Molecular characterization of the symbionts associated with marine nematodes of the genus *Robbea*†

Christoph Bayer,1*† Niels R. Heindl,1*† Christian Rinke,1 Sebastian Lücker,2 Joerg A. Ott1 and Silvia Bulgheresi1*†

Departments of 1Marine Biology and 2Microbial Ecology, University of Vienna, Althanstrasse 14, 1090 Vienna, Austria.

Summary

Marine nematodes that carry sulfur-oxidizing bacteria on their cuticle (*Stilbonematinae, Desmodoridae*) migrate between oxidized and reduced sand layers thereby supplying their symbionts with oxygen and sulfide. These symbionts, in turn, constitute the worms' major food source. Due to the accessibility, abundance and relative simplicity of this association, stilbonematids may be useful to understand symbiosis establishment. Nevertheless, only the symbiont of *Laxus oneistus* has been found to constitute one single phylootype within the *Gammaproteobacteria*. Here, we characterized the symbionts of three yet undescribed nematodes that were morphologically identified as members of the genus *Robbea*. They were collected at the island of Corsica, the Cayman Islands and the Belize Barrier Reef. The surface of these worms is covered by a single layer of morphologically indistinguishable bacteria. 18S rDNA-based phylogenetic analysis showed that all three species belong to the *Stilbonematinae*, although they do not form a distinct cluster within that subfamily. 16S rDNA-based analysis of the symbionts placed them interspersed in the cluster comprising the sulfur-oxidizing symbionts of *L. oneistus* and of marine gutless oligochaetes. Finally, the presence and phylogeny of the *aprA* gene indicated that the symbionts of all three nematodes can use reduced sulfur compounds as an energy source.

Introduction

Marine nematodes that live a few centimetres below the surface of sandy bottoms may carry sulfur-oxidizing bacteria (SOB) within their body as endosymbionts (*Astomonema* (Ott *et al.*, 1982; Vidakovic and Boucher 1987; Giere *et al.*, 1995; Musat *et al.*, 2007) and *Parastomonomema* (Kito, 1989)) or on their surface as ectosymbionts. The latter belong to the subfamily *Stilbonematinae* and consist of the genera *Adelphus* Ott 1997, *Catanema* Cobb 1920, *Eubostrichus* Greef 1869, *Laxus* Cobb 1894, *Leptonemella* Cobb 1920, *Robbea* Gerlach 1956, *Squanema* Gerlach 1963 and *Stilbonema* Cobb 1920 (reviewed in Ott *et al.*, 2004a,b). The worms migrate between oxygenated, upper sand layers and anoxic, sulfidic, deeper ones (Ott *et al.*, 1991) allowing the bacteria to obtain the oxygen they need as electron acceptor and the sulfur compounds (e.g. hydrogen sulfide, thiosulfate) as electron donor (Polz *et al.*, 1992; Hentschel *et al.*, 1999). Stable carbon isotope incorporation experiments showed that the ectosymbionts are the major components of their host diet (Ott *et al.*, 1991).

Symbionts are probably acquired from the environment because unhatched early embryos of *Laxus oneistus* are symbiont-free (Silvia Bulgheresi and Joerg A. Ott, in preparation). Environmental transmission would also enable nematodes to re-establish their symbiotic coat every time they replace their cuticle with a newly synthesized one. This process, known as molting or ecdysis, occurs several times during worm development. Moreover, *Robbea* sp.1 and sp.3 symbiont 16S rDNAs were detected in sand and seawater by polymerase chain reaction (PCR) and fluorescence in *situ* hybridization (FISH) with 16S rRNA-specific primers. As for the mechanisms of symbiont recruitment from the environment, we showed that the Ca²⁺-dependent lectin Mermaid mediates symbiont–symbiont and worm–symbiont attachment in L. oneistus (Bulgheresi *et al.*, 2006).

Up to the present study only the symbionts of *L. oneistus* have been shown to belong to one single phylotype of *Gammaproteobacteria* closely related to the endosymbionts of marine gutless oligochaetes (Polz *et al.*, 1994) and of *Astomonema* sp. (Musat *et al.*, 2007). Although a molecular characterization of the large, multinucleated, filamentous symbiont of *Eubostrichus dianae*...
has been attempted, its 16S rDNA could not be amplified by PCR (Polz et al., 1999).

