environmental microbiology

Environmental Microbiology (2010) **12**(8), 2312–2326 doi:10.1111/j.1462-2920.2010.02256.x

# Gamma- and epsilonproteobacterial ectosymbionts of a shallow-water marine worm are related to deep-sea hydrothermal vent ectosymbionts

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## Summary

**The marine oligochaete worm *Tubificoides benedii* is often found in high numbers in eutrophic coastal sediments with low oxygen and high sulfide concentrations. A dense biofilm of filamentous bacteria on the worm's tail end were morphologically described over 20 years ago, but no further studies of these epibiotic associations were done. In this study, we used fluorescence *in situ* hybridization and comparative sequence analysis of 16S rRNA and protein-coding genes to characterize the microbial community of the worm's tail ends. The presence of genes involved in chemoautotrophy (*cbbL* and *cbbM*) and sulfur metabolism (*aprA*) indicated the potential of the *T. benedii* microbial community for chemosynthesis. Two filamentous ectosymbionts were specific to the worm's tail ends: one belonged to the *Leucothrix mucor* clade within the Gammaproteobacteria and the other to the Thiovulgaceae within the Epsilonproteobacteria. Both *T. benedii* ectosymbionts belonged to clades that consisted almost exclusively of bacteria associated with invertebrates from deep-sea hydrothermal vents. Such close relationships between symbionts from shallow-water and deep-sea hosts that are not closely related to each other are unusual, and indicate that biogeography and host affiliation did not play a role in these associations. Instead, similarities between the dynamic environments of vents and organic-rich mudflats with their strong fluctuations in reductants and oxidants may have been the driving force behind the establishment and evolution of these symbioses.**

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## Introduction

Until recently, the epibiotic microflora of animals was often thought to be just a consortium of casual associates, but it is now becoming increasingly clear that it consists of a highly specialized community of microbes that form specific and stable symbioses with their hosts (McFall-Ngai, 2008). In chemosynthetic environments such as hydrothermal vents or sulfide-rich sediments, some marine animals are regularly colonized by ectosymbiotic bacteria (Dubilier *et al*., 2008; Goffredi, 2010). In coastal sediments these include nematode worms, amphipods and colonial ciliates all of which generally host only a single sulfur-oxidizing gammaproteobacterial symbiont (Gillan and Dubilier, 2004; Bayer *et al*., 2009; Rinke *et al*., 2009). Hosts from deep-sea hydrothermal vents such as shrimps, polychaete worms, crabs and barnacles often have a more diverse epibiotic community that is dominated by Gamma- and Epsilonproteobacteria (Goffredi *et al*., 2008; Suzuki *et al*., 2009; Watsuji and Takai, 2009; Petersen *et al*., 2010). The role of these epibiotic bacteria is generally not well understood. They have been hypothesized to contribute to their hosts' nutrition (Rieley *et al*., 1999; Rinke *et al*., 2006; Suzuki *et al*., 2009), detoxify sulfide and heavy metals (Alayse-Danet *et al*., 1987; Prieur *et al*., 1990), or provide defense against pathogenic microbes and predators (Gil-Turnes and Fenical, 1992; Goffredi *et al*., 2004a).

The tubificid oligochaete *Tubificoides benedii* (d'Udekem, 1855) is a marine worm that is commonly found in coastal mudflats of the North Atlantic, especially those with a high input of organic matter (Timm and Erséus, 2009). As other tubificids (Guérin and Giani, 1996), *T. benedii* feeds head down in the sediment and uses its tail end for respiration by holding it above the sediment in the oxygenated seawater and moving it in a swaying motion (Dubilier *et al*., 1995a). The worms can tolerate extended periods of low oxygen concentrations or anoxia by switching to an anaerobic metabolism, a strategy that is also used when sulfide concentrations become too high (Dubilier *et al*., 1994).

*Tubificoides benedii* from mudflats in the Wadden Sea off the coast of Germany are covered with a morphologically diverse assemblage of ectobacteria in

the mucus layer covering their body wall (Giere and Rhode, 1987). Their tail ends are regularly colonized by a dense film of filamentous bacteria. Some of these penetrate the body wall and the basal cells are anchored in the cuticle just above the worm's epidermis, suggesting a highly specific association (Dubilier, 1986). Seasonal 'blooms' of the filamentous bacteria have been observed, with the densest abundance occurring in the summer and fall months when sulfide concentrations are high in the worm's habitat (Dubilier, 1986). This observation together with the morphological similarity of the filamentous bacteria to sulfur-oxidizing bacteria such as *Thiothrix* lead to the hypothesis that these ectofilaments might be chemoautotrophic sulfur oxidizers (Dubilier, 1986), but no further studies on this association followed. The purpose of this study was therefore to characterize the phylogeny and metabolic potential of the filamentous ectobionts of *T. benedii* using fluorescence *in situ* hybridization (FISH) and comparative sequence analysis of 16S rRNA genes as well as genes involved in chemoautotrophy (*cbbL, cbbM* and *aclB*) and sulfur metabolism (*aprA* and *soxB*).

## Results and discussion

### *General bacterial diversity*

Comparative 16S rRNA gene analyses and FISH of *T. benedii* tail ends revealed a diverse bacterial community. The filamentous ectosymbionts were identified as Gamma- and Epsilonproteobacteria (see below). As this study focuses on these ectosymbionts, all other bacteria associated with this worm are only briefly described here.

Gammaproteobacterial sequences dominated the 16S rRNA clone libraries (52.4%), Deltaprotobacteria (20.5%) and Cytophaga/Flavobacterium/Bacteroides (CFB; 19.2%) were also abundant, and 4% of the sequences belonged to the Epsilonproteobacteria (Table 1 and Figs S1 and S2). FISH analyses with group specific probes for Deltaproteobacteria and CFB (Table 2) confirmed that bacteria from these phyla colonized the mucus layer covering *T. benedii* tail ends (Fig. S3). Sequences found in only low abundance in single individuals made up only 2.7% of all 16S rRNA clone library sequences and belonged to the Acidobacteria, Verrucomicrobia, Planctomycetes and candidate Division OD1 (Table 1).

Phylogenetic analyses revealed that many of the gamma- and epsilonproteobacterial sequences from the *T. benedii* 16S rRNA clone libraries were most closely related to symbiotic and free-living bacteria from chemosynthetic environments (Fig. 1). Specific FISH probes were developed for all dominant gamma- and epsilonproteobacterial phylotypes (Tables 2 and S1). Aside from the filamentous ectosymbionts described below, the only other gamma- or epsilonproteobacterial phylotype from the 16S rRNA clone libraries that could be found with FISH was a rod- to cocci-shaped bacterium named the Gamma 2 ectobiont (Fig. S3). This phylotype was highly abundant in the 16S rRNA clone libraries (23% of all clones, Table 1), but with FISH, the Gamma 2 ectobiont was only observed occasionally in the mucus membrane of the worm's tail end. Phylogenetic analyses placed this ectobiont in a clade that included the Gamma 3 endosymbionts of the gutless oligochaete worms *Olavius ilvae* and *Olavius algarvensis* from Mediterranean seagrass sediments (Fig. 1) (Ruehland *et al*., 2008). Such a close phylogenetic relationship between ecto- and endosymbionts of hosts that are separated by large geographic distances, live in different habitats, and are not closely related to each other have rarely been described but are not unprecedented. They have, for example, also been observed between the ectosymbionts of nematodes and the Gamma 1 endosymbionts of gutless oligochaetes (Bayer *et al*., 2009).

