick layer between the cuticle and the epidermal cells of the worm. Enzyme assays, immunohistochemistry, uptake experiments with inorganic carbon and the presence of sulfur globules indicate that at least some of the bacterial symbionts are thiotrophic, using reduced sulfur compounds as electron donors, and fix CO_2 autotrophically to organic carbon compounds (Dubilier *et al*., 2006). It is unclear if the transfer of these organic compounds to the host is the main mode of energy transfer or if digestion of the bacteria supplies the host with nutrients.

All gutless oligochaete species examined to date harbour thiotrophic *Gammaproteobacteria* called Gamma 1 symbionts that are related to free-living sulfur oxidizers of the family *Chromatiaceae* (Dubilier *et al*., 1995; 1999; 2001; Blazejak *et al*., 2005; 2006). Recently, the gutless oligochaete *Olavius algarvensis* (Giere *et al*., 1998) from the Mediterranean island of Elba was discovered to harbour a second thiotrophic *Gammaproteobacterium*, called the Gamma 3 symbiont, as well as two deltaproteobacterial sulfate reducers, called Delta 1 and Delta 4 symbionts (Dubilier *et al*., 2001; Woyke *et al*., 2006). These symbionts are engaged in a syntrophic sulfur cycle

in which the sulfate reducers produce reduced sulfur compounds that are used by the sulfur oxidizers as an energy source. Sulfide concentrations are extremely low and rarely exceed 80 nM in the habitat of *O. algarvensis* (Dubilier *et al*., 2001). This means that internal sulfide production from the sulfate-reducing symbionts greatly exceeds the flux of sulfide from the environment into the worms, showing the importance of the sulfate reducers for the thiotrophic symbiosis (Dubilier *et al*., 2001).

The discovery of a second gutless oligochaete species, *Olavius ilvae* (Giere and Erséus, 2002), that co-occurs with *O. algarvensis* in the Elba sediments provides an ideal opportunity to examine if sulfate-reducing symbionts are common to chemosynthetic hosts living in these sulfide-poor environments. Using the full cycle rRNA approach that includes comparative 16S rRNA sequence analysis and fluorescence *in situ* hybridization (FISH), we examined the distribution, diversity and phylogeny of bacterial symbionts in *O. ilvae*. In addition, we re-examined the *O. algarvensis* symbiosis because only four symbiont phylotypes were identified in the metagenomic library of this host (Gamma 1 and 3, and Delta 1 and 4) while PCR analyses of the 16S rRNA gene indicated the presence of an additional spirochete phylotype (Woyke *et al*., 2006). To better understand the metabolism of the symbionts in these hosts, we examined genes coding for enzymes diagnostic of sulfur metabolism and chemoautotrophy. For autotrophic CO_2 fixation via the Calvin–Benson–Bassham (CBB) cycle, *cbbL* and the *cbbM* genes, coding for the form I and II of ribulose-1,5-bisphosphate carboxylase/oxygenase (RubisCO), were used as diagnostic markers (Elsaied and Naganuma, 2001; Blazejak *et al*., 2006). The *aprA* gene, encoding the alpha subunit of adenosine-5′-phosphosulfate (APS) reductase, was used as an indicator for reductive and oxidative sulfur metabolism, as this gene is characteristic for both sulfate-reducing and many sulfur-oxidizing bacteria (Hipp *et al*., 1997; Friedrich, 2002; Blazejak *et al*., 2006). Finally, we examined the *dsrAB* genes, coding for the alpha and beta subunits of the dissimilatory (bi)sulfite reductase, as characteristic markers for dissimilatory sulfate/sulfite reduction (Wagner *et al*., 2005; Zverlov *et al*., 2005).

Results and discussion

The 16S rRNA gene clone libraries from eight *O. ilvae* and five *O. algarvensis* individuals showed a dominance of gamma- and deltaproteobacterial sequences. In addition, spirochete sequences were found in the *O. algarvensis* clone library (see Table S1 for clone library data and Table S2 for sequence identities to closest relatives).

By combining comparative 16S rRNA sequence analysis with FISH, we were able to clearly assign the dominant 16S rRNA sequences in the *O. ilvae* and *O. algarvensis* clone libraries to bacterial symbionts in these worms. The assignment of the metabolic indicator genes, *cbbL*, *aprA* and *dsrAB*, to a given symbiont was clear when sequences were identical or highly similar ($\geq$ 93.3% amino acid identity) to genes whose origin had been determined through metagenomic binning analyses (Woyke *et al*., 2006). Otherwise, the phylogenetic position of a sequence was used to predict its origin. This approach is justified by the observation that in relatively closely related species (which is the case for oligochaete symbionts) the phylogeny of their *dsrAB* and *aprA* genes generally corresponds well with their 16S rRNA phylogeny (Zverlov *et al*., 2005; Meyer and Kuever, 2007a,b). However, several studies have shown that phylogenetically diverse copies of a metabolic indicator gene can be present in a single species (e.g. for the *aprA* gene see Meyer and Kuever, 2007b; for *cbbL* see Scott *et al*., 2006). Furthermore, the 16S rRNA phylogeny of a species is not always reflected in the phylogeny of its other genes, because of lateral gene transfer (Boucher *et al*., 2003). We therefore distinguished between sequences whose assignment to a corresponding symbiont was unambiguous (based on their high similarity or identity to sequences whose origin was determined through metagenomic binning analyses) and sequences whose origin was inferred through their phylogeny, by placing the symbiont names of the latter in parentheses in the corresponding trees in Figs 3, 4 and 6.

Gammaproteobacterial endosymbionts

The dominant 16S rRNA sequences in both the *O. ilvae* and *O. algarvensis* clone libraries originated from their Gamma 1 symbionts (see FISH results below). The Gamma 1 sequences from both *O. algarvensis* and *O. ilvae* consistently fell in a monophyletic group of 16S rRNA sequences from the sulfide-oxidizing Gamma 1 symbionts of other gutless oligochaete species such as *Inanidrilus leukodermatus* and *Olavius crassitunicatus*, as well as symbionts of the marine nematodes *Laxus* sp. (Polz *et al*., 1994) and *Astomonema* sp. (Musat *et al*., 2007) (Fig. 1).

The Gamma 1 symbionts of *O. ilvae* and *O. algarvensis* were not most closely related to each other despite the fact that their hosts co-occur in the same sediments of the Mediterranean. Instead, their Gamma 1 symbionts were most closely related to symbionts from geographically distant oligochaete species: the *O. ilvae* Gamma 1 symbiont was most closely related to the Gamma 1 symbionts of *Olavius loisae* from the Australian Great Barrier Reef and *O. crassitunicatus* from the Peru margin, while the *O. algarvensis* Gamma 1 symbiont was most closely related to the Gamma 1 symbionts of *I. leukodermatus* from Bermuda and *Inanidrilus makropetalos* from the

