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Novel Epibiotic *Thiothrix* Bacterium on a Marine Amphipod

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Comparative analysis of the 16S rRNA gene and fluorescent in situ hybridization (FISH) was used to identify epibiotic filamentous bacteria living on the marine amphipod crustacean *Urothoe poseidonis*. The epibionts belong to the gamma proteobacteria and represent a novel marine phylotype within the genus *Thiothrix*. FISH and denaturing gradient gel electrophoresis revealed that the *Thiothrix* filaments are present on the majority of the amphipods examined.

Bacteria of the genus *Thiothrix* are filamentous sulfur-oxidizing organisms that produce gliding gonidia, form holdfast structures called rosettes, and deposit intracellular sulfur granules when grown in the presence of sulfide or thiosulfate (12, 18). *Thiothrix* spp. have been described from a number of habitats, ranging from sulfide-containing natural waters and irrigation systems (3, 4, 18) to activated-sludge wastewater treatment plants, where they contribute to the problem of filamentous sludge bulking (15, 21).

Some authors have suggested that *Thiothrix* spp. can also occur as epibionts on aquatic invertebrates such as mayfly larvae (17), tadpoles (7), hydrothermal vent organisms (14), intertidal sediment dwellers (9, 22), and the sea urchin *Echinocardium cordatum* (2, 26). However, these *Thiothrix*-like bacteria were mainly identified on the basis of their morphology (2, 7, 9, 17) or by using immunological methods (2). It is now known that morphology alone is of little use in identifying *Thiothrix* spp. because supposed defining features such as rosettes and gonidia are also found in other filamentous bacteria (12). To illustrate that morphology can be misleading, a recent report demonstrated that the filamentous bacteria in the digestive tract of *E. cordatum*, assumed for years to be *Thiothrix*-like bacteria (2, 26), are in fact *Desulfonema* spp. (28).

Comparative sequence analysis of the 16S rRNA gene has revealed that the true members of the genus *Thiothrix* form a well-defined monophyletic group within the gamma subdivision of the *Proteobacteria* (12, 15, 24, 27). To date, none of the *Thiothrix* spp. within this clade have been isolated from marine habitats or have been described as epibionts of aquatic invertebrates.

Urothoe poseidonis is a small amphipod crustacean (±4 mm in length) that lives freely in marine sediments or as a commensal in the burrows of various endofaunal invertebrates (10, 16, 29). Three morphotypes of filamentous bacteria have been described as epibionts on the walking appendices (the fifth pair of pereopods) of *U. poseidonis*, one of which shows the typical *Thiothrix* morphology (10). To date, these *Thiothrix*-like bacteria could not be cultivated using the various media described earlier for *Thiothrix* spp. (30). The aim of the present work is to identify these *Thiothrix*-like bacteria living on *U. poseidonis* by using molecular methods and in situ hybridization.

16S rRNA cloning and phylogenetic analysis. Individuals of *U. poseidonis* were collected from burrows of the sea urchin *E. cordatum* at Wimereux, Pas de Calais, France, in January of 2002. The bacteria from several amphipods were pooled and DNA was extracted using the freeze-thaw method (11). The PCR products, amplified with the bacterial primers 8F and 1492R for the 16S rRNA gene (5), were cloned into the pCR4-TOPO vector (Invitrogen). Thirty-seven clones were partially sequenced with the bacterial primer 518F (5) on an ABI Prism 3100 genetic analyzer and submitted to BLAST (20). Only one partial sequence grouped with known *Thiothrix* species, clone UP23b. This clone was sequenced completely (1,464 bp) by using the described bacterial primers for the 16S rRNA gene (5). The complete UP23b sequence was then aligned manually to the 16S rRNA sequences of close evolutionary relatives by using the sequence alignment editor SeqPup v0.6f (8). Phylip v.3.6a3 (6) was used to estimate maximum-likelihood trees on the basis of a data matrix of 1,265 characters (we used empirical base frequencies, a Ti/Tv ratio of 2, one category of unweighted sites, and a constant rate of variation among sites). Parsimony and maximum-likelihood bootstrap analyses were performed with 100 replicates using Phylip and the ARB software package (19). As shown in Fig. 1, clone UP23b grouped with other known *Thiothrix* species with bootstrap values of 99% (parsimony) and 77% (maximum likelihood). The highest similarity value observed was between clone UP23b and *Thiothrix eikelboomii* AB042819 (93.2%). The 16S rRNA sequence signature of the genus *Thiothrix*, a characteristic deletion in the stem-loop structure corresponding to positions 455 to 477 (*Escherichia coli* numbering), was found in clone UP23b (12).