### *Morphology and phylogeny of the filamentous ectosymbionts*

Transmission electron microscopy (TEM) showed that two filamentous morphotypes co-occurred on *T. benedii* tail ends (Fig. 2). Thinner filaments of 0.4–0.65 μm penetrated the worm's cuticle, with the basal cells embedded within the cuticle just above the host's epidermis (Fig. 2B–D). Vesicle-like structures were regularly observed between the basal cell of these filaments and the worm's epidermal cells (Fig. 2D), indicating interactions between the filaments and host tissues (Dubilier, 1986). Thicker filaments of 0.9–1.1 μm were attached to the cuticle but were never observed penetrating it (Fig. 2B). FISH with probes specific to the Gamma 1 and Epsilon 1 sequences shown in Fig. 1 revealed that these originated from two filamentous morphotypes on the worm's tail (Fig. 2B–D). The diameters of these two filament types in FISH micrographs corresponded to those measured with TEM in the thinner and thicker morphotypes (0.4–0.7 μm for the Epsilon 1 and 0.7–1.3 μm for the Gamma 1). This indicates that the thinner filaments observed with TEM in the worm's cuticle belonged to the Epsilon 1 ectosymbionts. This conclusion is supported by FISH analyses that show the Epsilon 1 ectosymbionts embedded within the cuticle (Fig. 2H). The distribution and abundance of the two filamentous ectosymbionts varied considerably within and between individual worms. In some cross-sections only one filament type was observed, in others, especially towards the posterior end of the tail, both the Gamma 1 and Epsilon 1 ectosymbionts co-occurred in thick patches (Fig. 2E). Overall, the abundance of both ectosymbionts was equal based on

**Table 1.** Clone library sequences from *T. benedii* tail ends.

| Gene/ protein | Clone group | Accession number | % in clone library | Closest relative | Identity BLAST (%) | Closest cultured relative | Identity BLAST (%) |
|---|---|---|---|---|---|---|---|
| 16S rRNA | Gamma 1 ectosymbiont | GU197434 | 12.8 | *Shinkaia crosnieri* setae associated clone, AB440175 | 95 | *Leucothrix mucor*, X87277 | 90 |
| | Gamma 2 | GU197441 | 22.6 | Sulfide-microbial incubator clone, DQ228657 | 97 | *Dechloromarinus chlorophilus*, AF170359 | 90 |
| *n* = 298 | Gamma 3 | GU197425 | 1.7 | *Urothoe poseidonis* ectosymbiont, AY426613 | 95 | *Thiothrix eikelboomii*, AB042541 | 91 |
| | Gamma 4 | GU197445 | 1.3 | *Solemya velum* endosymbiont, M90415 | 94 | *Thiobacillus prosperus,* EU653291 | 91 |
| | Gamma 5 | GU197422 | 12.0 | Environmental clones, e.g. hydrothermal vent, AB252427 | 97 | n.a. | |
| | Other Gamma | | 3.4 | | | | |
| | Epsilon 1 ectosymbiont | GU197436 | 1.3 | *Rimicaris exoculata* Epsilon 3 ectosymbionts, e.g. FM203395 | 97 | *Sulfurovum lithotrophicum*, AB091292 | 94 |
| | Epsilon 2 | GU197448 | 1.7 | Environmental clones, e.g. methane seep, FJ264676 | 99 | *Sulfurovum lithotrophicum*, AB091292 | 94 |
| | Other Epsilon | | 1.0 | | | | |
| | Delta | | 20.5 | | | | |
| | CFB | | 19.2 | | | | |
| | Other | | 7.1 | | | | |
| CbbL | CbbL 1a | GU197482 | 54.0 | *Solemya velum* endosymbiont, AAT01429 | 98 | *Mariprofundus ferrooxydans*, EAU53852 | 89 |
| | CbbL 1b | GU197481 | 10.3 | *Solemya velum* endosymbiont, AAT01429 (prot) | 96 | *Mariprofundus ferrooxydans*, EAU53852 | 90 |
| *n* = 165 | CbbL 1c | GU197476 | 24.8 | *Solemya velum* endosymbiont, AAT01429 | 93 | *Mariprofundus ferrooxydans*, EAU53852 | 89 |
| | CbbL 2 | GU197479 | 4.8 | –> | | *Mariprofundus ferrooxydans*, EAU53852 | 94 |
| | CbbL 3 | GU197478 | 3.6 | –> | | *Ralstonia metallidurans*, ABF08384 | 92 |
| | CbbL 4 | GU197480 | 2.4 | –> | | *Ralstonia metallidurans*, ABF08384 | 97 |
| | CbbL 5 | GU197477 | 0.6 | –> | | *Thiobacillus denitrificans*, AAB70697 | 96 |
| CbbM | CbbM 1 | GU197394 | 2.3 | *Lamellibrachia* sp. endosymbiont 2, BAA94433 | 93 | *Thiobacillus denitrificans*, AAA99178 | 85 |
| | CbbM 2 | GU197398 | 4.0 | *Lamellibrachia* sp. endosymbiont, CAQ63475 | 95 | *Halothiobacillus neapolitanus*, AAD02442 | 91 |
| *n* = 175 | CbbM 3a | GU197396 | 90.3 | *Lamellibrachia* sp. tube associated clone, CAQ63479 | 94 | *Thiomicrospira crunogena*, DQ272535 | 86 |
| | CbbM 3b | GU197397 | 2.3 | *Lamellibrachia* sp. tube associated clone, CAQ63479 | 90 | *Thiomicrospira halophila*, ABD52283 | 87 |
| | cbbM 4 | GU197395 | 1.1 | –> | | *Rhodopseudomonas palustris*, ABD88443 | 86 |
| AprA | AprA I1 | GU197400 | 0.4 | *O. ilvae* Gamma 3 symbiont, CAJ81245 | 96 | *Thiobacillus thioparus*, ABV80092 | 94 |
| | AprA II1 | GU197399 | 0.4 | *Ifremeria nautilei* symbiont, ABV80045 | 95 | *Thiothrix nivea*, ABV80025 | 92 |
| *n* = 242 | AprA II2 | GU197401 | 32.5 | *Lamellibrachia* sp. endosymbiont, CAQ63492 | 91 | *Thiothrix nivea*, ABV80025 | 90 |
| | AprA II3 | GU197406 | 0.8 | Sea urchin gut clone, CAT03603 | 96 | *Thiocapsa pendens*, ABV800015 | 89 |
| | AprA II4 | GU197404 | 60.1 | *Oligobrachia haakonmosbiensis* endosymbiont, CAP03143 | 90 | *Thiobacillus thioparus*, ABV80027 | 87 |
| | AprA II5 | GU197403 | 1.2 | *Lucinoma* aff. *kazani* symbiont, CAJ85653 | 94 | *Thiobacillus plumbophilus*, ABV80021 | 91 |
| | AprA II6 | GU197402 | 0.4 | *Lucinoma* aff. *kazani* symbiont, CAJ85653 | 90 | *Thiobacillus denitrificans*, ABV80031 | 88 |
| | AprA SRB1 | GU197405 | 4.1 | –> | | *Desulfotomaculum thermoacetoxidans*, ABR92588 | 78 |

Four worms were prepared and analysed individually for the 16S rRNA, three for the *cbbL*, *cbbM* and *aprA* clone libraries (numbers in the first column show the total number of clones sequenced for each gene). Sequences were grouped together if they had at least 99.0% nucleotide identity (16S rRNA gene) or 95% amino acid identity (*cbbL*, *cbbM* and *aprA* genes). Only 16S rRNA gene sequences found in at least four clones and two individuals are shown (sequences at lower abundances are grouped under 'other').
n.a., not applicable.