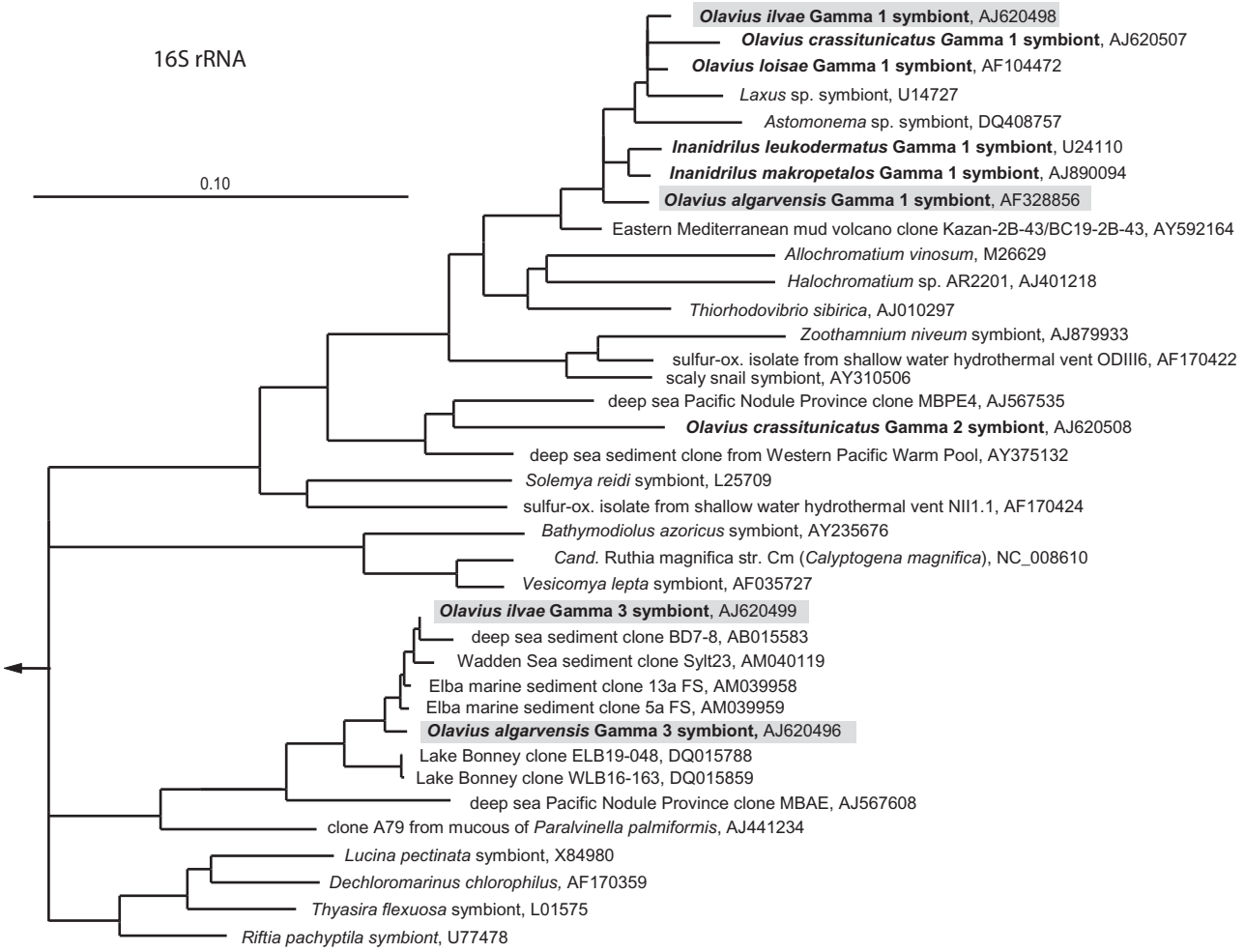

Fig. 1. Phylogenetic placement of the gammaproteobacterial symbionts from *O. algarvensis* and *O. ilvae* based on maximum likelihood (ML) analyses of 16S rRNA sequences (sequences from this study highlighted in grey; symbionts from gutless oligochaetes in bold). Nodes that differed between parsimony, neighbour-joining and ML analyses were collapsed to a consensus branch. Five deltaproteobacterial sequences were used as an out-group (arrow, AJ620497, AJ620509, M34407, AF418180, AJ620511). Scale bar = 0.10 estimated substitutions per site. The names of the oligochaete symbionts are derived from the bacterial group to which they belong (e.g. *Gamma-* or *Deltaproteobacteria*) with the number after their name denominating their position within each bacterial group. Symbionts with at least 95% sequence identity between their rRNA genes that were monophyletic in all three treeing analyses were given the same number. Alignments and phylogenetic analyses were performed with the ARB program (Ludwig *et al.*, 2004).

Bahamas (Fig. 1). This indicates that geography does not play an important role in determining the phylogenetic relationships of these symbionts. Interestingly, phylogenetic analyses based on host-specific mitochondrial cytochrome oxidase subunit I (COI) genes show that this is also true for their hosts (C. Erséus, A. Blazejak and N. Dubilier, unpubl. data). *Olavius ilvae* and *O. algarvensis* are more closely related to gutless oligochaetes from the Atlantic and Pacific Oceans than to each other. We are currently examining if co-speciation caused the phylogenetic patterns observed in oligochaete hosts and their Gamma 1 symbionts.

A second group of gammaproteobacterial sequences found in both host species originated from their Gamma 3 symbionts (Table S1). The Gamma 3 sequences of *O. ilvae* and *O. algarvensis* formed a clade separate from the Gamma 1 symbionts, and grouped with environmental clone sequences including those from sediments at the *O. ilvae* and *O. algarvensis* collection site (Fig. 1).

Fluorescence *in situ* hybridization with probes specific to the Gamma 1 and 3 16S rRNA sequences from *O. ilvae* and *O. algarvensis* (Table 1) confirmed their origin from symbionts in the layer between the cuticle and epidermis. Hybridization signals for probes specific to the Gamma 1 *O. algarvensis* and *O. ilvae* phylotypes were observed in all examined specimens and were consistent with the size, shape and distribution of the large bacterial morphotype (approximately 2.3–3 μm length and 1.4 μm width) observed in these hosts with transmission electron microscopy (Giere and Erséus, 2002) (Fig. 2B and F).

Table 1. Oligonucleotide probes used in this study.

Probe	Specificity	Probe sequence (5′–3′)	Position[a]	FA [%][b]	Reference organism	Literature reference
NON338	Antisense	ACT CCT ACG GGA GGC AGC	338–355	10 30 (HRP)	See Literature reference	Wallner *et al.* (1993)
Gam42a	*Gammaproteobacteria*	GCC TTC CCA CAT CGT TT	1027–1043[c]	30–35	See Literature reference	Manz *et al.* (1992)
DSS658	*O. algarvensis*/*O. ilvae* Delta 1, Delta 3 symbiont, *O. algarvensis* Delta 4 symbiont, *Desulfosarcina* spp. *Desulfofaba* sp., *Desulfococcus* spp., *Desulfofrigus* spp.	TCC ACT TCC CTC TCC CAT	658–685	30–60	See Literature reference	Manz *et al.* (1998)
OalgGAM1	*O. algarvensis* Gamma 1 symbiont	CTC GAG ATC TTT CTT CCC	445–462	10	*Inanidrilus leukodermatus* Gamma 1 symbiont, U24110 *O. ilvae* Gamma 1 symbiont, AJ620498	Dubilier *et al.* (2001)
OilvOcraGAM1	*O. ilvae*/*O. crassitunicatus* Gamma 1 symbiont	CAT ACT CTA GCC GAA CAG	643–660	10	*Inanidrilus triangulatus* Gamma 1 symbiont *O. algarvensis* Gamma 1 symbiont, AF328856	This study
Oalg/OilvGAM3	*O. algarvensis*/*O. ilvae* Gamma 3 symbionts, environmental clones such as: AB077346, AB015583, AB239038, AF424056, AF424077, AY133404, AJ567608, AM039958 (9), AM040119, AJ966594, AJ633950, AY580816, AY533996, DQ228658, DQ351743	CCG GAA TTC CAC TTG CCT	665–682	30	*Ifremeria nautilei* Gamma symbiont, AB189713	This study
OalgDEL1	*O. algarvensis* Delta 1 symbiont, environmental clone DQ395018	GTT ATC CCC GAC TCG GGG	136–153	10	*O. ilvae* Delta 1 symbiont, AJ620500 *Desulfonema magnum* (DSM 2077), U45989, *Rhodothermus marinus* (DSM 4252), X80994	Dubilier *et al.* (2001)
OilvDEL1	*O. ilvae* Delta 1 symbiont, environmental clone AY907763, AY710878	GTT ATC CCC GAT TCG GGG	136–153	30	*O. algarvensis* Delta 1 symbiont, AF328857, *D. magnum* (DSM 2077), U45989	This study
Oalg/OilvDEL3	*O. algarvensis*/*O. ilvae* Delta 3 symbiont	GTG CCT GCC TCC TGA AAG	1449–1465	30	*Desulfuromonas thiophila* (DSM 8987), Y11560	This study
OalgDEL4	*O. algarvensis* Delta 4 symbiont, environmental clones: DQ394892, DQ395063, EF061975, AB121109, AY499745, AY500008, AY822307, AY822331, DQ395004	GCC CAA CAA CTT CCG GTA	1427–1444	30	*Desulfobacterium indolicum* (DSM 3383), AJ237607	This study
SPIRO	*O. algarvensis*/*O. crassitunicatus*/*O. loisae* spirochete symbionts, clones from termite gut: AB192148, AB192203, AB192256	GCT ATC CCC AAC CAA AAG	136–153	30 (HRP)	*Spirochaeta stenostrepta*, DSM 2028, M88724	This study

