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Phylogenetic Affinity of a Wide, Vacuolate, Nitrate-Accumulating *Beggiatoa* sp. from Monterey Canyon, California, with *Thioploca* spp.

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Environmentally dominant members of the genus *Beggiatoa* and *Thioploca* spp. are united by unique morphological and physiological adaptations (S. C. McHatton, J. P. Barry, H. W. Jannasch, and D. C. Nelson, Appl. Environ. Microbiol. 62:954–958, 1996). These adaptations include the presence of very wide filaments (width, 12 to 160 μm), the presence of a central vacuole comprising roughly 80% of the cellular biovolume, and the capacity to internally concentrate nitrate at levels ranging from 150 to 500 mM. Until recently, the genera *Beggiatoa* and *Thioploca* were recognized and differentiated on the basis of morphology alone; they were distinguished by the fact that numerous *Thioploca* filaments are contained within a common polysaccharide sheath, while *Beggiatoa* filaments occur singly. Vacuolate *Beggiatoa* or *Thioploca* spp. can dominate a variety of marine sediments, seeps, and vents, and it has been proposed (H. Fossing, V. A. Gallardo, B. B. Jorgensen, M. Huttel, L. P. Nielsen, H. Schulz, D. E. Canfield, S. Forster, R. N. Glud, J. K. Gundersen, J. Kuver, N. B. Ramsing, A. Teske, B. Thamdrup, and O. Ulloa, Nature [London] 374:713–715, 1995) that members of the genus *Thioploca* are responsible for a significant portion of total marine denitrification. In order to investigate the phylogeny of an environmentally dominant *Beggiatoa* sp., we analyzed complete 16S rRNA gene sequence data obtained from a natural population found in Monterey Canyon cold seeps. Restriction fragment length polymorphism analysis of a clone library revealed a dominant clone, which gave rise to a putative Monterey *Beggiatoa* 16S rRNA sequence. Fluorescent in situ hybridization with a sequence-specific probe confirmed that this sequence originated from wide *Beggiatoa* filaments (width, 65 to 85 μm). A phylogenetic tree based on evolutionary distances indicated that the Monterey *Beggiatoa* sp. falls in the gamma subdivision of the class *Proteobacteria* and is most closely related to the genus *Thioploca*. This vacuolate *Beggiatoa—Thioploca* cluster and a more distantly related freshwater *Beggiatoa* species cluster form a distinct phylogenetic group.

Among the numerous conspicuous sulfur-oxidizing bacteria, the genera *Beggiatoa* and *Thioploca* have similar morphological and physiological characteristics, including disk-shaped or cylindrical cells arranged in long filaments, gliding motility, intracellular globules of elemental sulfur, and the occurrence of both freshwater and marine representatives that have a wide range of cell diameters (widths). Despite long-standing interest in both of these genera, there are pure cultures of only the narrowest *Beggiatoa* strains (width, less than 5 μm), while no *Thioploca* species has been cultured. Although some ultrastructural differences between certain *Thioploca* and *Beggiatoa* strains have been reported at the electron microscopy level (21, 22) and although some differentiation may be possible based on filament widths (Table 1), a single *Thioploca* filament cannot be reliably differentiated from a *Beggiatoa* filament by light microscopy. In addition, all wide (cell diameter, 12 to 160 μm) uncultured marine representatives of both genera examined to date have a massive central vacuole and accumulate nitrate, presumably in the vacuole and presumably for use as an electron acceptor that allows anaerobic sulfide oxidation (5, 24). Morphologically, *Beggiatoa* spp. are distinguished from *Thioploca* spp. only by the fact that in members of the genus *Thioploca* up to 100 separate filaments are contained within a single common polysaccharide sheath to form a bundle (22). Within each genus, filament width, which seems to divide natural populations into largely nonoverlapping groups, is the basis of species differentiation (37).

Beggiatoa and *Thioploca* filaments have been observed to form dense mats on sediments in estuarine, shelf, seep, and deep-sea hydrothermal vent environments (7, 10, 12). The biomass densities of vacuolate forms of members of these genera can be especially impressive, up to 1 kg (wet weight)/m^2 of sediment surface (5, 25). Although narrow nonvacuolate *Beggiatoa* spp. proliferate in a narrow zone whose vertical dimension is less than 1 mm, where both oxygen and H_2S occur (13, 30), the densities of the wider, vacuolate forms of both genera are high over a greater vertical distance (e.g., 10 cm), even in the absence of oxygen. Presumably, these organisms employ internal nitrate as an electron acceptor, which allows anaerobic oxidation of sulfide 10 to 15 cm below the sediment surface.

In a recent study Teske et al. reported that there is a relatively close phylogenetic relationship between *Thioploca* spp. and *Beggiatoa* spp. based on 16S rRNA gene sequence data (38), but that study included only freshwater, nonvacuolate, heterotrophic representatives of the genus *Beggiatoa*. In the current study we focused on a very wide (width, 65 to 85 μm), vacuolate, uncultured *Beggiatoa* sp. from Monterey Canyon, California. The filaments of this organism are ideal for study because they occur at extraordinary biomass densities and can be harvested with minimal contamination from other prokaryotes (24, 25). The Monterey Canyon *Beggiatoa* sp. is also among the best-characterized representatives having the vacuolate phenotype. Enzymatic studies have shown that it is a

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TABLE 1. Summary of properties of vacuolate *Beggiatoa* and *Thioploca* spp.