 

**Table 2.** Oligonucleotide probes used in this study (probes specific to the Gamma 1 and Epsilon 1 ectosymbionts in bold). Additional probe details are available at probeBase (Loy *et al.*, 2007).

| Probe | Specificity | Probe sequence (5′-3′) | Position[a] | FA [%][b] | Reference organism |
|---|---|---|---|---|---|
| EUBI-III | Eubacteria | GCW GCC WCC CGT AGG WGT | 338–355 | 40 | (Daims *et al.*, 1999) |
| NON338 | Antisense | ACT CCT ACG GGA GGC AGC | 338–355 | 40 | (Wallner *et al.*, 1993) |
| GAM42a | Gammaproteobacteria | GCC TTC CCA CAT CGT TT | 1027–1043[c] | 30–40 | (Manz *et al.*, 1992) |
| EP404 | *T. benedii* Epsilon 1 ectosymbiont and Epsilon 2 Epsilonproteobacteria (most Campylobacterales), various uncultured bacteria including associated bacteria of *C. squamiferum*, *R. exoculata ectosymbionts*, *R. pachytila* | AAA KGY GTC ATC CTC CA | 404–420 | 40 | (Macalady *et al.*, 2006) |
| **TbGAM1-138** | *T. benedii* Gamma 1 ectosymbiont associated bacteria of *V. osheai*, AB239761, *S. crosnieri*, e.g. AB440174, *K. hirsuta*, EU265799, vent clone AB464819 | GGC TTG TCC CCC ACT ACT | 138–155 | 40 | *Ectothiorhodospira mongolicum*, other *T. benedii* epibionts |
| **TbGAM1-444** | *T. benedii* Gamma 1ectosymbiont associated bacteria of *S. crosnieri*, e.g. AB440175, *K. hirsuta*, EU265799, some environment clones e.g. AM778459, FJ169979 | CTT AAC CCC TTC CTC ACA | 444–461 | 40 | n.d. |
| TbGAM2-447 | *T. benedii* Gamma 2, invertebrate burrow clones, FJ753075 and FJ753097 | AAG CTT AGG CTT TTC GTC | 447–464 | 40 | n.d. |
| **TbEP1-744** | *T. benedii* Epsilon 1 Epsilonproteobacteria, most *Sulfurovum*, including invertebrate associated clade, *C. squamiferum*, *V. osheai*, *A. pompejana* and *R. exoculata* Epsilon 1–5 ectosymbionts, few unculitvated, e.g. AF407203 | TCT CAG CGT CAG TAC TGT | 744–761 | 35 | n.d. |
| **TbEP1-94** | *T. benedii* Epsilon 1 ectosymbiont associated bacteria of *S. crosnieri*, e.g. AB440164, *Rimicaris* sp., FM203397-99, environ. clones most deep sea, e.g. FJ905659 | CCG TTC GCC ACT CGA CAG | 95–99 | 40 | *T. benedii* Gamma 1 |
| CF319a | Most Flavobacteria, some Bacteroidetes, some Sphingobacteria | TGG TCC GTG TCT CAG TAC | 319–336 | 40 | (Manz *et al.*, 1996) |
| DELTA495a competitor | Most Deltaproteobacteria and most Gemmatimonadetes | AGT TAG CCG GTG CTT CCT<br>AGT TAG CCG GTG CTT CTT | 495–512 | 40 | (Loy *et al.*, 2002) |

**a.** Position in the 16S rRNA of *Escherichia coli.*
**b.** Formamide concentrations used in the FISH and CARD FISH hybridization buffer in % (v/v).
**c.** Position in the 23S rRNA of *E. coli.*
nd, not determined.

 

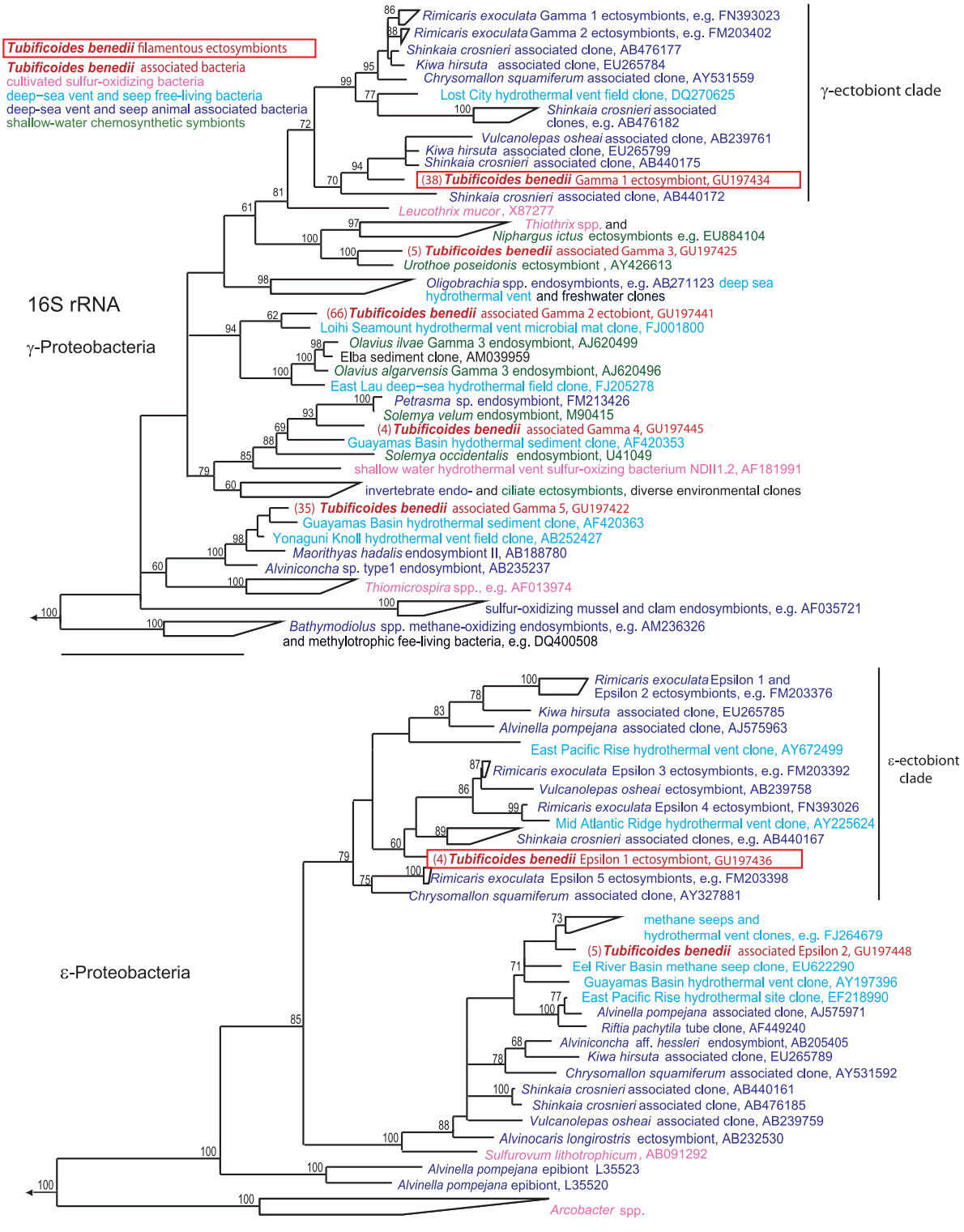


**Fig. 1.** Phylogenetic placement of Gamma- and Epsilonproteobacteria associated with *T. benedii* tail ends. Consensus tree based on maximum likelihood (ML) analyses of 16S rRNA sequences with nodes that differed in other treeing analyses collapsed to a consensus branch. Sequences from this study in red, in parentheses the number of sequences with > 99.0% to the given sequence. Bacterial sequences from the clone libraries that were not found with FISH on the worms were named '*T. benedii* associated'. Five deltaproteobacterial sequences were used as an outgroup (arrow). Scale bar = 0.10 estimated substitutions per site. Numbers next to nodes correspond to bootstrap values based on 1000 ML replicates (only values above 60% are shown).