Probe design and fluorescent in situ hybridization (FISH). Oligonucleotide probes were constructed using the PROBE_DESIGN tool of the ARB software package (13, 19). The designed probe (UP23b: 5′-CTCGGCATCCTGTCCACG-3′) targeted positions 1033 to 1050 (*E. coli* numbering) of the 16S rRNA molecule and had at least one central mismatch to all other known 16S rRNA sequences as determined by Check Probe 2.1r from the Ribosomal Database Project II (http://rdp.cme.msu.edu/). The probe was labeled at the 5′ end with the indocarbocyanin dye CY3. For FISH, whole animals were fixed

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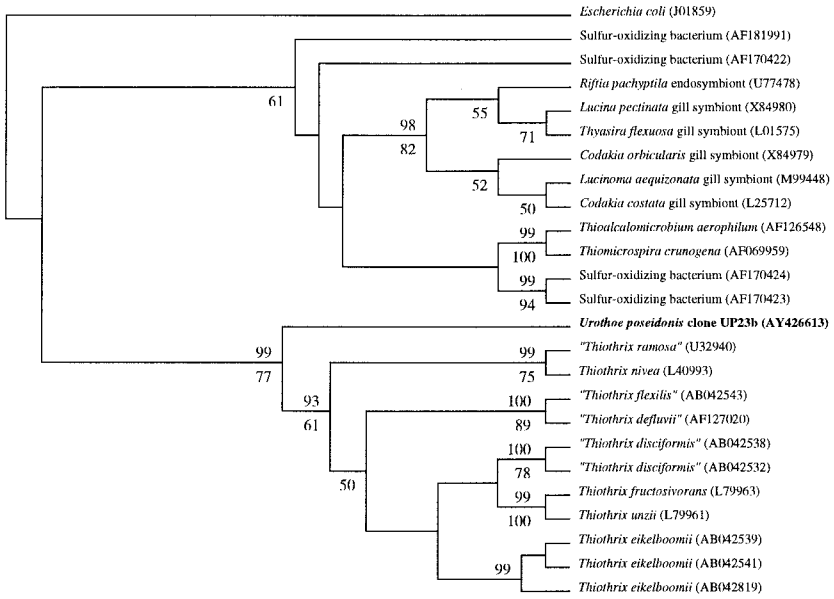

FIG. 1. Phylogenetic relationship of the *U. poseidonis* clone 23b based on comparative 16S rRNA sequence analysis using maximum likelihood (matrix of 1,265 nucleotides). Branch lengths do not represent the true distances in the consensus tree. Members of the gamma subdivision of the *Proteobacteria* are shown and GenBank accession numbers are listed for all sequences. Bootstrap values above 50% (obtained with 100 resamplings) are shown, with upper and lower values representing those from parsimony and maximum likelihood, respectively. *E. coli* served as the outgroup.

in 4% paraformaldehyde, rinsed, and kept in ethanol-sterile seawater (50:50) at −20°C. Pereopods with the attached bacteria were then separated, placed in 500 μl of ethanol-sterile seawater, and sonicated briefly. Five microliters of the suspension were deposited on gelatin-coated slides, hybridized at 46°C, and viewed as described elsewhere (23). Hybridizations were performed under stringent conditions with 60% formamide in the hybridization buffer. The probes EUB338, NON338, and GAM42a were used as positive and negative controls (1). In situ hybridization with probe UP23b yielded a strong fluorescent signal (Fig. 2). The labeled filaments were composed of disk-like cells of about 3 μm in diameter and about 1 μm in length. *Thiothrix* filaments were detected on 7 out of 10 amphipods examined. The *Thiothrix* filaments, which were also labeled with the EUB338 and GAM42a probes, were never abundant on individual amphipods (0 to 5 filaments of about 25 to 300 μm in length on each pereopod no. 5). Although other morphotypes of epibiotic bacteria are known to occur on *U. poseidonis* (10), only the *Thiothrix*-like filaments hybridized to the UP23b probe. Epibiotic filamentous bacteria have also been described from the shells of the bivalve *Montacuta ferruginosa*, another invertebrate living in the burrow of *E. cordatum* (9, 11), and have been reported to occur in the nodules of the digestive tube of *E. cordatum* (2, 26). These filamentous bacteria did not hybridize with the UP23b probe. Furthermore, *Thiothrix*-like filaments were never found in the sediments of the *E. cordatum* burrow. These results indicate that at least at the site investigated in this study, the *Thiothrix* phylotype is only associated with *U. poseidonis* and does not occur in or on other invertebrates or free-living in the sediment.