Organism(s)	Filament width (μm)	Common sheath	Vacuole vol. (% of biovolume)	Evidence for vacuole[a]	Length of 16S ribosomal DNA sequence (bp)	References
Beggiatoa sp. (Monterey Canyon)	65–85	No	80	P/V	1,493	24; this study
Beggiatoa spp. (Guaymas Basin vents)	17–35	No	68–85	P/V, EM	None	29, 31
	32–50	No	83	P/V, EM	None	
	88–140	No	92	P/V, EM	None	
Thioploca ingrica	2–4.5	Yes	39–42[b]	EM	1,491	18, 19, 38
Thioploca chileae	12–20	Yes	89[b]	EM	550	20, 21, 38
Thioploca araucae	30–43	Yes	>80	EM	620	5, 20, 21, 38

[a] P/V, protein biovolume ratios (31); EM, electron microscopy.
[b] Calculated from previously published electron micrographs of *Thioploca ingrica* (19) and *T. chileae* (21).

chemoautotrophic sulfide oxidizer with the ability to reduce its internal store of nitrate to ammonia while the nitrate serves as a presumptive electron acceptor (24, 25). The results reported here are the first phylogenetic results obtained for a vacuolate marine *Beggiatoa* sp. from any environment and provide data for an important comparison with previously published partial sequences (38) attributed to marine *Thioploca* spp. and finer resolution of the phylogeny of vacuolate, nitrate-accumulating, chemoautotrophic, marine, sulfide-oxidizing filaments. Confirmation by fluorescent in situ hybridization (FISH) that our sequence derives from the vacuolate *Beggiatoa* sp. sequence strongly supports the tight clustering of the genera *Beggiatoa* and *Thioploca*.

MATERIALS AND METHODS

***Beggiatoa* sampling.** Native filaments of a wide uncultured *Beggiatoa* sp. were collected at a depth of 900 m in Clam Field Seep (1) in Monterey Canyon in April 1997. Plexiglas cores were used for sediment sampling from this sulfide-rich cold seep; the cores were collected by the remotely operated vehicle Ventana. Samples, including at least 10 cm of overlying water, were transported on ice to Davis, Calif., where they were stored at 4°C for 24 h, a period which allowed filaments to glide through the disturbed surface layer and extend into the overlying water. Filaments were gently removed with a Pasteur pipette and transferred to 1.5-ml Eppendorff tubes. As determined by examination with a microscope, the material collected generally consisted of more than 99% (by biovolume) *Beggiatoa* filaments having widths ranging from 65 to 85 μm. The filaments were pelleted by centrifugation at 5,000 × *g* for 10 s and kept at −20°C until they were used. Chromosomal DNA was extracted as described by Wilson (44).

***Thiomicrospira* strains.** *Thiomicrospira* sp. strain L-12 was obtained from Holger Jannasch, Woods Hole Oceanographic Institution (32). *Thiomicrospira* sp. strain XCL-2 was cultured from the Galapagos Rift vents in 1988 (28); the DNA base composition, growth rate at 33°C, and morphology of this strain indicate that it is a *Thiomicrospira crunogena* strain (11). The culture conditions used for both strains were the conditions described previously.

Clone library construction. Small-subunit 16S rRNA genes were amplified from the potentially mixed DNA by PCR by using *Taq* DNA polymerase and standard methods (34). The two universal eubacterial 16S ribosomal DNA primers used were based on primers described by Weisburg et al. (43), primers 8fpl (5′-AGAGTTTGATCCTGGCTCAG-3′, corresponding to *Escherichia coli* positions 8 to 27) and 1492rpl (5′-GGTTACCTTGTTACGACTT-3′, corresponding to positions 1510 to 1492), and contained added polylinkers. The reaction mixtures were overlaid with mineral oil and were incubated in a Perkin-Elmer model 480 DNA thermal cycler. Three control reaction mixtures (one lacking template DNA, one lacking forward primer, and one lacking reverse primer) were prepared. The amplification conditions were as follows: denaturation at 94°C for 5 min, annealing at 45°C for 1 min, and extension at 70°C for 4 min for 30 cycles. Following the final cycle, the reaction mixture was incubated at 72°C for 10 min. The amplified products were inserted into the TA vector and transformed into INVαF′ cells (Invitrogen Corp.). Positive transformants (white colony morphotype) were streaked for isolation and were screened by using a miniprep kit (Qiagen, Chatsworth, Calif.). A total of 111 clones containing the full-length 16S rRNA gene inserts from the *Beggiatoa*-enriched DNA were obtained from three independent PCR and subsequent multiple cloning reactions.

RFLP analysis and sequencing. All of the clones were characterized by an *Eco*RI restriction fragment length polymorphism (RFLP) analysis in which standard methods were used (34). Restriction fragments were resolved by gel electrophoresis (1% agarose in 1× Tris-acetate-EDTA buffer) and stained with ethidium bromide (0.5 μg ml^{-1}). Three representatives of the dominant restriction pattern, which was produced by 76% of the clones, were partially sequenced (approximately 250 bases), which confirmed the sequence identity. A single representative of the dominant operational taxonomic unit (OTU) was then selected for complete bidirectional sequencing by Sanger's dideoxynucleotide chain termination method (34), in which a Sequenase, version 2.0, kit (U.S. Biochemicals Corp., Cleveland, Ohio) was used. The following sequencing primers were used: forward primer −40 and reverse primer −21 (U. S. Biochemicals Corp.); universal reverse primers 519r, 907r, and 1392r (16); and custom forward primers MBF1 (positions 346 to 363; GGGAGGCAGCAGTAGGGA), MBF2 (positions 666 to 683; GGGAAGCGGAATTCTTAG), and MBF3 (positions 1174 to 1191; GGAGGAAGGTGGGGATGA). The manually obtained sequence data were also confirmed by an automated sequencing analysis in which we used ABI PRISM dye terminator cycle sequencing with dRhodomine terminator chemistry. Reactions were performed by using an ABI PRISM DNA sequencer (model 377) and a 5% Long Ranger gel. Sequence data were edited and analyzed by using ABI PRISM sequencing 2.1.1 software.