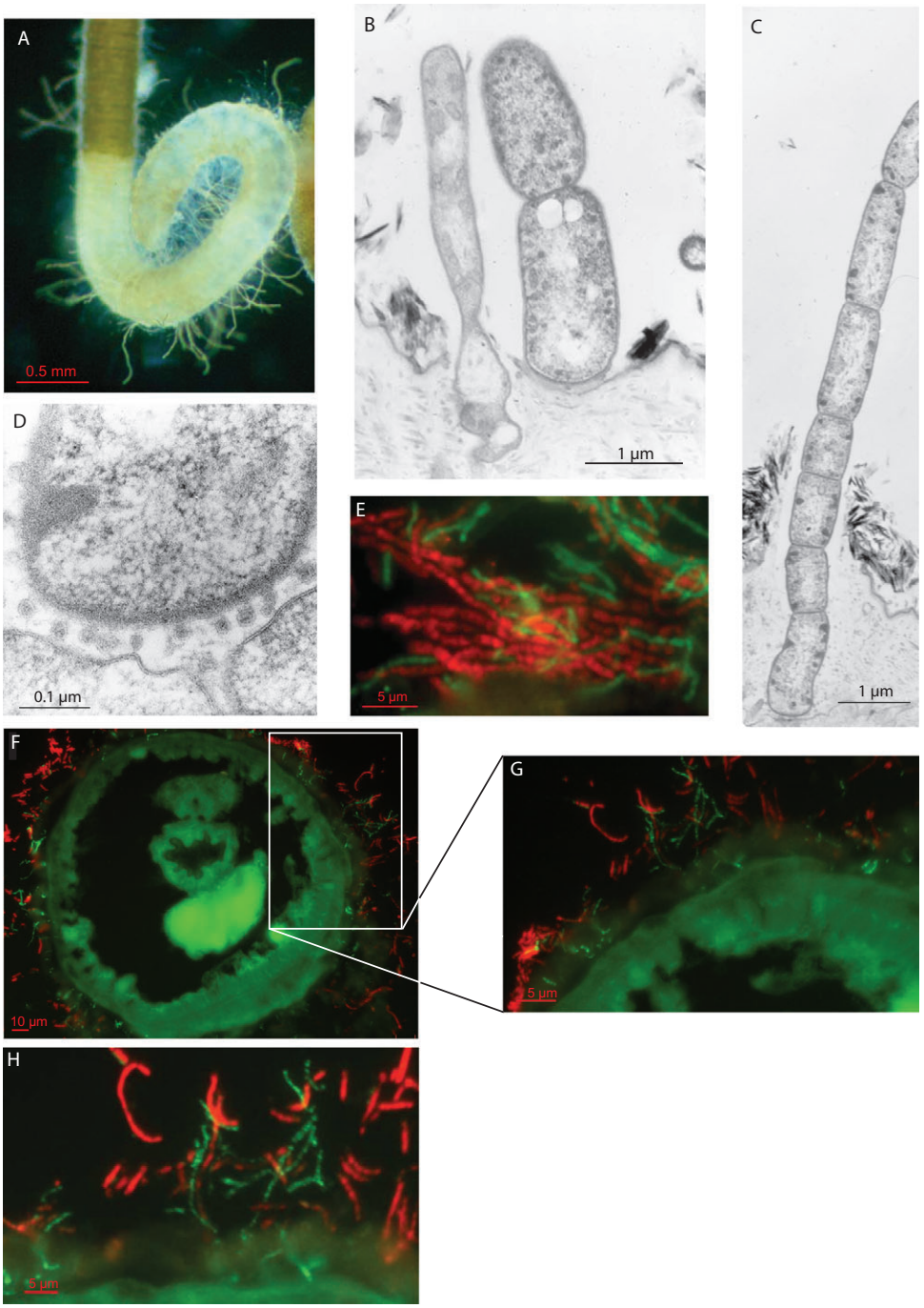


**Fig. 2.** Microbial community of *T. benedii* tail ends.
A. Light microscopy showing the numerous filaments on the worm's tail.
B–D. Transmission electron microscopy. Only the thinner filaments penetrate the cuticle (B, C, D), while the thicker filaments (B) are attached to the surface of the worm. D. Vesicle-like structure between the worm's epidermis and the basal cell of a thin filament.
E–H. Epifluorescence microscopy using FISH (E) and CARD-FISH (F–H). The thinner Epsilon 1 ectosymbionts are shown in green with the general epsilonproteobacterial probe EP404 (E) and the specific Epsilon 1 symbiont probe TbEP1-94 (F–H). The thicker Gamma 1 ectosymbionts are shown in red with the specific Gamma 1 symbiont probe TbGAM1-138 (E–H).

FISH analyses. The much lower abundance of Epsilon 1 sequences in the 16S rRNA clone libraries could have been caused by differences in the extraction efficiency of their DNA, primer mismatch and/or polymerase chain reaction (PCR) bias, as described from many other studies of mixed microbial communities (Gonzalez and Moran, 1997; Kanagawa, 2003; Sipos *et al*., 2007; Hong *et al*., 2009).

Phylogenetic analyses revealed that the Epsilon 1 ectosymbiont belonged to the newly established family Thiovulgaceae within the Epsilonproteobacteria (Campbell *et al*., 2006) and the Gamma 1 ectosymbiont to the *Leucotrix/Thiothrix* group within the Gammaproteobacteria (Fig. 1). Both *T. benedii* ectosymbionts belonged to clades consisting nearly exclusively of bacteria associated with invertebrate hosts from deep-sea hydrothermal vents (γ- and ε-ectobiont clades in Fig. 1). These hosts included the galatheid crab *Shinkaia crosnieri* (Watsuji and Takai, 2009), the Yeti crab *Kiwa hirsuta* (Goffredi *et al*., 2008), the scaly foot snail *Chrysomallon squamiferum* (Goffredi *et al*., 2004b), the stalked barnacle *Vulcanolepas osheai* (Suzuki *et al*., 2009), and the shrimp *Rimicaris exoculata* (Petersen *et al*., 2010). Filamentous ectobionts have been described from all of these hosts but a clear assignment of sequences from the γ- and ε-ectobiont clades to bacteria with a filamentous morphology has only been shown for *R. exoculata* (Petersen *et al*., 2010) and *V. osheai* (Suzuki *et al*., 2009). Free-living filamentous bacteria of the genus *Thiothrix* fall in a more distantly related but neighbouring clade to the *T. benedii* Gamma 1 ectosymbionts (Fig. 1), and within this clade filamentous ectosymbionts have been identified from two other animal hosts, the marine amphipod *Urothoe poseidonis* (Gillan and Dubilier, 2004) and the freshwater cave amphipod *Niphargus ictus* (Dattagupta *et al*., 2009).

The closest cultured relative of the *T. benedii* Gamma 1 ectosymbiont is the filamentous bacterium *Leucothrix mucor*, while the Epsilon 1 ectosymbiont is most closely related to the coccoid- to oval-shaped *Sulfurovum lithotrophicum* (Table 1, Fig. 1). Both *L. mucor* and *S. lithotrophicum* are sulfur oxidizers (Grabovich *et al*., 1999; Inagaki *et al*., 2004) and there is evidence for autotrophy and the use of reduced sulfur compounds as energy sources by the filamentous ectobionts of *R. exoculata* (Polz *et al*., 1998) and *N. ictus* (Dattagupta *et al*., 2009). The close relationship of the *T. benedii* ectosymbionts to symbiotic and free-living chemosynthetic bacteria and the sulfidic environment of the worms therefore led us to examine the potential of the *T. benedii* association to gain its energy through chemoautotrophic sulfur oxidation.