a. Position in the 16S rRNA of *E. coli.*

b. Formamide concentrations used in the FISH and catalysed reporter deposition (CARD) FISH (SPIRO, NON338 30%) hybridization buffer in percentage (v/v).

c. Position in the 23S rRNA of *E. coli.*

Further properties of the probes are available at probeBase (Loy *et al.*, 2007). Nine *O. algarvensis* and three *O. ilvae* specimens were prepared for FISH with monolabelled probes and CARD FISH with horseradish peroxidase (HRP)-labelled probes and tyramide signal amplification as described previously (Blazejak *et al.*, 2006). Specific and group oligonucleotide probes targeting the dominant 16S rRNA sequences found in *O. algarvensis* and *O. ilvae* were created with the ARB program (Ludwig *et al.*, 2004) and evaluated *in silico* by using BLAST (Altschul *et al.*, 1990) and the probe match tool of RDPII (Cole *et al.*, 2007). The specificity of the symbiont probes was tested against reference bacteria with 16S rRNA sequences containing one or more mismatches. General probes for the *Bacteria*, *Gammaproteobacteria* and a subgroup of the *Deltaproteobacteria* served as positive controls and the nonsense probe NON338 as a negative control, and hybridizations were performed at the formamide concentration ensuring specificity.

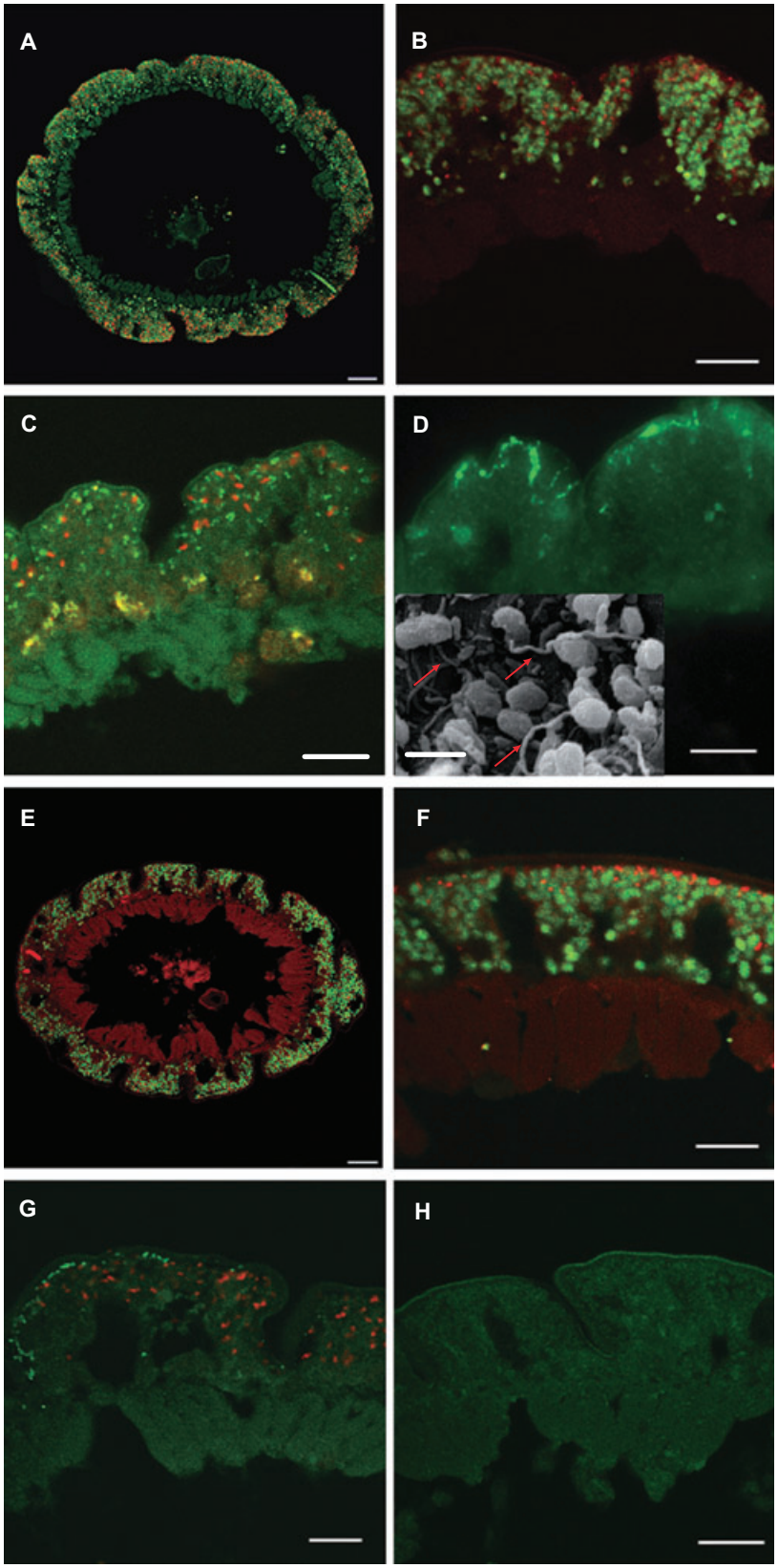

Fig. 2. Fluorescence *in situ* hybridization (FISH) identification of bacterial symbionts in *O. algarvensis* (A–D) and *O. ilvae* (E–H).
A and E. Cross-section through entire worm showing all gammaproteobacterial (green, Gam42a) and deltaproteobacterial (red, DSS658) symbionts.
B and F. The gammaproteobacterial symbionts. (B) *Olavius algarvensis* Gamma 1 (green, OalgGAM1) and Gamma 3 (red, Oalg/OilvGAM3) symbionts. (F) *Olavius ilvae* Gamma 1 (green, Oilv/OcraGAM1) and Gamma 3 (red, Oalg/OilvGAM3) symbionts.
C and G. The deltaproteobacterial symbionts. (C) *Olavius algarvensis* Delta 1 (red, OalgDEL1) and Delta 4 (green, OalgDEL4) symbionts. (G) *Olavius ilvae* Delta 1 (red, OilvDEL1) and Delta 3 (green, OilvDEL3) symbionts.
D and H. Spirochetes were found in *O. algarvensis* (shown in green using the probe SPIRO) but not in *O. ilvae* (H). Inset in (D) shows scanning electron microscopy image with red arrows showing spirochetes.
Scale bars: 20 μm in (A) and (E), 2 μm in inset (D), 10 μm in all other images. Fixation for FISH was carried out as described previously (Blazejak *et al.*, 2005). For scanning electron microscopy specimens of *O. algarvensis* were fixed in 4% glutaraldehyde buffered in 0.1 M Na-cacodylate, pH 7.3 and 3% sucrose, stored in fixative and post-fixed in buffered 1% OsO_4 solution.