Denaturing gradient gel electrophoresis (DGGE). DNA from 10 individuals (5 males, 5 females) was extracted as described above. PCR products, amplified with the bacterial primers GM5F-GC-clamp and 518R (11), were separated on 25 to 75% gradient gels (11). DGGE analysis revealed a complex epibiotic microbial community. Of the 26 band positions

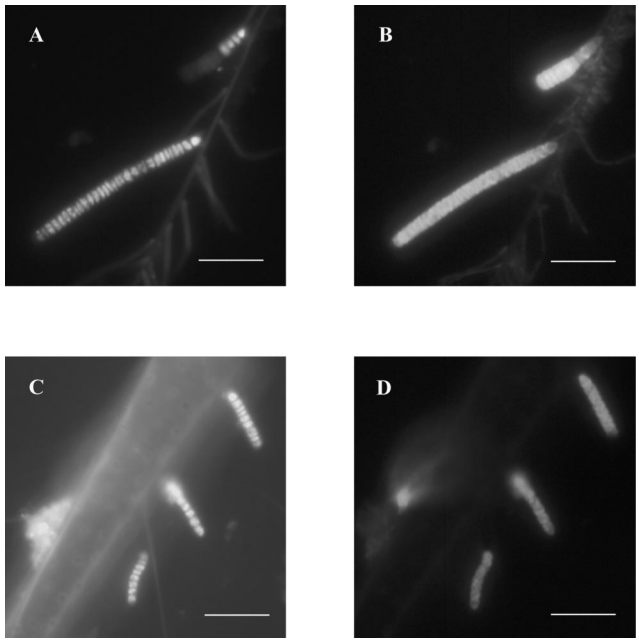

FIG. 2. FISH of the *Thiothrix*-like bacteria from *U. poseidonis* with the specific probe UP23b. The filamentous bacteria are attached to setae (A and B) or to spines (C and D) of the amphipod. Identical microscopic fields were visualized with an epifluorescence microscope using filter sets specific for DAPI (4′,6′-diamidino-2-phenylindole) (A and C) and the CY3 fluorochrome (B and D). Scale bars, 10 μm.

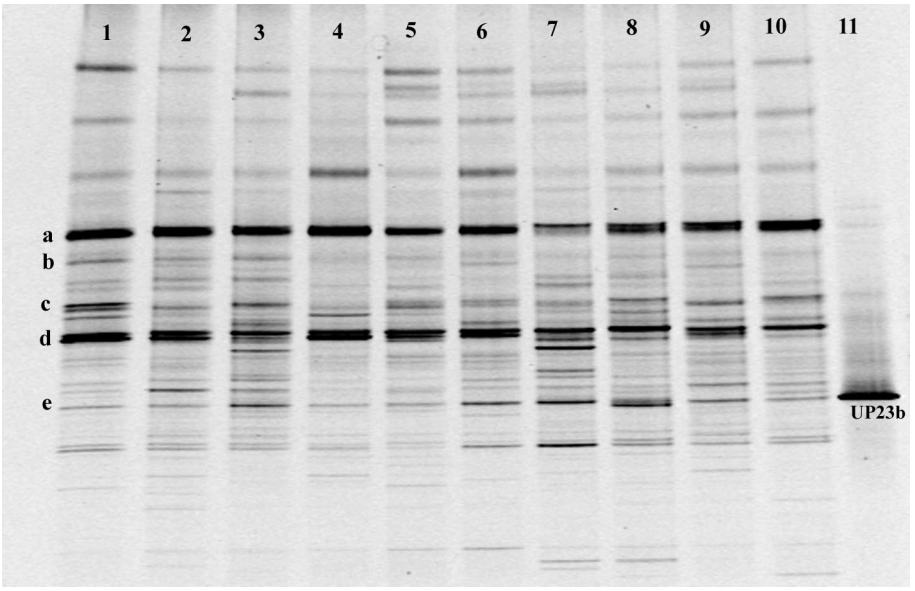

FIG. 3. DGGE of PCR-amplified 16S rDNA fragments from the epibiotic microbial communities of 10 amphipods (lanes 1 to 10). The same 16S rDNA fragment was amplified by PCR for clone UP23b (lane 11). The five DGGE bands that are present in every profile are indicated on the left (a to e).

detected with DGGE, only 5 bands were present in all 10 DGGE profiles (Fig. 3, bands a to e). These could represent common epibiotic bacteria but could also represent seawater bacteria. Band e migrated to the same position as clone UP23b. To check the identity of band e, it was excised from three DGGE profiles and reamplified using primer GM5F without a GC clamp (11). The sequences of band e were 100% identical to the sequence of clone UP23b (±150 bp of the DGGE fragments could be sequenced). This indicates that the *Thiothrix* phylotype was present on all 10 amphipods examined.

Concluding remarks. This study demonstrates for the first time that a *Thiothrix* species thrives in the marine environment as an epibiont of an amphipod. These bacteria have the characteristic *Thiothrix* 16S rRNA sequence signature and the key morphological features of *Thiothrix* spp. As the rRNA sequence of clone UP23b differs from other *Thiothrix* species by more than 2.5%, clone UP23b probably represents a novel *Thiothrix* species (12, 25). The ability of this new *Thiothrix* species to oxidize sulfur compounds and its role in the microbial community associated with *U. poseidonis* remains to be shown.

Nucleotide sequence accession number. The 16S rRNA gene sequence UP23b has been deposited in the GenBank database under accession no. AY426613.

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