### *Metabolic potential for chemoautotrophy and sulfur oxidation*

The metabolic potential of the *T. benedii* bacterial community was assessed by analysing protein-coding genes for sulfur metabolism (*aprA* and *soxB*) and chemoautotrophy (*cbbl* and *aclB*) (Table 1). The *aprA* gene, coding for the alpha-subunit of adenosine-5′-phosphosulfate (APS) reductase, is widespread in sulfur-oxidizing microorganisms including many Gammaproteobacteria, but has not yet been found in Epsilonproteobacteria (Meyer and Kuever, 2007). The majority of AprA sequences from the *T. benedii* microbial community (95% or 231 clones) belonged to the AprA lineage II of sulfur-oxidizig bacteria that includes sequences from both symbiotic and free-living Gammaproteobacteria (Meyer and Kuever, 2007). Two sequences were highly abundant within this lineage (*T. benedii* AprA II2 and II4) and both were closely related to AprA sequences from the sulfur-oxidizing gammaproteobacterial endosymbionts of tubeworms from cold seeps (Table 1, Fig. 3). Given the absence of the *aprA* gene from Epsilonproteobacteria, it is most likely that the AprA lineage II sequences from *T. benedii* originated from their gammaproteobacterial ectobionts, thus indicating their potential for the use of reduced sulfur compounds as an energy source. However, because of the incongruence of AprA phylogeny with 16S rRNA phylogeny, it is not clear which AprA sequence(s) might have originated from the *T. benedii* Gamma 1 ectosymbiont.

A small number of *T. benedii* AprA sequences (4%) belonged to the lineage of AprA sequences from sulfate-reducing bacteria (Fig. 3). In sulfate reducers, the enzyme functions in the opposite direction as in sulfur oxidizers (Meyer and Kuever, 2007). The presence of AprA sequences in *T. benedii* from the sulfate reducer lineage corresponds well with our 16S rRNA results showing that deltaproteobacterial sequences related to sulfate-reducing bacteria were abundant in the clone libraries (20%, see Table 1 and Fig. S2). Sulfate-reducing bacteria are well known as endosymbionts in gutless marine oligochaetes where they co-occur with sulfur-oxidizing endosymbionts and engage in syntrophic cycling of reduced and oxidized sulfur compounds (Dubilier *et al*., 2001; Woyke *et al*., 2006). Evidence for co-occurring sulfur-oxidizing and sulfate-reducing bacteria has also been described for the ectosymbiotic community of the Yeti crab *K. hirsuta* (Goffredi *et al*., 2008).

To determine the potential of the Epsilon 1 ectosymbiont for sulfur oxidation, we attempted to amplify the *soxB* gene, coding for the SoxB component of the Sox enzyme complex. This gene is widespread among sulfur-oxidizing bacteria and has been found in all epsilonproteobacterial and some gammaproteobacterial sulfur oxidizers (Meyer *et al*., 2007; Ghosh *et al*., 2009). We were not able to amplify this gene from DNA extracted from *T. benedii* tail ends, despite the use of degenerate primers that work well for a phylogenetically wide range of bacteria (Petri *et al*., 2001; Meyer *et al*., 2007). It is possible that the concentrations of Epsilon 1 ectosymbiont DNA were too low for successful amplification. This symbiont was underrepresented in the 16S rRNA clone libraries (1.3%, Table 1), despite our FISH studies showing its high abundance (Fig. 2E,H).

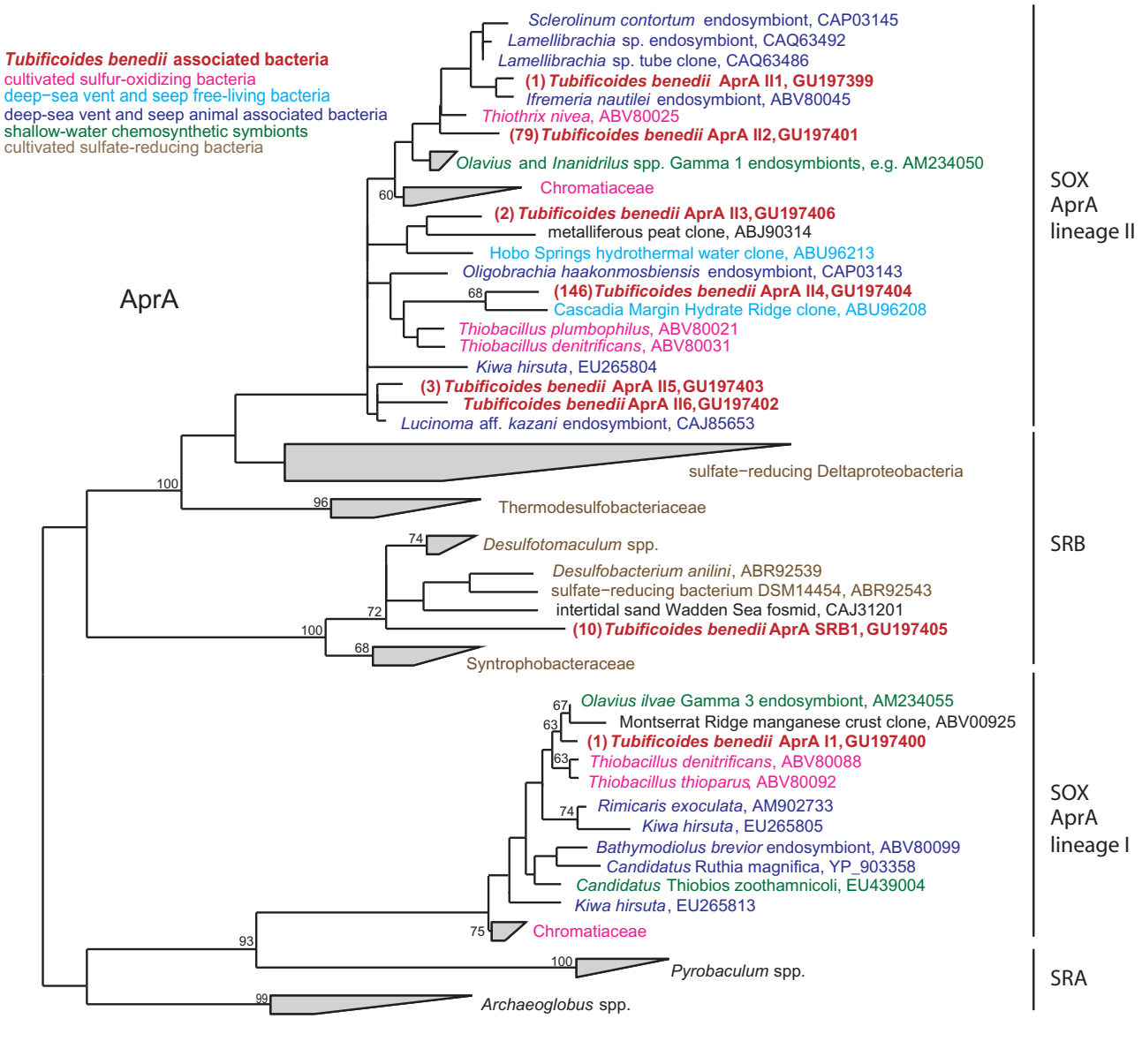


**Fig. 3.** AprA phylogeny based on ML analyses of deduced amino acid sequences of the *aprA* gene. Sequences from this study in red, in parentheses the number of sequences with > 95.0% to the given sequence. Scale bar = 0.10 estimated substitutions per site. Numbers next to nodes show bootstrap values based on 100 ML replicates (only values above 60% are shown). SOX, sulfur-oxidizing bacteria; SRB, sulfate-reducing bacteria; SRA, sulfate-reducing archaea.