Probe design and labeling. An 18-mer Monterey *Beggiatoa*-specific probe, MBSP1RC (Table 2), was targeted to variable region 29 (6, 9, 39) of the dominant 16S rRNA clone sequence (OTU 3) for use in FISH. The probe was obtained from OPERON Technologies with the 5′ amino modifier, 6-(4-monomethoxytritylamino)hexyl-(2-cyanoethyl)-(*N*,*N*-diisopropyl)-phosphoramide. The 5′ amino groups of this probe and other probes were labeled with the

TABLE 2. Fluorescent rRNA-specific oligonucleotide probes used in this study

Probe	Target positions	Sequence	Specificity
16S rRNA probes			
MBSP1RC	850–833	5′-AGGATCAATCTCCCCCAA-3′	Wide *Beggiatoa* sp.
MBSP1C[a]	833–850	5′AACCCCCTCTAACTAGGA-3′	None
Eub-338[b]	338–355	5′-GCTGCCTCCCGTAGGAGT-3′	Eubacteria
ALF1b[c]	19–35	5′-CGTTCG(C/T)TCTGAGCCAG-3′	Alpha subdivision
Thioploca-829[d]	829–849	5′-GGATTAATTTCCCCCAACATC-3′	*Thioploca* spp.
23S rRNA probe[c]			
BET42a	1027–1043	5′-GCCTTCCCACTTCGTTT-3′	Beta subdivision
GAM42a	1027–1043	5′-GCCTTCCCACATCGTTT-3′	Gamma subdivision

[a] Nonsense probe designed as the reverse of MBSP1RC.
[b] Probe described by Wagner et al. (41).
[c] Probe described by Manz et al. (23).
[d] Probe described by Teske et al. (38).

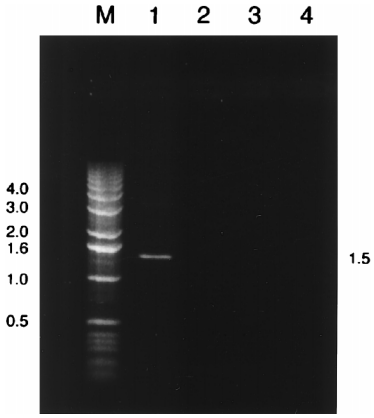

FIG. 1. PCR amplification products based on chromosomal DNA extracted from *Beggiatoa* sp. filaments from Monterey Canyon, California, as revealed by ethidium bromide staining and agarose gel electrophoresis. Each PCR mixture contained a 3-μl sample. Lane M, 1.0-kb DNA ladder; lane 1, PCR amplification product obtained with universal eubacterial 16S rRNA gene primers; lanes 2 through 4, three separate control reactions (lane 2, no DNA template; lane 3, no forward primer; lane 4, no reverse primer). DNA sizes (in kilobase pairs) are indicated on the left and right.

fluorophore BODIPY-TMR (excitation at 542 nm, emission at 574 nm) by using the instructions provided by the supplier (Molecular Probes Inc.). Unlabeled probe was removed by using a spin column purification kit. Aliquots (50 μl; 25 ng/μl) of labeled probe were distributed into nuclease-free tubes, dried in a SpeedVac apparatus, and stored at −20°C in the dark. The probe's optimal hybridization parameters were calculated as described by Stahl and Amann (36).

FISH. Filament samples from Monterey Canyon were fixed, prehybridized, and hybridized with fluorescent probes by previously described methods (38), with the following modifications. Filaments fixed in paraformaldehyde (1.4% in filtered [pore size, 0.2 μm] natural seawater) were consecutively dehydrated in 50, 80, and 100% ethanol. After air drying, the slides were prehybridized with 40 μl of prehybridization buffer at 37°C for 2 h. Then the prehybridization buffer was replaced with 40 μl of hybridization buffer and an appropriate oligonucleotide probe at a final concentration of 5 to 10 ng μl^{-1}.

Hybridization was carried out at 37°C (10, 15, or 20% [vol/vol] formamide) or at 37 to 43°C (20% [vol/vol] formamide) for 16 h to determine the conditions for specific binding of probes. The slides were incubated with wash buffer at 37°C for 20 min to remove unbound fluorescent probe, rinsed with water, air dried, and mounted in 100% glycerol. Fluorescence was detected with a Zeiss Axioskop microscope equipped with an epifluorescence filter set (Narrow X HQ 545/565/610; Chroma Technology, Brattleboro, Vt.). Micrographs were taken with a Zeiss model MC 100 camera and Kodak Ektachrome 1600 film. A composite (see Fig. 5) was edited by using Photoshop, version 4.01, and was printed by using a Fujix Pictrography 3000 printer.

Phylogenetic analysis. All of the sequences used for comparison were retrieved from the Ribosomal Database Project (17). Sequences were manually aligned and edited by using the SeqLab program included in the Wisconsin package, version 9.1 (7a). Evolutionary trees were constructed by distance, maximum-parsimony, and maximum-likelihood methods by using programs contained in the phylogeny inference package (PHYLIP, version 3.5c) (4). Two data sets that included only regions in which the alignment was unambiguous were used for phylogenetic analysis. The large set (small mask) consisted of 1,203 aligned positions for the Monterey *Beggiatoa* sp. and previously published sequence data. The small set (large mask) consisted of only 534 positions that were required to accommodate the partial sequences available for *Thioploca araucae* and *Thioploca chileae*. For each alignment, 100 bootstrapped replicate resampling data sets were generated by using the SEQBOOT program with random sequence addition and global rearrangement. We estimated evolutionary distances with the program DNADIST by using the option for Kimura's two-parameter model for nucleotide change and a transition/transversion ratio of 2.0 (15). We also tested the Jukes-Cantor model (14) for nucleotide substitution. The resulting evolutionary distance matrices were used to reconstruct phylogenetic trees by the neighbor-joining method (33) by using NEIGHBOR. Parsimony and maximum-likelihood trees were reconstructed with the programs DNAPARS and DNAML, respectively. We edited the phylogenetic trees with the program TREECON for Windows 95, version 1.3b (40).