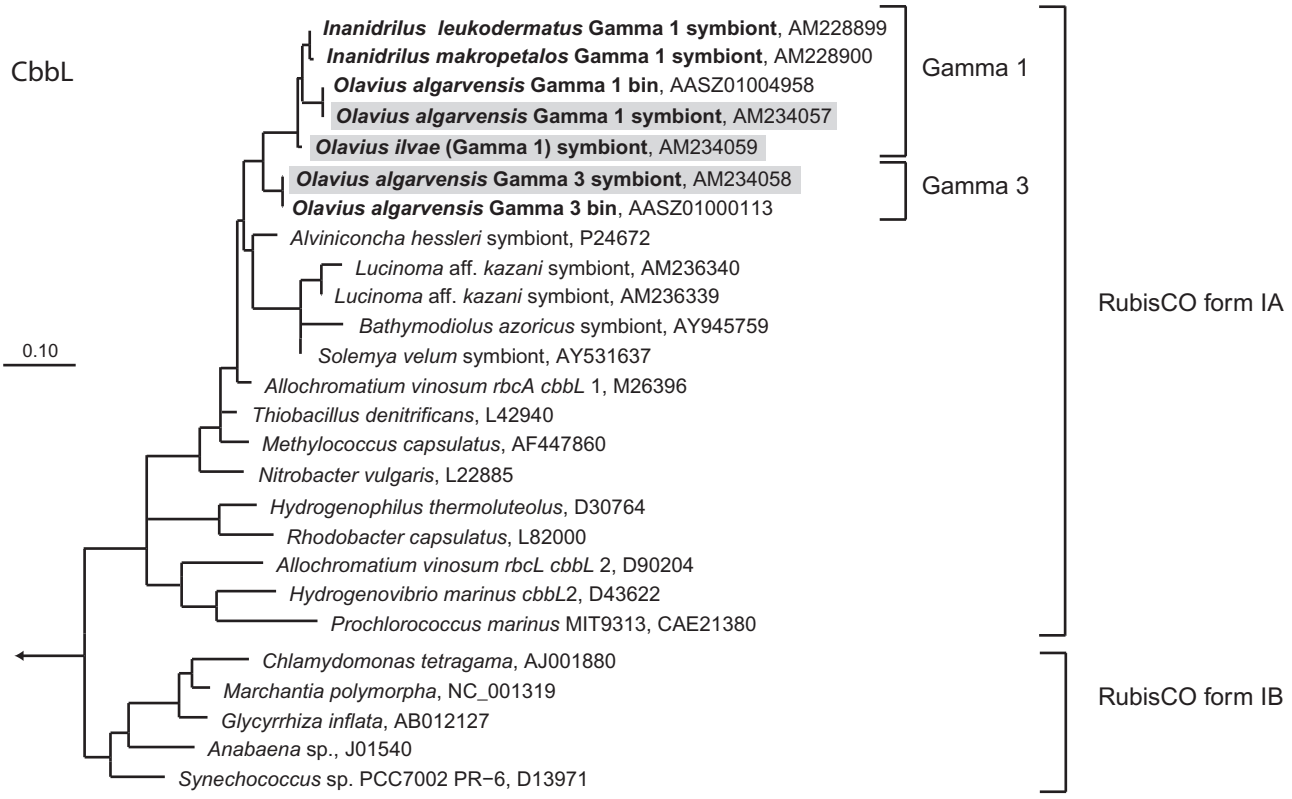

Fig. 3. CbbL consensus tree based on deduced amino acid sequences of the *cbbL* gene coding for the large-subunit of RubisCO (sequences from this study highlighted in grey; symbionts from gutless oligochaetes in bold; symbiont name of sequence whose origin was inferred through phylogeny is in parentheses). The *O. ilvae* (Gamma 1) symbiont sequence is assumed to have originated from the Gamma 1 symbiont of this host, because the sequence falls in a clade containing only sequences from Gamma 1 symbionts. CbbL sequences from form IC, ID and form II were used as out-groups (arrow, AJ001880, NC_001319, AB012127, J01540, D13971, M17744, M64624, M59080, X61918, X55372, X14171, AF047688, AF442518, D28135, X00286). Scale bar = 0.10 estimated substitutions per site. The phylogenetic tree was generated from sequences of 230 amino acids using the ML algorithm and a JTT model with a 25% positional conservation filter. Polytomic nodes were set for *sequences* for which positions varied with different filters.

Fluorescence *in situ* hybridization analyses with probes specific to the Gamma 3 phylotypes of *O. ilvae* and *O. algarvensis* showed that these symbionts were much smaller than the Gamma 1 symbionts (approximately 1 μm diameter; Fig. 2B and F). Their distribution and abundance varied in the two host species (Table 2). In *O. algarvensis*, the Gamma 3 symbionts were observed in seven of the nine specimens examined, occurred in roughly equal amounts as the Gamma 1 symbionts and were distributed evenly throughout the symbiotic region (Fig. 2B). In *O. ilvae*, all three individuals examined contained the Gamma 3 symbionts, but these were much less abundant than the Gamma 1 symbionts and occurred mainly just below the cuticle of the worms (Fig. 2F).

Genes characteristic for chemoautotrophic sulfur oxidation were found in both host species (Table S3), namely *cbbL* coding for RubisCO form I (Fig. 3) and *aprA* coding for APS reductase (Fig. 4), indicating that the Gamma 1 and 3 symbionts are thiotrophs. While the presence of two thiotrophic symbionts has been shown for *O. algarvensis* (Woyke *et al*., 2006), this was not previously known for *O. ilvae*. Unexpectedly, we were not able to find a *cbbL* gene that we could clearly assign to the Gamma 3 symbiont of *O. ilvae* (Fig. 3), despite the analysis of more than 200 clones from three individuals (Table S3). However, the close phylogenetic relationship between the 16S rRNA and *aprA* genes of the *O. ilvae* Gamma 3 symbiont with those of the thiotrophic Gamma 3 symbiont of *O. algarvensis* supports the identification of the *O. ilvae* Gamma 3 symbiont as a sulfur oxidizer.