We assessed the potential of the *T. benedii* microbial community for autotrophic carbon fixation by analysing key genes of the Calvin Benson Bassham (CBB) cycle (*cbbL* and *cbbM*) and the reductive tricarboxylic acid (rTCA) cycle (*aclB*). The *cbbL* and *cbbM* genes, coding for the large subunits of the form I and II ribulose-1,5-bisphosphate carboxylase/oxygenase (RubisCO), respectively, are widespread among autotrophic organisms including many Gammaproteobacteria, but have not been found in Epsilonproteobacteria. Both genes could be amplified in DNA extracted from *T. benedii* tail ends, indicating the potential of its microbial community for autotrophy. The dominant sequences in the *cbbL* clone libraries were most closely related to sequences from the chemoautotrophic sulfur-oxidizing endosymbiont of the clam *Solemya velum*, while for *cbbM*, the dominant sequences grouped with sequences from bacteria associated with the seep tubeworm *Lamellibrachia* sp. (Fig. 4, Table 1). As with the *aprA* gene, given the many inconsistencies between phylogenetic trees based on *cbbL* and

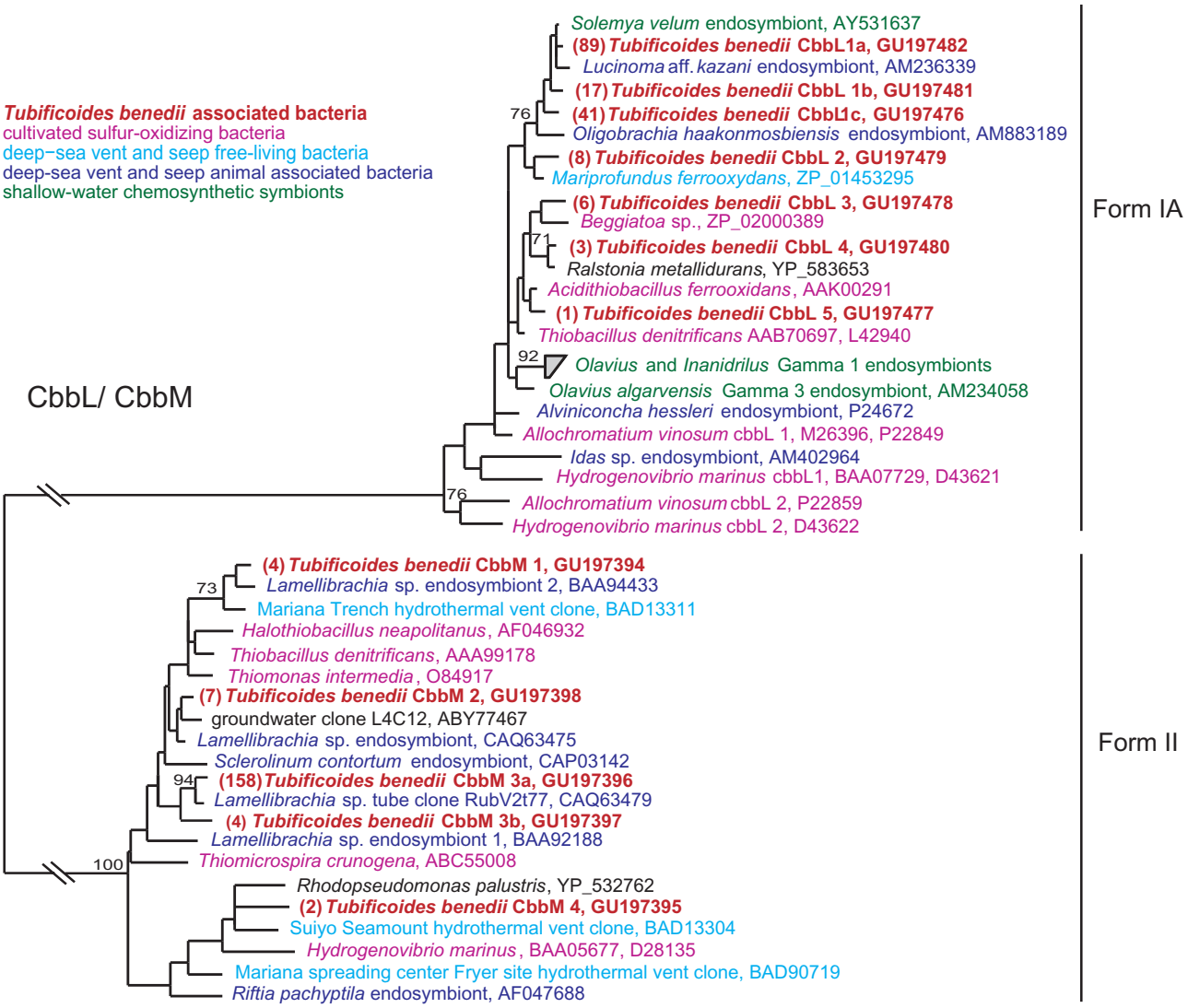


**Fig. 4.** RubisCO consensus tree based on ML analyses of deduced amino acid sequences of *cbbL* and *cbbM* genes (sequences from this study in red, in parentheses the number of sequences with > 95.0% to the given sequence). Scale bar = 0.10 estimated substitutions per site. Numbers next to nodes show to bootstrap values based on 100 ML replicates (only values above 60% are shown).

*cbbM* genes versus the 16S rRNA gene, it is not possible to identify which of these genes might have originated from the Gamma 1 ectosymbiont.

To examine the potential of the Epsilon 1 ectosymbiont for autotrophy, we examined the *aclB* gene, coding for the beta subunit of ATP citrate lyase. This gene is widespread in Epsilonproteobacteria including those commonly found at deep-sea hydrothermal vents but is not known from Gammaproteobacteria (Campbell *et al*., 2006; Nakagawa and Takai, 2008). We were not able to amplify this gene from DNA extracts of *T. benedii* tail ends, paralleling the lack of amplification products for the indicator gene *soxB* for sulfur oxidation in Epsilonproteobacteria. The metabolism of *T. benedii's* epsilonproteobacterial ectobionts therefore remains unclear. Epsilonproteobacteria are highly versatile and can live autotrophically, as well as mixo- or heterotrophically (Campbell *et al*., 2006). In the organic and sulfide-rich sediments in which *T. benedii* lives, any of these metabolisms are conceivable.

### *Nature of the* T. benedii *ectosymbiont association*

The ectosymbiotic community associated with *T. benedii* has the potential for chemoautotrophic sulfur oxidation and it is possible that the symbionts fix inorganic carbon to organic carbon compounds that they pass on to their host. This has been suggested (but rarely proven) for other hosts with chemoautotrophic ectosymbionts such as stilbonematinid nematodes (Polz *et al*., 1992; Ott *et al*., 2004), the vent shrimp *R. exoculata* (Rieley *et al*., 1999;

Zbinden and Cambon-Bonavita, 2003), the vent barnacle *V. osheai* (Suzuki *et al*., 2009), the vent crab *S. crosnieri* (Watsuji and Takai, 2009) and the cave amphipod *N. ictus* (Dattagupta *et al*., 2009). As mixo- or heterotrophs, the *T. benedii* ectosymbionts could use the waste products the host excretes during anaerobic metabolism such as succinate, acetate and propionate (Dubilier *et al*., 1994) and recycle these back to the host as suggested for gutless oligochaetes (Dubilier *et al*., 2001; Woyke *et al*., 2006). They could also take up organic compounds directly from the environment and pass these or essential amino acids and vitamins to the host. However, *T. benedi* has a fully functional digestive system and like other aquatic oligochaetes ingests sediment and the organic material and microorganisms therein to gain nutrition. It is therefore hard to imagine that its ectobiotic community can play an important role in its nutrition in comparison to the organic matter available in its surroundings. Furthermore, only the basal cells of the filamentous ectosymbionts are in direct contact with the worm, and thus provide very little surface area for the exchange of organic compounds.