Nucleotide sequence accession numbers. The nucleotide sequence of the 65- to 85-μm-wide Monterey *Beggiatoa* sp. has been deposited in the GenBank database under accession no. AF064543. The nucleotide sequences of *Thiomicrospira* sp. strain L-12 and *Thiomicrospira* sp. strain XCL-2 have been deposited under accession no. AF064544 and AF064545, respectively.

RESULTS

Collection of environmental sample. Sediment cores obtained from a sulfide-rich cold seep (Clam Field Seep) off the California coast in Monterey Canyon (depth, 900 m) typically had surface mats consisting of macroscopically visible *Beggiatoa* sp. filaments that projected 1 to 2 cm above the sediment surface. Microscopic examination of harvested filaments showed that the wide *Beggiatoa* sp. dominated; all of the unicellular contaminants comprised less than 1% of the biovolume (24). Although the diameters of most of the filaments harvested ranged from 65 to 85 μm, a few of the *Beggiatoa* sp. filaments were narrower, with diameters ranging from 20 to 30 μm. All of the filaments had sulfur inclusions and moved by gliding.

Extraction of chromosomal DNA. The initial conventional extraction of bacterial DNA (34) from the wide filaments resulted in a poor yield or degraded DNA. We presumed that a large amount of extracellular polysaccharide in the samples affected the efficiency of cell breakage and separation of DNA from the exopolymers. In order to solve this problem, a modified method in which cetyltrimethylammonium bromide was used (44) allowed removal of cell wall debris and denatured protein and polysaccharide, while the intact nucleic acid remained in solution, as demonstrated by agarose gel electrophoresis (data not shown).

PCR amplification and construction of 16S rRNA gene clone library. PCR amplification performed with universal eubacterial 16S rRNA gene primers and mixed template DNA from the *Beggiatoa*-dominated population was successful and yielded a single 1.5-kb DNA fragment (Fig. 1), suggesting that the PCR was specific for the target region. Cloning of the PCR products yielded 111 positive clones having the complete 1.5-kb insert. After positive clones were screened by performing an RFLP analysis, five different OTUs were defined. The dominant restriction pattern (OTU 3) was produced by 76% of the clones screened (Fig. 2).

Sequencing. The first 250 bases (5′ to 3′) of three representatives that were picked randomly from OTU 3 were sequenced with primer −40F. All three representatives had identical sequences in this region that included two regions known to be highly variable within the gamma subdivision of the *Proteobacteria* (7a). The complete 16S rRNA gene sequence of the Monterey *Beggiatoa* sp. was confirmed by manual and automated sequencing. There were no unresolved mismatches. The 16S rRNA gene sequence of the dominant clone

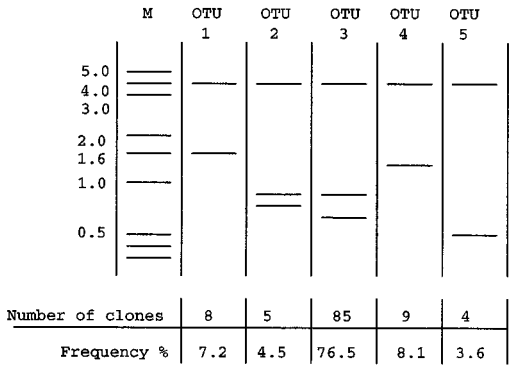

FIG. 2. Diagram of RFLP patterns (after *Eco*RI digestion) of cloned 16S rRNA genes, resolved by agarose gel electrophoresis. All five different restriction patterns obtained, defined as OTUs, are shown along with their proportional representation in the 111 clones screened. Molecular weight standards (lane M) were included for comparison. Fragment sizes (in kilobase pairs) are indicated on the left.

was not a product of chimeric artifacts, as determined by the CHECK_CHIMERA program. To eliminate misincorporation PCR errors as a major source of variations, we used secondary-structure models (9, 45) to examine the nature and position of the sequence variation. In this analysis we assumed that base substitutions caused by DNA polymerase errors should be randomly distributed throughout the sequences. In fact, the secondary-structure analysis confirmed that all substitutions compared to the *E. coli* sequence were restricted to highly variable regions of the 16S rRNA sequence, were largely compensated for by corresponding substitutions in the complementary stem region, and did not disturb the highly conserved secondary structure.

Database search and alignment of 16S rRNA gene sequence. Searches of databases were performed with the BLAST program (Wisconsin package, version 9.1 [7a]) in order to identify partial and complete sequences similar to the putative 16S rRNA gene sequence of the Monterey *Beggiatoa* sp. *Beggiatoa* sp. strain B1401-13 had the highest score, and the excellent matches included matches with sequences from *Thioploca* spp., *Thiobacillus* spp., and a number of free-living and endosymbiotic sulfur-oxidizing bacteria belonging to the gamma subdivision of the *Proteobacteria*.

Phylogenetic analysis. Tree construction analyses performed with both distance and parsimony methods and bootstrapping unambiguously placed the Monterey *Beggiatoa* sequence in the gamma subdivision of the *Proteobacteria*. The phylogenetic trees that were inferred from the distance matrix data by neighbor-joining tree reconstruction methods are shown in Fig. 3. Although the number of nucleotide positions analyzed was only 534 when partial sequences of *T. araucae* and *T. chileae* were included in the alignment (Fig. 3B), this small number of positions did not result in instability of the overall tree topology or significant changes in the bootstrap values compared to the values obtained for the small mask (1,203 positions) (Fig. 3A).