It is intriguing that both *O. algarvensis* and *O. ilvae*, as well as *O. crassitunicatus* from the continental margin of Peru, harbour two thiotrophic symbionts (Blazejak *et al*., 2005). These three host species occur in abiogenic, silicate sediments. In contrast, the other host species we have examined to date, *O. loisae*, *I. leukodermatus* and *I. makropetalos*, live in biogenic, calcareous sediments and only have a single thiotrophic symbiont, the Gamma 1 symbiont found in all gutless oligochaetes (Dubilier *et al*., 1999; Blazejak *et al*., 2006). Analyses of additional host species from silicate and calcareous sediments

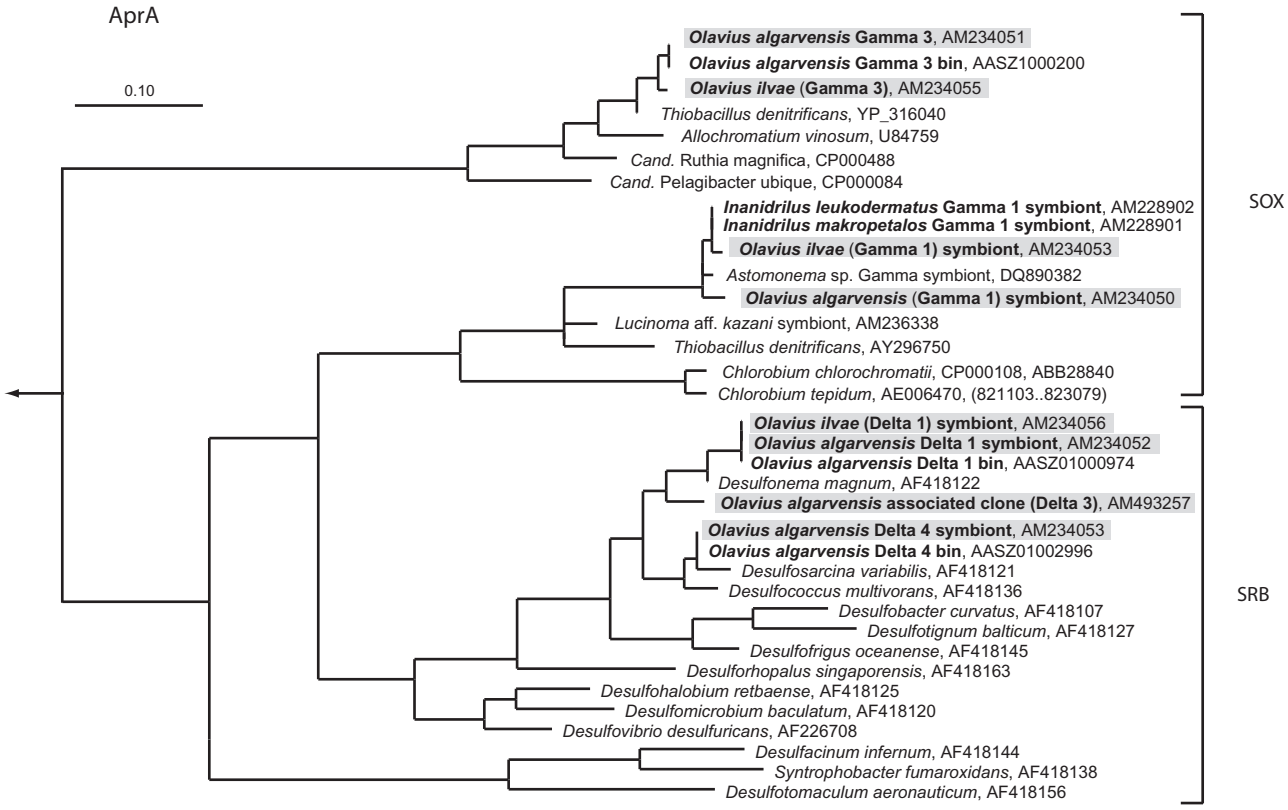

Fig. 4. AprA phylogeny based on deduced amino acid sequences of the *aprA* gene coding for the alpha subunit of APS reductase. The tree was generated from sequences of 131 amino acids using the ML algorithm and a JTT model with a 25% positional conservation filter. Sequences from this study are highlighted in grey, with those originating from symbionts in bold; symbiont names of sequences whose origin was inferred through phylogeny are in parentheses. Three AprA sequences from *Archaeoglobus* spp. served as an out-group (arrow, AE000782, AF418132, AF418134). The polytomic nodes were set for sequences whose positions varied with different filters. Scale bar = 0.10 estimated substitutions per site. SOX, sulfide-oxidizing bacteria; SRB, sulfate-reducing bacteria.

will reveal if the presence or absence of a second thiotrophic symbiont is related to their abiogenic or biogenic habitat.

Deltaproteobacterial endosymbionts

The dominant deltaproteobacterial sequences of *O. ilvae* and *O. algarvensis* belong to three distinct lineages called Delta 1, Delta 3 and Delta 4 (Fig. 5 and Table S1). The Delta 1 16S rRNA sequence from *O. ilvae* was most closely related to the *O. algarvensis* Delta 1 sequence (99.6% sequence identity). These two sequences formed a clade with the Delta 1 symbiont from *O. crassitunicatus* and a clone sequence from Arabian Sea picoplankton (Fig. 5). As for the Delta 1 sequences from *O. ilvae* and *O. algarvensis*, the Delta 3 16S rRNA sequences found in

Table 2. Morphology, abundance and distribution of the symbionts in *O. algarvensis* and *O. ilvae* observed with FISH.

		No. of individuals in which detected by FISH		% estimated abundance[b]		Distribution in symbiont region	
Symbiont	Cell dimension[a] (μm)	*O. algarvensis* ($n = 9$)	*O. ilvae* ($n = 3$)	*O. algarvensis*	*O. ilvae*	*O. algarvensis*	*O. ilvae*
Gamma 1	2.3–3 × 1.4	9	3	25–45%	70%	Even	Even
Gamma 3	1	7	3	25–30%	5–10%	Even	Mostly below cuticle
Delta 1	1–1.8	9	3	10–45%	20%	Even	Even
Delta 3	0.9–1.2	n.d.	3	n.d.	≤ 5%	n.d.	Mostly below cuticle
Delta 4	0.8–1.2	2	n.d.	25–30%	n.d.	Even	n.d.
Spirochete	0.2–0.3 × 5–12	9	n.d.	10%	n.d.	Patchy	n.d.

a. Cell dimensions are influenced by fixation and dehydration.
b. These numbers are based on non-quantitative observations.
n.d., not detected.