Another function hypothesized to play a role in symbioses with sulfur-oxidizing bacteria is that these detoxify hydrogen sulfide, a potent inhibitor of aerobic respiration, for their hosts (Somero *et al*., 1989; Dattagupta *et al*., 2009). However, our calculations show that diffusion rates of sulfide through the body wall of the worms are so fast that bacterial sulfide oxidation would not be able to outcompete it (Dubilier *et al*., 1995a). Finally, it is also unlikely that the ectosymbionts are involved in pathogenic interactions, as we saw no differences in the vitality of worms lightly and heavily covered with the filaments in this study and an earlier one (Dubilier, 1986).

While the benefit of the ectosymbionts for *T. benedii* remains unclear, the advantages for these in associating with the worm are obvious. The sulfur-oxidizing ectosymbionts have access to an ideal environment on the worm's tail with access to sulfide rising up from the sediments and oxygen available from the water above the sediment surface. There is a strong selective advantage for sulfur-oxidizing bacteria in associating with animals that can provide them with both sulfide and oxygen. For example, Røy and colleagues (2009) showed that the sulfur-oxidizing symbionts of the ciliate *Zoothamnium niveum* take up 100 times more sulfide than bacteria on flat inert surfaces. This selective advantage is so strong that these types of associations have evolved multiple times in numerous lineages of sulfur-oxidizing bacteria and in a wide array of host groups (Dubilier *et al*., 2008). For the mixo- and heterotrophic members of the *T. benedii* microbial community, the association with an animal that excretes large amounts of carbon and nitrogen waste compounds provides a rich source of nutrition, and this advantage is hypothesized to play a role in many associations between bacteria and marine invertebrates (Carman and Dobbs, 1997; Robidart *et al*., 2008).

### *Specificity of the association*

The *T. benedii* Gamma 1 and Epsilon 1 ectosymbionts belong to clades that consist almost exclusively of bacteria associated with hydrothermal vent invertebrates. This suggests that bacteria from these clades have developed an adaptive trait that enables them to easily colonize marine invertebrates. In the symbiotic associations between bioluminescent *Vibrio fischeri* and their marine hosts, Mandel and colleagues (2009) hypothesize that only a single regulatory gene was needed to confer free-living *V. fisheri* with the ability to colonize their hosts, possibly by 'switching "on" pre-existing capabilities for interacting with an animal'. Intriguingly, the closest cultured relative to the *T. benedii* Gamma 1 ectosymbiont, the filamentous sulfur oxidizer *L. mucor*, forms a basal node to the γ-ectobiont clade (Fig. 1). *Leucothrix mucor* has been described from surfaces as diverse as marine algae, fish eggs, and dead and live aquatic invertebrates (Johnson *et al*., 1971; Sieburth, 1975; Carman and Dobbs, 1997; Payne *et al*., 2007), suggesting a less specific interaction between these bacteria and the surfaces they colonize. This could indicate that there is a progression from these possibly ancestral *L. mucor* associations to the highly stable and specific associations within the γ-ectobiont clade. However, given that most studies used only morphological characteristics to identify the filamentous epibionts as *L. mucor*, unambiguous identification through 16S rRNA sequencing and FISH is needed to test this hypothesis.

### *Conclusions*

Symbiotic associations with Epsilonproteobacteria have only been described from deep-sea invertebrates, making *T. benedii*, to our knowledge, the first invertebrate from shallow environments with symbiotic Epsilonproteobacteria. *Tubificoides benedii* is an opportunistic species that is well adapted to estuaries and mudflats with organic-rich sediments and rapid environmental fluctuations, including low oxygen and high sulfide concentrations (Giere, 2006). Similarly, Epsilonproteobacteria have been described as uniquely suited to thrive in extreme environments such as deep-sea hydrothermal vents, where they can rapidly colonize dynamic environments with suboxic to anoxic conditions (Campbell *et al*., 2006). Thus, *T. benedii* and its epsilonproteobacterial ectosymbionts make good partners in sharing similar ecological niches.

Symbiotic associations with Gammaproteobacteria are widespread in both shallow-water and deep-sea chemosynthetic environments (Dubilier *et al*., 2008). However,

the *T. benedii* Gamma 1 ectosymbiont belongs to a clade that consists exclusively of symbiotic and free-living bacteria from deep-sea hydrothermal vents, while the chemosynthetic symbionts of other hosts from shallow waters belong to clades distant from the γ-ectobiont clade (Fig. 1). Thus, *T. benedii* is, to date, unique in its symbiotic association with gamma- and epsilonproteboacterial symbionts related to those from deep-sea hydrothermal vents.

The discovery of the *T. benedii* ectosymbiosis shows that factors other than biogeography and host affiliation must have been the driving force behind the associations within the γ- and ε-ectobiont clades. Instead, the environment appears to have been crucial for the establishment and evolution of these ectosymbiotic associations, namely highly dynamic environments with strong fluctuations of oxidants and reductants. We hypothesize that symbioses with bacteria from these clades may be more widespread in shallow-water environments such as sulfide-rich intertidal mud flats than currently recognized. Morphological and molecular analyses of ectobionts from coastal marine sediments combined with analyses of their metabolic potential will be useful in providing a better understanding of the factors defining distribution patterns and function of associations between marine hosts and their symbiotic bacteria.

## Experimental procedures

### *Specimen collection*

*Tubificoides benedii* were collected in 1998 from Wadden Sea sediments at the Lister Haken in the Königshafen Bay on the Island of Sylt (55.03 N 8.10 E). The collection site is characterized by eutrophication and in the warmer months, massive green algal mats cover the sediment and lead to high sulfate reduction rates and sulfide concentrations (Kristensen *et al*., 2000; Reise and Kohlus, 2008).

To ensure as little contamination as possible, worms were allowed to defecate their gut contents before fixation, and only worms with visibly clear guts were used. Individual specimens were rinsed three times in 0.2 μm filtered seawater and fixed as described below.

### *Electron microscopy*

For TEM specimens were fixed and prepared as described previously (Dubilier, 1986).

### *Cloning and sequencing of 16S rRNA,* aprA*,* soxB*,* cbbL*,* cbbM *and* aclB *genes*

The tail ends of four *T. benedii* individuals were prepared individually for PCR as described previously (Dubilier *et al*., 1999) using the isolation protocol of Schizas and colleagues (1997). Briefly worms were digested with Proteinase K and DNA was extracted with Gene Releaser (BioVentures, Murfreesboro, TN, USA). Amplification, cloning and sequencing of the 16S rRNA, *cbbL*, *cbbM* and *aprA* genes was carried out as described previously (Blazejak *et al*., 2006) with the following modifications: only 28–30 cycles and an extra reamplification procedure of five cycles. The annealing temperature for *cbbL* was set to 56°C instead of 48°C, for *aprA* to 58°C instead of 54°C. For the amplification of *cbbM*, the primers cbbMF_Els (Elsaied and Naganuma, 2001) and a modified cbbM1R (Blazejak *et al*., 2006) with the sequence 5′ MGA GGT SAC SGC RCC RTG RCC RGC MCG RTG 3′ were used with an annealing temperature of 62°C. PCR of the *soxB* gene was carried out with soxB1446b and soxB432f with an annealing temperature of 47°C as described (Petri *et al*., 2001). For *aclB* the primer combinations used were aclB892F or aclB275F with aclB1204R with an annealing temperature of 42°C (Campbell *et al*., 2003; Takai *et al*., 2005).