In this study, we molecularly characterized three associations involving stilbonematids which we assigned to the genus *Robbea* (Gerlach, 1956; 1963) based on their morphological characteristics. We collected *Robbea* sp.1 in the Mediterranean Sea from a subtidal sand patch close to a *Posidonia oceanica* seagrass meadow near Calvi (Corsica, France), and *Robbea* sp.2 and *Robbea* sp.3 in the Caribbean Sea from shallow back-reef sandbars at Little Cayman Island (Cayman Islands) and Carrie Bow Cay (Belize) respectively. We first analysed the phylogenetic position of the worms by making clone libraries of their 18S rRNA genes. We then characterized the symbionts associated with each species by cloning their respective 16S rRNA genes. To confirm that the latter were indeed derived from the ectosymbionts, we applied FISH on whole worms. Finally, the cloning and phylogenetic analysis of a gene that is involved in sulfur metabolism support the sulfur-oxidizing nature of the *Robbea* symbionts.

**Results and discussion**

**Morphological and 18S rDNA-based molecular characterization of Robbea nematodes**

The genus *Robbea* was established by Gerlach (1956). It is characterized by a clearly set off and muscle-rich distal part (corpus) of the tripartite pharynx. Moreover, all males, except in *Robbea caelestis*, are provided by a row of ventromedian suckers in the postpharyngeal region which are supposed to be copulation-helping organs (J.A. Ott, unpubl. data; Fig. 1A, C, E and G and asterisks in Fig. 1F). The number of suckers is constant and species-specific. Because each of the three nematodes characterized in this study had a tripartite, muscle-rich pharynx and carried a row of ventromedian suckers, we assigned them to the genus *Robbea*. Nevertheless, they did not form a monophyletic lineage within the *Stilbonematinae* (*Chromadorea*) in our 18S rDNA-based phylogenetic reconstruction (Fig. 2). It is therefore conceivable that their distinctive morphological traits evolved several times independently. Alternatively, supplementary sequence information from the 28S or Internal Transcribed Spacers (ITS) rDNA or from mitochondrial genes might be needed to support the genus *Robbea* at the molecular level.

*Robbea* sp.3 and *Stilbonema majum* were the only stilbonematids which showed the highest 18S rDNA sequence similarity with one another, while co-occurring in the same collection site, the Belize Barrier Reef. The 18S rDNA of *Robbea* sp.2, however, showed the highest sequence similarity with that of stilbonematids collected in an extremely distant geographical location.

Each nematode is covered by a single morphotype of symbionts: *Robbea* sp.1 and sp.2 display coccosoid bacteria c. 1.5 μm wide (Fig. 1B and D respectively) whose shape and arrangement are reminiscent of kernels on a
Robbea sp.3 is covered by spindle-shaped rods 0.2 mm long (Fig. 1F). These assume different orientations with respect to the worm’s surface, with some standing perpendicularly, as observed for L. oneistus, and some laying horizontally. In Robbea sp.1 and sp.2, the symbionts appear to divide transversally (arrows in Fig. 1B and D respectively). In Robbea sp.3 they divide longitudinally (arrows in Fig. 1H and I), a special mode of binary fission also exhibited by L. oneistus symbionts (Polz et al., 1992; 1994). Concerning the length of the microbial coat, only the anterior-most region of Robbea sp.3 and the very tip of the tail are symbiont-free. In Robbea sp.1 and Robbea sp.2, instead, the coat starts a short distance behind the anterior end, coinciding with a reduction in the worm diameter to accommodate the symbionts. This last feature is also displayed by L. oneistus.

As in all other known stilbonematids, the Robbea symbionts are densely packed and appear bright white in incident light, probably due to inclusions of elemental sulfur (Himmel et al., 2009).

Robbea symbionts belong to the marine nematode and oligochaete symbionts cluster

Robbea symbiont 16S rDNA clones were randomly picked and comparison of their complete sequences showed that they could be assigned to three distinct clone groups belonging to the *Gammaproteobacteria*, with a sequence similarity within each clone group ≥ 99.8%. In our 16S rDNA-based phylogenetic reconstruction (Fig. 3) the three obtained gammaproteobacterial 16S rDNAs clustered with those of the symbionts of L. oneistus, of the nematode Astomonema sp., and of all known marine gutless oligochaetes (*Inanidrilus* and *Olavius* spp.). This nematode–oligochaete symbiont cluster is most closely related to the SOB from the family *Chromatiaceae* (> 90%). It is intriguing that, although free-living, some of these sulfur purple bacteria engage in symbiotic associations with unrelated bacteria in phototrophic consortia (Tonolla et al., 2000; Overmann, 2002).