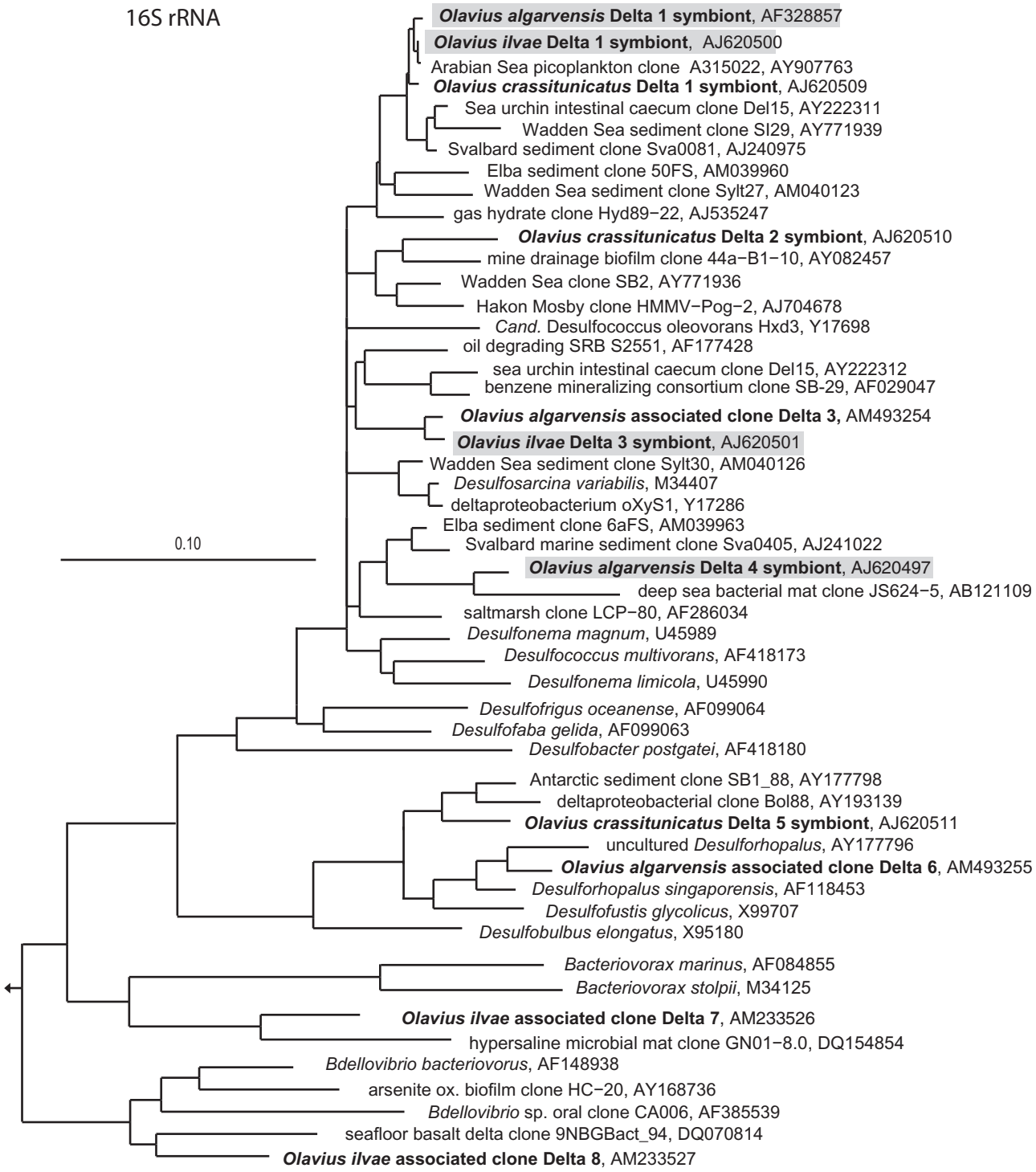

Fig. 5. Phylogenetic placement of the deltaproteobacterial 16S rRNA gene sequences from *O. algarvensis* and *O. ilvae*. Sequences from this study are highlighted in grey, with those confirmed to originate from symbionts using FISH highlighted in bold. Sequences not confirmed to originate from symbionts using FISH are called *O. algarvensis*- or *O. ilvae*-associated clones. Consensus tree based on maximum likelihood (ML) analyses of 16S rRNA sequences. Nodes that differed between parsimony, neighbour-joining and ML analyses were collapsed to a consensus branch. The relationships of the Delta 1, 3 and 4 sequences from *O. ilvae* and *O. algarvensis* to each other and to the closest cultivated *Desulfobacteraceae* were not stable and are therefore shown as a consensus branch. The *O. crassitunicatus* Delta 5 symbiont was formerly called the *O. crassitunicatus* Delta 3 symbiont in Blazejak and colleagues (2005). Five *Sphingomonas* spp. 16S rRNA sequences were used as an out-group (arrow, AJ416411, AB024289, U37341, AF281032, X97776). Scale bar = 0.10 estimated substitutions per site.

these two hosts were also closely related to each other (98.2% sequence identity). The Delta 3 sequences were most closely related to a group of environmental clone sequences that included 16S rRNA genes from bacteria in hydrocarbon-degrading consortia (Phelps *et al*., 1998) and the gut nodules of the sea urchin *Echinocardium cordatum* (Da Silva *et al*., 2006). The Delta 4 16S rRNA sequence from *O. algarvensis* was not closely related to other sequences from oligochaete hosts, and belonged instead to a separate lineage that included environmental clone sequences, some of which were from the collection site of the worms.

Fluorescence *in situ* hybridization with specific probes for the Delta 1 sequences (Table 1) showed that the Delta 1 symbionts were present in all individuals of both host species (Fig. 2C and G; Table 2). The probe specific to the Delta 3 sequence showed a signal in all *O. ilvae* individuals, but only a very small percentage ($\leq$ 5%) of the symbiotic microbial community belonged to the Delta 3 symbionts in this host (Fig. 2G; Table 2). Despite the presence of a Delta 3 sequence in *O. algarvensis* clone libraries (although only a single clone from one individual, Specimen 7 in Table S1), no signals for a Delta 3 symbiont were observed in *O. algarvensis*. Instead, two out of nine *O. algarvensis* individuals examined with FISH harboured the Delta 4 symbiont (Fig. 2C). This corresponds with our previous metagenomic analysis of *O. algarvensis*, in which we only found 16S rRNA genes for the Delta 1 and 4 symbionts (Woyke *et al*., 2006).

Delta 1 symbionts have now been found in the two Mediterranean host species examined in this study as well as in the Peruvian species *O. crassitunicatus* (Blazejak *et al*., 2005). The close phylogenetic relationship between the Delta 1 symbionts of these three host species (Fig. 5) despite their large geographic distances indicates that these associations are highly specific and not just casual relationships between the worms and *Deltaproteobacteria* from their habitat.

In contrast to the Delta 1 symbionts which occur in all members of both host species, the distribution of the Delta 3 and 4 symbionts from *O. ilvae* and *O. algarvensis* was variable between individuals (Table 2). Similarly, the additional deltaproteobacterial symbionts of *O. crassitunicatus* (Delta 2 and 5 in Fig. 5) also varied intraspecifically. This suggests that these additional deltaproteobacterial symbionts play a less important role in the association than the Delta 1 symbionts.

The close phylogenetic relationship of the deltaproteobacterial symbionts from *O. ilvae* and *O. algarvensis* to free-living sulfate-reducing bacteria (Fig. 5) suggests that the symbionts are also sulfate reducers. While this was previously shown for the Delta 1 and Delta 4 symbionts of *O. algarvensis* (Dubilier *et al*., 2001; Woyke *et al*., 2006), this study indicates that *O. ilvae* also harbours two sulfate-reducing symbionts, Delta 1 and Delta 3. Corresponding to the close relationship of the 16S rRNA sequences of the Delta 1 symbionts of *O. ilvae* and *O. algarvensis*, both AprA and DsrAB sequences of *O. ilvae* were closely related to the AprA and DsrAB sequences from the *O. algarvensis* Delta 1 symbiont, suggesting that these originated from the *O. ilvae* Delta 1 symbiont (Figs 4 and 6). For the Delta 3 symbiont of *O. ilvae*, a corresponding *aprA* was not found (Table S3), but the presence of *dsrAB* assumed to have originated from its Delta 3 symbiont (Fig. 6) suggests that the *O. ilvae* Delta 3 symbiont is also a sulfate reducer.

In addition to the *aprA* genes from the Delta 1 and Delta 4 symbionts of *O. algarvensis*, we found a third *aprA* sequence in this host (called *O. algarvensis* associated clone Delta 3 in Fig. 4, and *aprA* $D3_{alg}$ in Table S3). This gene was only found in Specimen 7, and correspondingly, we only found a 16S rRNA Delta 3 sequence in Specimen 7 (Table S1; Fig. 5). As described above, we could not find the Delta 3 16S rRNA sequence in *O. algarvensis* using FISH, but it is possible that a Delta 3 symbiont is present in *O. algarvensis* that only occurs in very low numbers or in a small percentage of the host population.