The partial-sequence tree (Fig. 3B) and an evolutionary distance matrix (Table 3) showed that the vacuolate marine thioplocas (*T. araucae* and *T. chileae*) are the closest relatives of the Monterey *Beggiatoa* sp. (Fig. 3B) and that *T. araucae* is equally close to the Monterey *Beggiatoa* sp. and *T. chileae* in terms of evolutionary distance. Narrow, nonvacuolate, freshwater, filamentous bacteria (i.e., *Beggiatoa* spp. strains B15LD and B1401-13) and *Thioploca ingrica* are more distantly related to this cluster (Fig. 3). Even with the large mask, the bootstrap values gave complete (100%) support for (i) the three-species cluster that contains all known vacuolate, marine, filamentous sulfur bacteria (i.e., *Beggiatoa* and *Thioploca* spp.), (ii) the finding that the freshwater organism *T. ingrica* is the closest relative of this three-species cluster, and (iii) the monophyletic nature of the *Beggiatoa-Thioploca* lineage within the gamma subdivision.

FISH. We used FISH to confirm that the sequence which we retrieved is the sequence of the wide vacuolate *Beggiatoa* sp. from Monterey Canyon. The highlighted target region in the multiple alignment in Fig. 4 shows that the sequence of the 18-mer probe (MBSP1RC), which was designed to be specific for the Monterey *Beggiatoa* sp., differed from the sequences of the two marine *Thioploca* spp. by two or three nucleotides. Compared with the sequence of the narrow freshwater organism *T. ingrica*, there were eight mismatches. The sequence of MBSP1RC was checked, and negative results were obtained with the CHECK_PROBE program of the Ribosomal Database Project (updated 15 June 1997), which allowed two mismatches.

MBSP1RC bound specifically to wide *Beggiatoa* filaments under fairly stringent hybridization conditions (Fig. 5C). The probe did not hybridize to the occasional narrower *Beggiatoa* filaments (width, 20 to 30 μm) observed in some preparations. The intensity and yellow color of the hybridization signal were comparable to the intensity and color of the signal obtained when universal eubacterial probe was used with the same samples (Fig. 5B). The most intense fluorescence was observed in the presence of 20% formamide with both MBSP1RC and Eub-338. The filaments in these preparations also hybridized with the GAM42a probe, giving a signal that was stronger than the signal observed with either BET42a or ALF1b (data not shown). As a negative control, nonsense probe MBSP1C did not hybridize with the *Beggiatoa* rRNA (Fig. 5A), and filaments appeared exactly as if no probe had been added. When a mismatched probe or no probe was added, *Beggiatoa* filaments appeared orange-red due to autofluorescence conferred apparently by abundant cytochromes (data not shown). The *Thioploca* sp.-specific probe (*Thioploca*-829) did not hybridize to Monterey *Beggiatoa* sp. filaments at temperatures above 37°C (Table 4), but MBSP1RC hybridized with the target filaments at temperatures up to 42 or 43°C (Table 4). Since Monterey *Beggiatoa* cells are very large by bacterial standards, hybridization experiments revealed some internal details of filaments (Fig. 5D); the central vacuole lacking ribosomes appeared as a clear area when the microscope was focused in mid-filament.

DISCUSSION

Phenotypic and phylogenetic comparisons of the genera *Beggiatoa* and *Thioploca*. Until recently, massive natural occurrence of filaments of *Beggiatoa* or *Thioploca* spp. have been identified based solely on their characteristic morphologies by using (i) the presence (*Thioploca* spp.) or absence (*Beggiatoa* spp.) of a single sheath around multiple filaments and (ii) filament widths as the major criteria (Table 1). No strain of a wide marine *Beggiatoa* or *Thioploca* sp. has been obtained in pure culture. The physiological properties of these genera can, therefore, be determined only from observations of natural populations. Such studies have revealed several metabolic similarities. These similarities include chemoautotrophic carbon metabolism (20, 24, 29, 31), sulfide oxidation (20, 25), and concentration of nitrate in the vacuolate cells at levels several-thousand-fold above ambient nitrate levels (5, 24). Teske et al. (38) described the phylogenetic position of vacuolate, unusually wide *Thioploca* filaments and demonstrated that *Beggiatoa* spp. were their closest relatives. The study of Teske et al. included only two phenotypically similar, nonvacuolate, narrow (width, 2.8 to 3.0 μm), freshwater *Beggiatoa* isolates that appear to be obligate chemoheterotrophs (26). No marine strains were analyzed.

In the current study we examined the sequence of a marine or vacuolate *Beggiatoa* sp. for the first time. Phylogenetic trees (Fig. 3) showed that all of the *Beggiatoa* spp. examined so far fall into a coherent evolutionary cluster (bootstrap value, 100%) that includes as its only other members species of the genus *Thioploca*. One of the two clusters identified contains only the narrow, nonvacuolate, freshwater, chemoheterotrophic *Beggiatoa* strains. Somewhat surprisingly, the second cluster contains a narrow freshwater *Thioploca* strain in addition to all of the vacuolate, marine *Beggiatoa* and *Thioploca* strains whose sequences have been determined. Based on the monophyletic association of the wide marine vacuolate bacteria regardless of the presence of a sheath, an association between the freshwater organism *T. ingrica* and narrow freshwater *Beggiatoa* spp. might have been anticipated. The search for a common feature to unify the vacuolate *Beggiatoa-Thioploca*