Our phylogenetic reconstruction shows that the three Robbea symbionts (16S rDNA sequence identity ≥ 97.1%) do not form a distinct group within the nematode–oligochaete sulfur-oxidizing symbionts cluster (16S rDNA sequence identity ≥ 95.4%). Moreover, nematode symbionts cannot be consistently grouped according to the geographical origin of their hosts and probably did not speciate in concert with their hosts. Phylogenetic incongruence between host and symbiont is typical of horizontally transmitted symbioses (Moran and Baumann, 2000), and was also observed for marine gutless oligochaetes and their sulfur-oxidizing symbionts (Dubilier et al., 2001; Blazejak et al., 2006; Musat et al., 2007).

To confirm that the gammaproteobacterial 16S rDNA sequences derived from the Robbea symbionts, we carried out FISH with the symbiont-specific probes Rca470, Rss457 and Rhs465, for Robbea sp.1, sp.2 and sp.3 respectively (Table 1). All the bacteria attached to the worms were triple stained by the eubacterial probe EUB338, by the *Gammaproteobacteria*-specific probe GAM42a and by the respective specific probe (Fig. 4). In contrast, no FISH signal was detectable with the negative control probe NON338 or with a *Betaproteobacteria*-specific probe (data not shown). This indicates that the bacteria covering each of the three Robbea species belong to one single phylotype and that no additional bacteria are present. This is consistent with the electron microscopy analysis, which shows only one bacterial mor-
photype on each *Robbea* worm, and with our highly homogeneous 16S rDNA libraries.

**aprA** gene analysis of stilbonematid-associated bacteria

To gain evidence that *Robbea* symbionts are indeed SOB, we cloned a fragment of the gene encoding for the alpha subunit of the adenosine-5′-phosphosulfate (APS) reductase *(aprA)*, an enzyme involved in sulfur metabolism. The AprA protein reduces APS to sulfite in sulfate-reducing bacteria (SRB), but also catalyses the reverse reaction in SOB (Hipp et al., 1997; Sanchez et al., 2001; Friedrich, 2002). By using a set of *aprA*-specific primers, we PCR amplified and cloned a ~1400-nt-long fragment from *Robbea*- and *L. oneistus*-associated bacteria. Several clones from each *aprA* library were randomly picked (see *Experimental procedures*) and their predicted protein sequences used for tree calculation (Fig. 5).

The AprA sequences of bacteria associated with *Robbea* sp.2, sp.3 and *L. oneistus* clustered together with those of the *Bathybiotus brevior* symbiont, and of some free-living sulfur-oxidizing gammaproteobacteria and sulfur purple bacteria *(AprA-lineage I*; see Meyer and Kuever (2007b) for a definition of *AprA*-lineages I and II); *Robbea* sp.1-associated symbiont AprA, instead, clustered with those of gutless oligochaete sulfur-oxidizing symbionts *(AprA-lineage II)*. Notably, *Robbea* sp.2 and sp.3 symbionts cluster together in both the 16S rDNA- and *aprA*-based trees.

In conclusion, all the AprA sequences obtained in this study are most closely related to SOB AprAs. This suggests that *Robbea* symbionts oxidize sulfur compounds as an energy source.

**Conclusions**

We characterized three new nematode–bacteria associations with very different geographical origins — the island of Corsica, the Cayman Islands and the Belize Barrier Reef. Although we cannot exclude that the three *Robbea* symbionts could stably associate with other marine organisms, our data show that each *Robbea* sp. is always coated by one characteristic symbiont phylotype. The basis of this conclusion is that each 16S rDNA and each *aprA* library was highly homogeneous and that the symbionts of each species were reproducibly stained by a symbiont 16S rDNA-specific FISH probe. Accordingly, electron microscopic analysis revealed that individuals of each *Robbea* sp. are always coated by the same, characteristic bacterial morphotype.

Our 18S rDNA-based tree shows that all three nematode species are stilbonematids, albeit additional worm nuclear and/or mitochondrial DNA sequence information is needed to confirm the genus *Robbea* at the molecular level.