Three additional deltaproteobacterial 16S rRNA sequences were found in very low numbers in the clone libraries of *O. algarvensis* and *O. ilvae*, namely *O. algarvensis*-associated clone Delta 6, and *O. ilvae*-associated clones Delta 7 and 8 (Fig. 5 and Table S1). We did not develop FISH probes specific to these sequences because the amount of bacteria observed with the general eubacterial probe in the worms did not differ markedly from the sum of bacteria hybridized with specific probes for the symbionts described above. This indicates that these additional deltaproteobacterial sequences are rare or from non-symbiotic bacteria. The *O. algarvensis* Delta 6 sequence fell in a clade of sequences from sulfate-reducing bacteria of the family *Desulfobulbaceae* that also includes the *O. crassitunicatus* Delta 5 symbiont (formerly called *O. crassitunicatus* Delta 3 symbiont in Blazejak *et al*., 2005). The *O. ilvae*-associated clones Delta 7 and 8 fall in clusters that include bacteria belonging to the *Bacteriovoracaceae* and the genus *Bdellovibrio*. Bacteria from these groups are parasites of free-living and symbiotic Gram-negative bacteria (Davidov and Jurkevitch, 2004). It is therefore possible that the *O. ilvae* Delta 7 and 8 sequences originated from parasites of the gamma- or deltaproteobacterial symbionts. While bacteria with a typical *Bdellovibrio*-like morphology (Shemesh and Jurkevitch, 2004) have not yet been observed in oligochaete symbionts, these have not been specifically searched for in ultrastructural analyses.

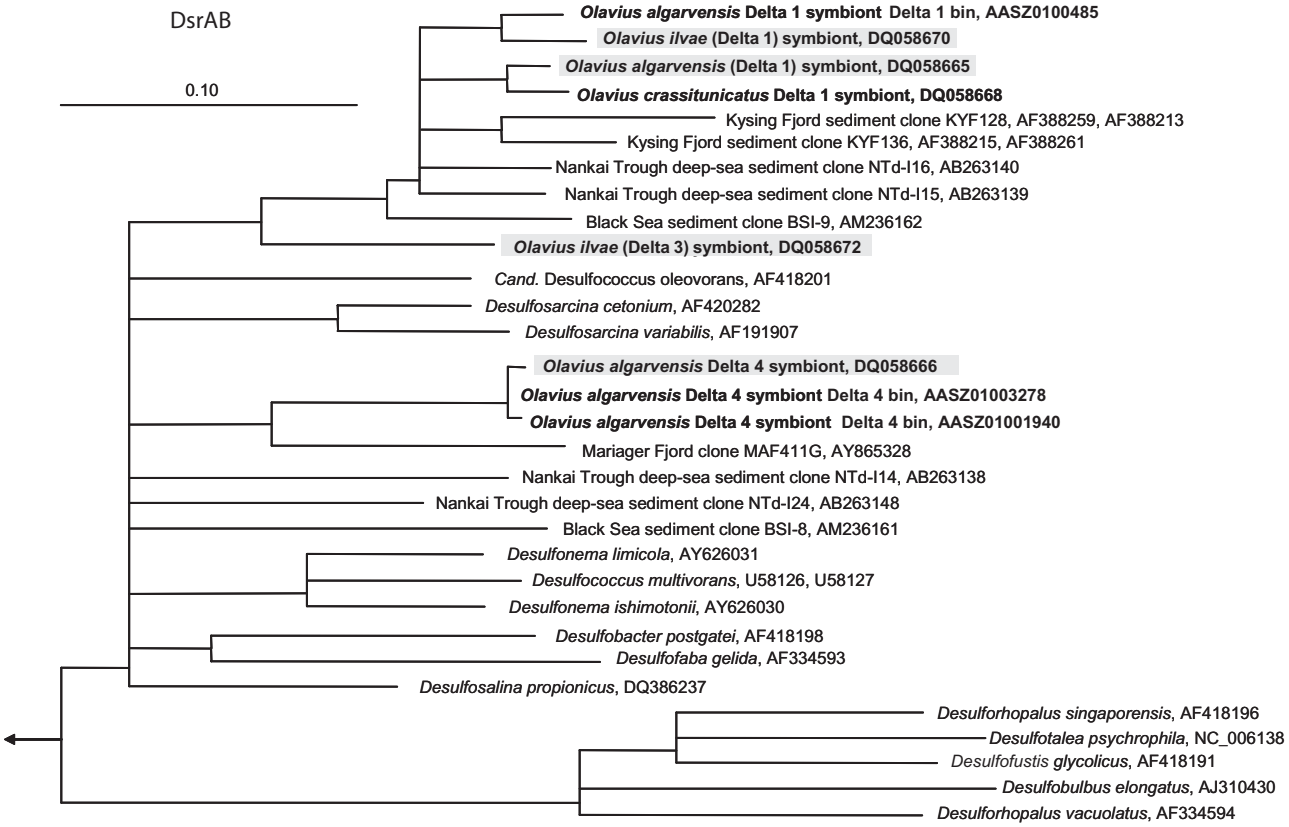

Fig. 6. DsrAB phylogeny based on deduced amino acid sequences of the *dsrAB* gene coding for the alpha and beta subunits of the dissimilatory (bi)sulfite reductase (sequences from this study highlighted in grey; symbionts from gutless oligochaetes in bold; symbiont names of sequences whose origin was inferred through phylogeny are in parentheses). Polytomic nodes in the DsrAB consensus tree connect branches for which a relative order could not be determined unambiguously by applying distance matrix, maximum parsimony and maximum likelihood treeing methods. The DsrAB sequence of *Desulfovibrio vulgaris* (U16723) was used as an out-group. Scale bar = 0.10 estimated substitutions per site as inferred from the distance matrix method.

Spirochete endosymbionts

The *O. algarvensis* spirochete 16S rRNA sequence from this study grouped with a monophyletic clade of spirochete symbiont sequences from the gutless oligochaetes *O. crassitunicatus* and *O. loisae* (Fig. 7). Their closest relatives were sequences from enrichment cultures of tubes from the hydrothermal vent polychaete *Alvinella pompejana*. Using a specific probe for all known oligochaete spirochete symbionts (Table 1), these bacteria were only observed in *O. algarvensis*, where they occurred regularly in all individuals examined (Fig. 2D, Table 2). Scanning electron microscopy analyses showed the elongated, spirochete-like shape of these bacteria (Fig. 2D, inset).

As discussed above for the Delta 1 symbionts, the close phylogenetic relationship of the spirochete symbionts from hosts found in geographically distant locations such as the Australian Great Barrier Reef (*O. loisae*), the Peru margin (*O. crassitunicatus*) and the Mediterranean suggests that these are widespread and specific to gutless oligochaetes and not just casual associates. However, the lack of spirochetes in *O. ilvae* that co-occurs in the same habitat with *O. algarvensis* as well as their absence in other gutless oligochaete species (Blazejak *et al.*, 2006) shows that these symbionts are not necessarily essential to the symbiotic association. Whether the presence of spirochetes provides a selective advantage to the host remains to be shown and cannot be clarified until their physiological role is known. As yet, there is no genomic analysis of oligochaete spirochetes.