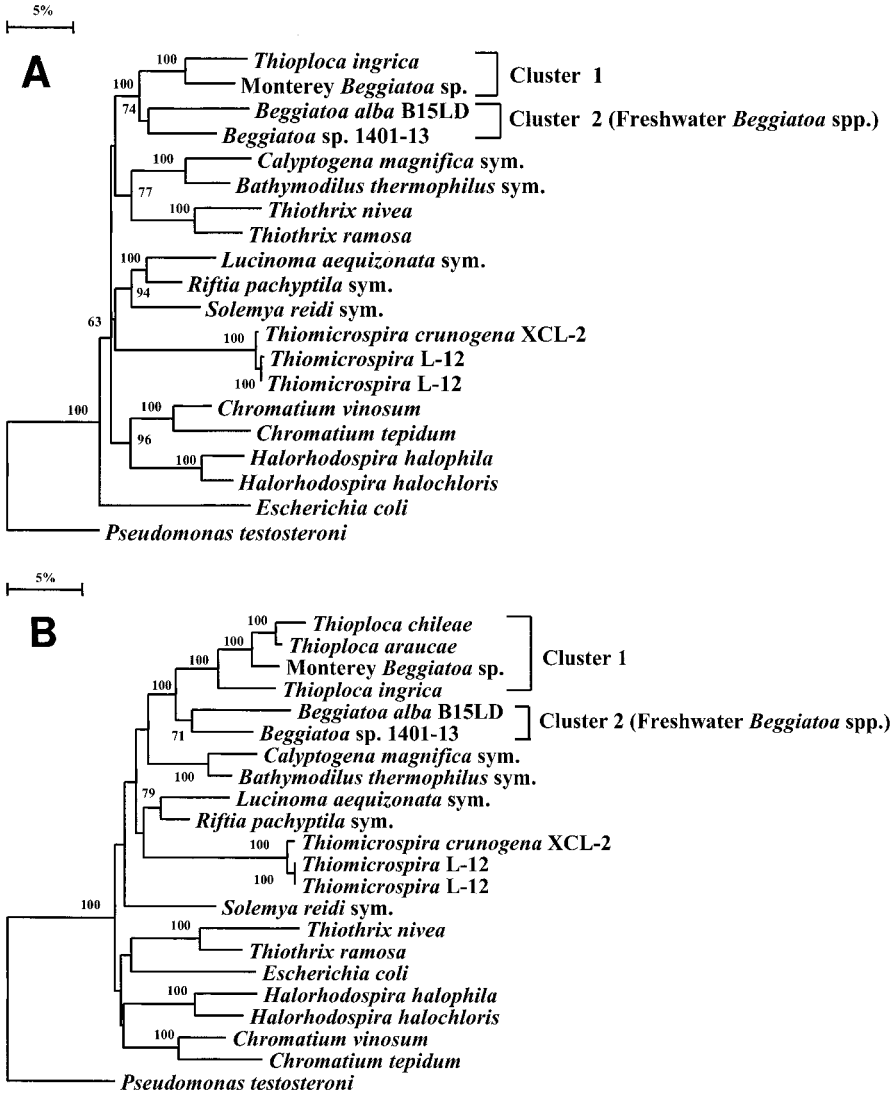

FIG. 3. Phylogenetic trees showing the positions of the Monterey *Beggiatoa* sp. and other representatives of the gamma subgroup of the *Proteobacteria*, as inferred by the neighbor-joining method. Distances were corrected with Kimura's two-parameter model. The sequence of *Pseudomonas testosteroni* (a member of the β subdivision of the *Proteobacteria*) was used to root the tree. *Halorhodospira halophila* and *Halorhodospira halochloris* (9a) sequence data were accessed as data for the corresponding *Ectothiorhodospira* species. The phylogenetic analyses were performed with programs contained in the PHYLIP package, version 3.5c. There are two main *Beggiatoa-Thioploca* clusters. Cluster 1 contains the Monterey *Beggiatoa* sp. sequence and all previously published *Thioploca* spp. sequences; cluster 2 contains all sequences belonging to freshwater *Beggiatoa* spp. (A) Small mask tree inferred from 1,203 nucleotide positions. Partial *T. araucae* and *T. chileae* sequences were not included. (B) Tree inferred with the full mask by using only 534 positions, which allowed inclusion of partial sequences of *T. araucae* and *T. chileae*. All of the sequences used except the new sequences were retrieved from the Ribosomal Database Project (17). Scale bar = 5 substitutions/100 nucleotide positions. *Thiomicrospira crunogena* XCL-2 and *Thiomicrospira* sp. strain L-12 were sequenced in this study; the sequence of *Thiomicrospira* sp. strain L-12 was also determined previously (31). sym, symbiont.

cluster placed emphasis on the presence of vacuoles in all of the members of the cluster (Table 1) and suggested that there should be a search for nitrate accumulation in *T. ingrica*.

The finer details of the evolutionary relationships between *Thioploca* spp. and the wide vacuolate *Beggiatoa* spp. may still be forthcoming because several additional representatives of the latter group have been identified, including strains whose widths overlap the widths of *T. ingrica* and *T. chileae* strains (Table 1). Of special interest is understanding whether these strains form two separate well-defined genera or are actually members of more closely related species of a single genus with variable phenotypic responses (e.g., perhaps they form sheaths only in particular environments). The observed transitions from unsheathed forms (*Beggiatoa* spp.) to sheathed forms (*Thioploca* spp.) in the Peruvian upwelling (35) and Monterey Canyon seeps (2) support this suggestion. On the other hand, sheathed forms have never been observed among the wide *Beggiatoa* spp. of the Guaymas Basin vents or Gulf of Mexico seeps (28). In any case, the taxonomy of the *Beggiatoa* and *Thioploca* spp. certainly requires revision. The cluster containing the Monterey *Beggiatoa* sp., *T. araucae*, and *T. chileae* (Table 3) is, on the basis of the criteria of Devereux et al. (3), narrow enough to warrant a single genus. In contrast, the distances between any two *Beggiatoa* spp. or between *T. ingrica* and the two other *Thioploca* spp. (Table 3) are greater than the acceptable range of distances for a coherent genus (3).

TABLE 3. Evolutionary distances between 16S rRNA sequences of *Beggiatoa* and *Thioploca* spp.