For all genes, PCR products were purified with the QIAquick PCR purification kit (QIAGEN, Hilden, Germany) and cloned using the pGEM-T/pGEM-T Easy Kit (Promega, Madison, WI, USA) or the TOPO Kit (Invitrogen, Paisley, UK) according to the manufacturers' protocols. Plasmid DNA was purified from overnight cultures using the QIAprep plasmid kit (QIAGEN, Hilden, Germany). Clones with correct insert size were partially sequenced (approximately 450–900 bp) and grouped according to phylogentic positioning and similarity values from a distance matrix in ARB (Ludwig *et al*., 2004).

Sequencing reactions were run on the ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). For each host individual, at least one representative clone from each dominant clone group was fully sequenced in both directions. Full sequences within each clone group shared at least 99.0% sequence similarity (% identical nucleotides) for the 16S rRNA gene and at least 95% sequence similarity (% identical amino acids) for the protein coding genes. Closely related sequences of representative sequences were identified with BLAST (Altschul *et al*., 1990) queries and through phylogenetic analyses.

### *Phylogenetic analyses*

16S rRNA chimeras were identified using CHIMERA_CHECK from the Ribosomal Database Project (Cole *et al*., 2007) and by eye in sequence alignments, and trimmed or excluded from further analyses. Alignments were based on the 16S rRNA secondary structure without partitioning into stem and loops (Pruesse *et al*., 2007). Phylogenetic analyses were performed with the ARB program (Ludwig *et al*., 2004) and the online version of RaxML (Stamatakis *et al*., 2008). For the 16S rRNA gene, phylogenetic trees were calculated with sequences no shorter than 1300 bp using neighbour-joining, parsimony and maximum likelihood (ML, HKY substitution model) as well as RaxML. Group filters for ML calculations (25%-, 30%-, 40%- and 50%-filters) were constructed from published gamma- and epsilonproteobacterial sequences in ARB. Bootstrap values for the gamma- and epsilonproteobacterial 16S rRNA trees were based on 1000 ML bootstraps calculated with ARB. Short sequences in the CFB and deltaproteobacterial 16S rRNA trees (Figs S1 and S2) were added to the ML-tree using parsimony with a positional variability filter, bootstraps resulted from whole tree ML calculations.

Phylogenetic trees of protein-coding genes were generated from translated gene sequences of 131 (*aprA*), 133 (*cbbM*) and 230 (*cbbL*) amino acids using the ML algorithm and a JTT model with a 25% positional conservation filter. Trees were reconstructed using the standard operating procedure for phylogenetic inference SOPPI (Peplies *et al*., 2008), by visually comparing different methods, parameters and filters to identify the most stable tree topologies. All trees shown in this study were constructed based on ML analyses with nodes that were not stable (i.e. differed in more than two methods) collapsed to a consensus branch.

### *Accession numbers*

The sequences from this study are available through GenBank under the accession numbers GU197394-GU197482.

### *Fluorescence* in situ *hybridization (FISH)*

Worm tail ends were fixed, embedded and sectioned as described previously for gutless oligochaetes (Dubilier *et al*., 1995b) with the omission of the redundant postfixation step with 4% paraformaldehyde during the rehydration process. Sections of *T. benedii* posterior ends were prepared for FISH with monolabeled cy3 and cy5 probes and catalyzed reporter deposition (CARD) FISH with horseradish peroxidase labeled probes and tyramide signal amplification (with the fluorescent dyes Alexa 488 and 633) as described previously (Blazejak *et al*., 2006) with the following modifications: To decrease the loss of FISH signal in the mucous layer of the worm, additional digestion procedures were added. After 12 min of 0.3 M HCl, instead of a 5 min digestion with Proteinase K, the slides were immersed for 30 min to 1 h in 0.1% lysozyme in 0.1 M Tris/HCl / 0.05 M EDTA, 30 min to 1 h in 0.005% amylase in 1 × PBS (60 U $ml^{-1}$) and 5 min in 0.0005% Proteinase K in 20 mM Tris/HCl (all enzyme incubations at 37°C). Washing for CARD-FISH in 1 × SSC buffer was increased to 1 h. Probe concentrations in the hybridization buffer were 3.3 ng $\mu l^{-1}$ in FISH and 0.05 ng $\mu l^{-1}$ in CARD-FISH)

Specific and group oligonucleotide probes targeting the dominant gamma- and epsilonproteobacterial 16S rRNA sequences found in *T. benedii* clone libraries (Table 1) were created with the ARB program and checked against sequences in GenBank with BLAST and in the RDP database with Probe Match (Cole *et al*., 2007) (Table 2). The specificity of the ectosymbiont probes was tested against reference bacteria with 16S rRNA sequences containing one or more mismatches unless otherwise noted. General probes for the Bacteria, Gammaproteobacteria, Deltaproteobacteria and the CFB served as positive controls and the nonsense probe NON338 as a negative control. Hybridizations were performed at formamide concentrations ensuring specificity for the targeted groups (Table 2). Images were recorded with the Axiovision camera (Zeiss) and optimized with the accompanying program AxioVision LE 4.5.

## Acknowledgements

We thank Sabine Lenk for her help with *soxB* amplifications, and Silke Wetzel and Lisa Drews for excellent technical assistance. We are grateful to Claudia Bergin, Jan Küver and Sabine Lenk for fruitful discussions. This work was supported by the Max Planck Society, Munich, Germany.

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## Supporting information

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

**Fig. S1.** Phylogenetic placement of Cytophaga/Flavobacterium/Bacteroides 16S rRNA bacterial sequences from *T. benedii* tail ends based on ML analyses (sequences from this study in red, in parentheses the number of sequences with > 99.0% to the given sequence). Scale bar = 0.10 estimated substitutions per site. Numbers next to nodes correspond to bootstrap values based on 100 ML replicates (only values above 60% are shown).

**Fig. S2.** Phylogenetic placement of deltaproteobacterial 16S rRNA sequences from *T. benedii* tail ends based on ML analyses (sequences from this study in red, in parentheses the number of sequences with > 99.0% to the given sequence). Five sphingobacterial sequences were used as an outgroup (arrow). Scale bar = 0.10 estimated substitutions per site. Numbers next to nodes correspond to bootstrap values based on 100 ML replicates (only values above 60% are shown).

**Fig. S3.** CARD-FISH images of epibacteria on *T. benedii* tail ends.

A. Cross section through the tail end showing the thick layer of bacteria covering the surface of the worm (eubacterial probe EUBI-III).

B. The general gammaproteobacterial probe (GAM42a, red) hybridized with rods and cocci in the mucus membrane, while the Gamma 1 ectosymbionts hybridized with specific probes (shown in green: TbGAM1-138) but not with the GAM42a probe.

C. The *T. benedii* Gamma 2 ectobionts (hybridized with the specific probe TbGAM2-447, green) were cocci-shaped and found occasionally in the mucous membrane. D.-E. Deltaproteobacteria (probe DELTA495a, red) and Bacteroidetes (probe CF319a, green) populated the worm's mucous layer. Deltaproteobacteria occurred singly as rods in groups of coccoid or oval shaped cells. Bacteroidetes mostly occurred in patches within the mucus and were often rod-shaped, and sometimes elongated or filamentous.

**Table S1.** Additional oligonucleotide probes used in this study that did not result in reproducible clear signals.

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