Conclusions

Differences in the abundance and distribution of the co-occurring oligochaete symbionts indicate a dynamic evolutionary process in which some bacteria have become well established as symbionts while others are less stable members of the association. Co-occurring Gamma 1 and Delta 1 symbionts have now been found in the two Mediterranean species examined here as well as in *O. crassitunicatus* from the Peru margin (Blazejak *et al.*, 2005). The regular and persistent occurrence of these

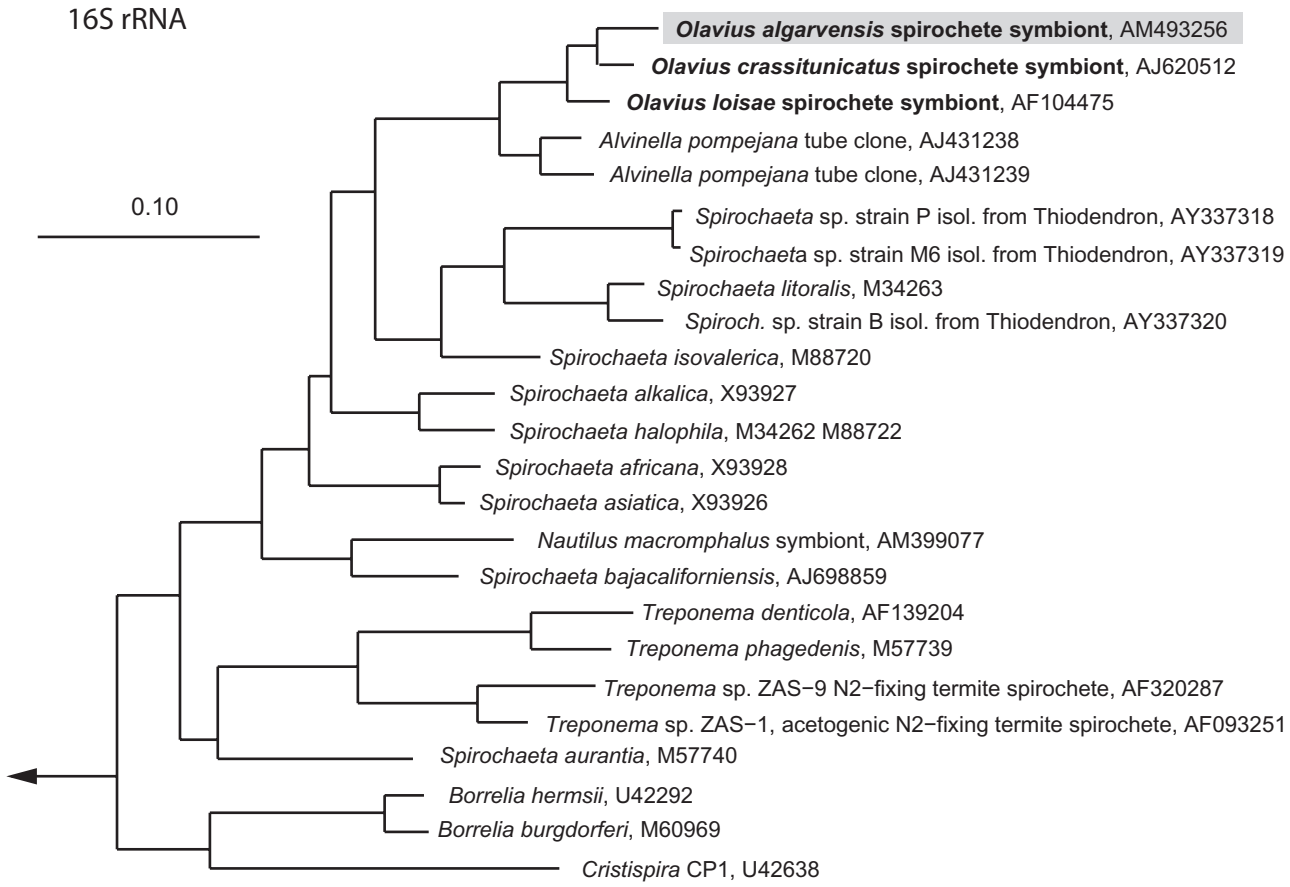

Fig. 7. Phylogenetic placement of the spirochete symbiont from *O. algarvensis* based on maximum likelihood (ML) analyses of 16S rRNA sequences (sequence from this study highlighted in grey; symbionts from gutless oligochaetes in bold face). *Escherichia coli* and *Vibrio fischeri* sequences were used as an out-group (arrow, AE016770, X56578). Scale bar = 0.10 estimated substitutions per site.

symbionts in these three host species suggests that they are essential for these hosts and that these associations are stable and well established. The additional gammaproteobacterial symbionts of these hosts (Gamma 2 and 3 in Fig. 1) occur fairly regularly within each host population, although some variability was observed in *O. algarvensis* (Table 2). The highest variability was observed in the abundance and distribution of the additional deltaproteobacterial symbionts of these three host species (Delta 3, 4 and 5 in Fig. 5), suggesting that these symbionts are less important, and that these associations are less stable. The phylogenetic diversity of these additional deltaproteobacterial symbionts (Fig. 5) indicates that these associations were established multiple times and independently of each other in convergent evolution.

As in other invertebrate hosts with multiple symbionts, little is currently understood about the selective advantage of harbouring different types of co-occurring bacteria. In gutless oligochaetes with sulfur-oxidizing and sulfate-reducing symbionts such as *O. algarvensis* and *O. ilvae*, it is clear that syntrophic sulfur cycling provides both the symbionts and their hosts with benefits (Dubilier *et al.*, 2001; Woyke *et al.*, 2006). Multiple symbionts with similar metabolic capabilities guarantee functional redundancy and increase the fitness of the host to respond to changes in the environment that might favour one symbiont species over the other. Particularly the provision of sulfide through sulfate-reducing symbionts is assumed to play a crucial role in these hosts, as sulfide concentrations in the Elba sediments are extremely low and rarely exceed a few micromolars (Dubilier *et al.*, 2001). It is intriguing that *O. algarvensis* and *O. ilvae* that are only distantly related to each other harbour a similar association of gamma- and deltaproteobacterial symbionts. This suggests that there was a strong selective pressure on these hosts to establish associations with sulfate-reducing bacteria to better supply their gammaproteobacterial symbionts with a continuous source of reduced sulfur compounds.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Table S1. 16S rRNA gene libraries from *O. ilvae* and *O. algarvensis* individuals. Worms were prepared individually for DNA extraction as described previously (Dubilier *et al*., 1999) using the isolation protocol of (Schizas *et al*., 1997). Amplification of the 16S rRNA genes was performed with the general bacterial primers 8F and 1492R (Muyzer *et al*., 1995). At least one representative clone from each clone group was fully sequenced in both directions. Clones were grouped based on a sequence identity threshold of 99% for full sequences.

Table S2. Sequence identities of genes from *O. algarvensis* and *O. ilvae.* The 16S rRNA, *dsrAB*, *cbbL* and *aprA* sequences isolated from *O. algarvensis* and *O. ilvae* were submitted either to EBI or to GenBank. Nucleotide sequence accession numbers are presented. Amino acid identity values for protein-coding genes were calculated in PFAAT (Johnson *et al*., 2003).

Table S3. Protein-coding gene libraries from *O. ilvae* and *O. algarvensis* individuals. Amplification, cloning and sequencing of the *dsrAB*, *cbbL*, *cbbM* and *aprA* genes were carried out as described previously (Loy *et al*., 2004; Wagner *et al*., 2005; Blazejak *et al*., 2006). Full sequences within each clone group shared at least 98.4% sequence identity (% identical amino acids).

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