Intriguingly, the 16S rDNAs of the stilbonematid symbionts are tightly grouped with those of mouthless oligochaetes. One explanation is that nematodes and oligochaetes co-occur in shallow-water sandy bottoms and they are all exposed to a similar pool of environmental bacteria. This habitat potentially promoted the establishment of these associations several times in the course of the evolution and at many different geographical locations. In this scenario, nematodes and oligochaetes recruited similar bacteria from this shared habitat as prospective symbionts. Sequencing of one or more stilbonematid symbiont metagenomes might unveil molecular adaptations shared by the oligochaete and nematode sulfur-oxidizing symbionts.
The fact that Robbea-associated bacteria harbour SOB-like aprA genes indicates that they gain energy from oxidation of reduced sulfur compounds. Moreover, their white appearance supports their capacity to store elemental sulfur. Migration of Robbea nematodes between deep and superficial sand layers, as observed for L. oneistus (Ott et al., 1991), would alternatively supply their symbionts with reduced sulfur compounds and oxygen. In the absence of oxygen, symbionts might use nitrate to respire sulfide (Hentschel et al., 1999), while they could resort to their sulfur stores when sulfide is unavailable in the environment.

In turn, the Robbea worms might feed on their symbionts. Stable isotope incorporation experiments and electron microscope analysis of the gut microbiome indicate that Robbea-associated bacteria harbour SOB-like aprA genes and gain energy from oxidation of reduced sulfur compounds. Moreover, their white appearance supports their capacity to store elemental sulfur. Migration of Robbea nematodes between deep and superficial sand layers, as observed for L. oneistus (Ott et al., 1991), would alternatively supply their symbionts with reduced sulfur compounds and oxygen. In the absence of oxygen, symbionts might use nitrate to respire sulfide (Hentschel et al., 1999), while they could resort to their sulfur stores when sulfide is unavailable in the environment.

### Table 1. Probes used for FISH.

| Probe    | Standard probe name | Specificity                                      | Sequence/5′ modification | Target RNA Position | Formamide percentage/incubation time (h)/probe concentration (ng ml−1) | Reference                      |
|----------|---------------------|-------------------------------------------------|--------------------------|---------------------|--------------------------------------------------------------------------|--------------------------------|
| EUB338   | S-*BactIV-0338-a-A-18 | Most bacteria                                   | 5′-GCT GCC TCC CGT AGG AGT-3′/fluorescein | 16S 338−355         | 35–40%/1.5–o.n./3                                                        | Amann et al. (1990)           |
| GAM42a   | L-C-gProt-1027-a-A-17 | Gammaproteobacteria                             | 5′-GCC TTC CCA CAT CGT TT-3′/Cy5         | 23S 1027−1043       | 35–40%/1.5–o.n./3                                                        | Manz et al. (1992)            |
| Rcs4a70  | S-Rob2s-0471-a-A-21  | Robbea sp.1 symbiont                            | 5′-TGC GTA ACG TCA AGA CCC TGG-3′/Cy9   | 16S 471−491         | 25%/1.5−3.8                                                             | This study                    |
| Rss456   | S-*Rob2s-0457-a-A-21 | Robbea sp.2 symbiont                            | 5′-ACC C1G AGC TAT TAA CCC AAG-3′/Cy3   | 16S 457−477         | 35%/o.n./4                                                              | This study                    |
| Inanidilus leukodermatus endosymbiont 1 (GenBank Accession No. AJ890100) | | | | | | |
| Rhs465   | S-Rob3s-a-A-21       | Robbea sp.3 symbiont                            | 5′-AAC GTC AGG ATC CCC CCG AGC TAT-3′/Cy3 | 16S 466−486         | 40%/3-2.3                                                               | This study                    |
| NON338   | Not named            | Negative control                                | 5′-ACT CCT ACG GGA GGC AGC-3′/Cy3       | 16S 338−355         | 35–40%/1.5–o.n./3                                                        | Wallner et al. (1993)         |
| BET42a   | L-C-bProt-1027-a-A-17 | Betaproteobacteria                              | 5′-GCC TTC CCA CTT CGT TT-3′/fluorescein | 16S 1027−1043       | 35–40%/1.5–o.n./3                                                        | Manz et al. (1992)            |

a. According to Alm and colleagues (1996).
b. 16S rRNA position, Escherichia coli numbering (Brosius et al., 1978).
c. 23S rRNA position, E. coli numbering (Brosius et al., 1981).
o.n., overnight.

**Fig. 4.** Fluorescence in situ hybridization (FISH) confocal microscopy photographs of Robbea sp.1 (A–D), Robbea sp.2 (E–H) and Robbea sp.3 (I–L) symbionts attached to the worm surface. Each single symbiont is triple stained with a eubacteria-specific probe (green), a Gammaproteobacteria-specific probe (blue), and a symbiont-specific probe (red). (D), (H) and (L) are overlay pictures of (A)–(C), (E)–(G) and (I)–(K), respectively. Scale bar is 2 μm.
cate this to be the case for other stilbonematids (Ott et al., 1991). Cloning of other symbiont genes involved in sulfur metabolism and carbon fixation, and transmission electron microscopy of the symbionts coupled with multi-isotope imaging mass spectrometry will shed light on their physiology.