Organism	Estimated no. of substitutions per 100 bases						
	Monterey *Beggiatoa* sp.	*Thioploca araucae*	*Thioploca chileae*	*Thioploca ingrica*	*Beggiatoa* sp. strain B15LD	*Beggiatoa* sp. strain B1401-13	*Escherichia coli*
Monterey *Beggiatoa* sp.	0.00	2.60	3.74	8.73	14.89	12.48	20.67
Thioploca araucae		0.00	2.41	9.14	14.91	12.26	20.91
Thioploca chileae			0.00	10.19	15.56	12.05	20.67
Thioploca ingrica				0.00	17.45	13.18	24.22
Beggiatoa sp. strain B15LD					0.00	12.70	22.62
Beggiatoa sp. strain B1401-13						0.00	22.13
Escherichia coli							0.00

[a] Values were corrected by the Jukes-Cantor model for nucleotide substitution (14).

FISH, gene copy, and ribosome density. The low number of OTUs observed (see above) suggests that the sample extracted was dominated by DNA from a narrow range of microorganisms. Based on the in situ hybridization results (Fig. 5), the vacuolate genus *Beggiatoa* is the dominant OTU and has the corresponding 16S rRNA sequence. At first glance, this might have been expected because microscopic examination revealed that the mat material collected was a virtual monoculture of wide *Beggiatoa* filaments; the volume of all of the other bacterial biomass was equal to less than 1% of the total *Beggiatoa* biovolume (24). However, because of the huge size of individual *Beggiatoa* cells (roughly 75 by 20 μm), much higher filament purity or a much high copy number of the *Beggiatoa* genome seems to be required to account for our findings. For example, if we assumed that unicellular contaminants (1 by 2 μm; same genome copy number and rRNA operon copy number as the Monterey *Beggiatoa* sp.) were present at a volume that was equivalent to 0.1% of the *Beggiatoa* biovolume, the contaminants would be expected (assuming no PCR bias) to contribute six times as many rRNA gene copies as the wide vacuolate *Beggiatoa* sp.

Due to low signal intensity attributed to low ribosome density, FISH signals of individual *Thioploca* sp. filaments were often difficult to detect (38). Only amplification of a signal emanating from overlying filaments within a bundle made detection straightforward. In contrast, individual *Beggiatoa* filaments could be readily detected. The presumptive higher density of the Monterey *Beggiatoa* sp. ribosomes may reflect optimal growth conditions compared to the Chilean sediments, where growth may be restricted for certain periods of time due to the absence of both nitrate and oxygen.

Specificity and fidelity of probe and sequence. The 16S rRNA sequence reported here appears to reflect the entire population of the 75-μm-wide, vacuolate *Beggiatoa* sp. from Monterey Canyon. Three independent PCR performed with the mixed DNA showed that the OTU corresponding to this sequence was always dominant, and the partial 16S rRNA sequences of three random representatives of this OTU were identical. A specific probe was designed for a variable region of the 16S rRNA sequence assigned to the Monterey *Beggiatoa* sp., and in situ hybridization experiments revealed the specificity of this probe for the target species (the probe hybridized

```
                                827                                 860
Escherichia coli                UUGGAGGUUGUGCC--CUUGA---GGCGUGGCUUCCGGA
Monterey Beggiatoa sp.          UAGAUG-UUGGGGGA-GAUUGA--UCCUUUAGUAUCGTA
Thioploca araucae               UAGAUG-UUGGGGGA-AAUUAA--UCCCUUAGUAUCGCA
Thioploca chileae               UAGAUG-UUGGGGGA-AAUUAA--UCCCUUAGUNUCGCA
Thioploca ingrica               UAGACG-UUGGAAGG-GUAUA---CCUUUUAGUGUCGCN
Beggiatoa alba B15LD            UAGAUG-UUGGGAGA-GACUG---UCUCUUACUAUCGCA
Beggiatoa sp. B1401-13          UAGAUG-UUGGGGGA-GUUAAG--UCCCUUAGUAUCGCN
Sym. Bathymodilus thermophilus  UAGCCG-UUGGGAGG-AUUUA---CCUCUUAGUGGCGAA
Sym. Calyptogena magnifica      UAGCCG-UUGGGGGG-AAGUG---CCCUUUAGUGGCGAA
Sym. Riftia pachyptila          UAGCCG-UUGGGCUN-AUUAUAA-GGGCUUAGUGGCGCA
Sym. Lucinoma aequizonata       UAGCCG-UUGGACUC-AUUAUAA-GGGUUUAGUGGCGCA
T. crunogena XCL-2(AA)          UAGCUG-UUGGCCUU-AUUAAA--AAGGUUAGUAGCGTA
Thiomicrospira L-12(AA)         UAGCUG-UUGGUCUU-AUUAAA--AAGAUUAGUAGCGTA
```

FIG. 4. 16S rRNA target region for the Monterey *Beggiatoa* sp.-specific probe (MBSP1RC; length 18 nucleotides) aligned with sequences from selected endosymbiotic and free-living sulfur-oxidizing bacteria. The specific target sequence of the Monterey *Beggiatoa* sp. (in boldface type) differs from the aligned sequences of all of the other sulfur-oxidizing bacteria by at least two nucleotides. The target of the *Thioploca*-829 probe is also shown in boldface type for *T. chileae*. The sequences correspond to variable region helix 29 of the *E. coli* 16S rRNA secondary structure model predicted by Van de Peer et al. (39). All predict a 12-base stem starting at position 829 and ending at position 857 with a five- to seven-base loop beginning at the aligned gap.