The geographical distribution of the three Robbea nematodes characterized in this study appears to be restricted to the respective collection sites. One future task will be to investigate if the stilbonematid symbionts can be found only in the host habitat, as in the case of tube worms (Harmer et al. 2008) and lucinid mussels symbionts (Gros et al., 2003) or, instead, are widely distributed throughout the oceans and can survive without their hosts.

Another key question is how specific ectosymbionts are recruited from the environment by different stilbonematid species. In this respect, we plan to identify which repertoire of Mermaid isoforms are expressed by the Robbea worms and to compare them with each other and with those of L. oneistus. An exciting outcome could be that expression of a characteristic lectin repertoire by each stilbonematid species underpins acquisition and maintenance of a specific bacterial coat.

**Experimental procedures**

**Specimen collection**

Robbea sp.1 was collected in July 2007 from a subtidal sand patch close to a P. oceanica seagrass meadow in c. 2 m depth in the harbour of the Station de Recherches Sous-Marines et Océanographiques (STARESO), Calvi, France (42°34′49″N, 8°43′27″W). Robbea sp.2 was collected in October 2006 in c. 1 m depth from a shallow water back-reef sand bar off Point of Sand Beach on Little Cayman, Cayman Islands (19°42′08″N, 79°57′46″W). Robbea sp.3 was collected in November 2007 in c. 1 m depth from a shallow water back-reef sand bar off Carrie Bow Cay, Belize (16°48′11″N, 88°04′55″W). The worms were extracted from the sand by shaking it in seawater and pouring the supernatant through a 63-μm-pore-size mesh screen. Single individuals were then picked by hand under a dissecting microscope.

Robbea sp.1 and Robbea sp.3 worms were fixed either in ethanol, for DNA extraction, or in 1% osmium tetroxide in seawater, for FISH (Rinke et al., 2006), and then stored in ethanol at -80°C. Robbea sp.2 worms were flash frozen in liquid N2 and stored at -80°C either unfixed (for DNA extraction) or upon methanol fixation (for FISH).

**Scanning electron microscopy**

Worms were pre-fixed in a 2.5% glutaraldehyde, 0.1 M sodium cacodylate, 2% sucrose solution, rinsed with 0.1 M sodium cacodylate buffer, and post-fixed in a 1% osmium tetroxide, 0.1 M sodium cacodylate, 2% sucrose solution. After alcohol dehydration, worms were gold sputter coated and viewed through a Philips XL 20 scanning electron microscope.

**DNA extraction and PCR amplification of 18S rDNA**

We extracted and purified the DNA from single Robbea worms as described previously (Schizas et al., 1997) and 2 μl was used as a template for each PCR. A fragment of the Robbea sp.1 18S rRNA gene was amplified by PCR with the general eukaryotic primers 1f (5′-CTGGTTGATYCTGCCAGT-3′; Winnepenninckx et al., 1995) and 2023r (5′-GGTTACCTTCCGAGAAC-3′; Pradillon et al., 2007). Cycling conditions were: 94°C for 5 min; 94°C for 45 s, 48°C for 45 s, 72°C for 2 min 35s; 72°C for 10 min. The PCR product was 1779 nt. Robbea sp.2 18S rRNA was amplified with the general eukaryotic primers 1f (see above) and 18SE (5′-ATGTACCTTCCGAGAAC-3′; Pradillon et al., 2007) and Robbea sp.3 18S rRNA was amplified with primers 1f and 2023r. Cycling conditions were: 95°C for 5 min; 95°C for 45 s, 48°C for 45 s, 72°C for 2 min 35s; 72°C for 10 min. The PCR product was 1779 nt for Robbea sp.2 and 1783 nt for Robbea sp.3.
DNA extraction and PCR amplification of 16S rDNA

Symbionts were washed off a deep-frozen pellet of 500 Robbea sp. 2 individuals with 50 μl of ddH2O. The 50 μl was then transferred to a fresh 1.5 ml tube and incubated at 94°C for 10 min. Five microlitres of this solution was directly used as a template for PCR. For Robbea sp.1 and Robbea sp.3, DNA was extracted from single worms as described (Schizas et al., 1997), and 2 μl each was used as a template for PCR. For all Robbea worms, PCR was performed using the eubacterial primers 616FV (5'-AGAGTTTGATYMTGGCTC-3', Juretschko et al., 1998) and 1492R (5'-GATTTACCTTGGAGACACTT-3', Kane et al., 1993). The PCR programme for Robbea sp.2 and sp.3 was: 94°C for 5 min; 94°C for 45 s, 47°C for 45 s, 72°C for 1 min 30 s; 72°C for 10 min. Cycling conditions for Robbea sp.1 were: 94°C for 4 min; 94°C for 45 s, 49°C for 30 s, 72°C for 1 min 45 s; 72°C for 10 min. Each PCR product was 1499 nt.

DNA extraction and PCR amplification of APS reductase (aprA) gene

We extracted and purified the DNA from single Robbea worms as described previously (Schizas et al., 1997) and 2 μl was used as a template for each PCR. To amplify a c. 1400 nt aprA (adenosine phosphosulfate reductase alpha subunit) gene fragment we used the primers AprA-1-FW (5'-TGGCGAGATCAGTAYMGG-3') and AprA-10-RV for Robbea sp.1-associated bacteria (5'-CGCWAGTAGWAR CCRGGRTA-3') and AprA-11-RV (5'-CGKGYRTAGTAKCCS GCACA-3') for Robbea sp.2- and Robbea sp.3-associated bacteria, as described (Meyer and Kuever, 2007a,b).

Cloning

All PCR products were gel purified and cloned into pCR2.1-TOPO using the TOPO TA Cloning Kit (Invitrogen Life Technologies, Germany).

We randomly picked and fully sequenced: 8, 7 and 6 clones of the 18S rDNA fragments obtained by Robbea sp.1 (EU766870), sp.2 (EU76887) and sp.3 (EU784735) respectively; 13, 19 and 11 clones of the 16S rDNA fragments obtained by Robbea sp.1 (EU7711427), sp.2 (EU7711426) and sp.3 (EU7711428) respectively; 24, 31 and 21 clones of the aprA gene fragment from Robbea sp.1 (EU864035), sp.2 (EU864037) and sp.3 (EU864039), respectively. Sequences were aligned and compared with CodonCode Aligner 1.6.3 software.

Phylogenetic analysis

For each Robbea species, the sequences of the symbiont 16S rDNA and the worm 18S rDNA were compared with sequences in GenBank by using BLASTN, the AprA sequences by using BLASTP (Altschul et al., 1990). Phylogenetic analysis was carried out using the ARB program package (Ludwig et al., 2004). We used TreePuzzle 5.0 to evaluate the phylogenetic position of each Robbea worm and its respective symbiont. For 18S rDNA-based phylogenetic reconstruction, we also used the maximum parsimony method and constructed a consensus tree. Similarity matrices were calculated using the similarity matrix option in the neighbour joining field of the ARB software package.

For tree calculations, we applied a 50% conservation filter and we used only sequences longer than 1450 bp for host phylogeny and longer than 1325 bp for symbiont phylogeny. Sequences of Priapulus caudatus (AF025927) and Halycryptus spinulosus (AF342790) for the host 18S rDNA tree and sequences of Alkalimicrona halodurans (AJ404972), Nitrococcus mobilis (L35510) and Methylhalobius crimeensis (AJ581837) served as out-groups for the symbiont 16S rDNA tree.

For the AprA protein tree, we aligned selected members of SOB, sulfate-reducing prokaryotes (SRP) and the stilbomatin symbiont sequences using T-coffee (Notredame et al., 2000). We applied a 50% conservation insertion deletion (indel) filter for tree calculation and members of the AprA lineage I (Meyer and Kuever, 2007b) served as out-groups.

Fluorescence in situ hybridization (FISH)

We designed FISH probes by using the ARB PROBE DESIGN tool (see Table 1) and confirmed their specificity by comparing them with all available sequences in GenBank, SILVA, Greengenes. Probes were fluorescently labelled on their 3’ end (Thermo, Germany). FISH was performed according to Manz and colleagues (1992). Briefly, fixed nematodes (n = 30) of each Robbea sp. were incubated at 46°C in hybridization buffer containing the respective FISH probes [0.9 M NaCl, 20 mM Tris-HCl (pH 8.0), 0.001% SDS; refer to Table 1 for incubation time, formamide percentage and probe concentration]. Unspecific bound probe was subsequently removed by incubating at 48°C for 15 min in appropriate washing buffer. Nematodes were mounted in DAPI Vectashield (Vector Labs) and examined using a Leica TCS-NT confocal laser scanning microscope.

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