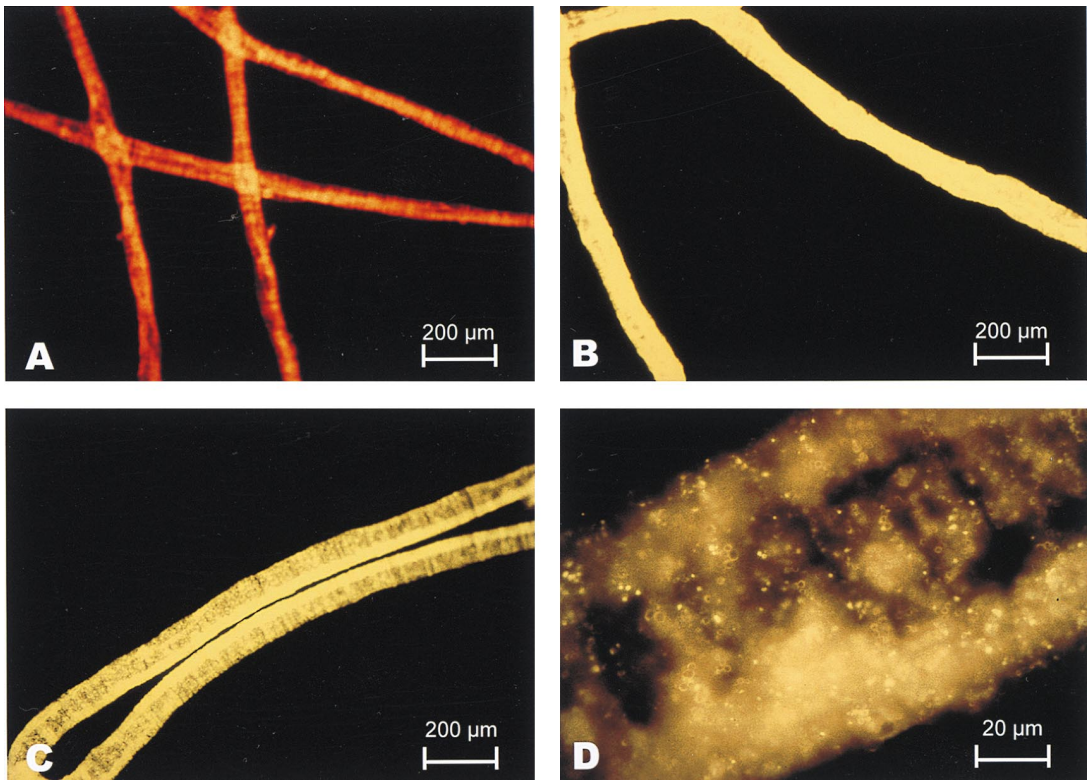

FIG. 5. Photomicrographs of fluorescent FISH results. (C and D) Hybridization (37°C, 20% formamide) of the MBSP1RC probe labeled with the fluorophore BODIPY-TMR in a single wide (width, approximately 75 μm) *Beggiatoa* filament. The intensity of the hybridization signal was comparable to the intensity of the signal obtained when the universal eubacterial probe (Eub-338) was used as a positive control (B). Nonspecific probe did not hybridize with the *Beggiatoa* rRNA (A). The orange-red coloration (A) (compared with the bright yellow of the probe [B through D]) appeared to be autofluorescence and was also detected in unstained filaments.

only with the *Beggiatoa* filaments that were 65 to 85 μm wide and did not hybridize with narrow *Beggiatoa* filaments or unicellular prokaryotes observed in the samples). The MBSP1RC probe hybridized with the Monterey *Beggiatoa* sp. under conditions stringent enough to eliminate hybridization of the *Thioploca*-specific probe (Table 4). Suggesting broader specificity, our probe also hybridized with a 70-μm-wide *Beggiatoa* sp. collected from sulfide-rich seeps (depth, 600 m) in the Gulf of Mexico.

When we sought evidence of a chimera, we observed no abnormalities in the secondary structure when the sequence assigned to the Monterey *Beggiatoa* sp. was examined for base complementarity within the helical regions of rRNA (8). In addition, a separate phylogenetic analysis of short sequence domains with the CHECK_CHIMERA program of the Ribosomal Database Project (17) gave negative results. We noted, however, that a chimeric sequence resulting from a fusion between two closely related species might go undetected (27). It has been shown (42) that a higher frequency of chimera formation is expected when very complex DNA is used for PCR. Because our environmental sample was dominated by a single 16S rRNA OTU, a low frequency of chimera formation was expected. We believe that the sequence which we obtained is unique and can be assigned to the wide vacuolate *Beggiatoa* sp. that dominated the Monterey Canyon sample.

Compared to the available partial sequences of closely related *Thioploca* spp., the complete 16S rRNA sequence of the wide vacuolate Monterey Canyon *Beggiatoa* sp. retrieved and examined in this study provides a more complete database for comparison with future sequences derived from natural populations of filamentous sulfur bacteria. In addition, the in situ hybridization studies with fluorescent probes which we performed can be extended to establish differences between single *Beggiatoa* and *Thioploca* filaments within mixed natural populations, perhaps revealing correlations between subtle sequence differences and morphological differences (e.g., differences in filament width, the presence or absence of a sheath, or niche differences).

TABLE 4. FISH of preserved Monterey *Beggiatoa* filaments with various probes at different temperatures

Probe[a]	Hybridization at[b]:				
	37°C	39°C	41°C	42°C	43°C
MBSP1RC	++++[c]	+++	+++	++	+
Thioploca-829	++	−	−	−	−
Eub-338	++++	+++	ND[d]	+++	ND
MBSP1C	−	−	−	−	−

[a] Probes MBSP1RC and *Thioploca*-829 are putatively specific for the Monterey *Beggiatoa* sp. and *Thioploca* spp., respectively (Fig. 4).
[b] All hybridizations were performed in the presence of 20% formamide, and the final concentration of the probe in the hybridization buffer was 5 ng/μl.
[c] The hybridization signal intensity ranged from very strong (++++) to no additional signal (−) compared with the background autofluorescence.
[d] ND, not determined.

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