Phylogenetic Relationships of the Filamentous Sulfur Bacterium *Thiothrix ramosa* Based on 16S rRNA Sequence Analysis†

MARTIN F. POLZ,¹ ELENA V. ODINTSOVA,^{2‡} AND COLLEEN M. CAVANAUGH^{1*}

*The Biological Laboratories, Harvard University, Cambridge, Massachusetts 02138,¹ and
Woods Hole Oceanographic Institution, Woods Hole, Massachusetts 02543²*

The phylogeny of *Thiothrix ramosa* based on 16S rRNA sequences was determined. This species is the first species in this genus that has been shown to be capable of autotrophic growth with reduced sulfur compounds as sole energy sources. *T. ramosa* forms a monophyletic clade with *Thiothrix nivea*, as determined by distance, parsimony, and maximum-likelihood methods. Both of these species clearly belong to the gamma subdivision of the *Proteobacteria*, where they are loosely associated with other sulfur-oxidizing chemoautotrophic organisms.

Bacteria belonging to the genus *Thiothrix* are characterized by their distinct morphological features (12, 13). These organisms form ensheathed filaments which accumulate sulfur internally in the presence of sulfide and have the ability to attach to substrates by means of holdfasts. The filaments often grow in a rosette-like array and can produce gliding gonidia from their unattached ends. The primary natural habitats of *Thiothrix* strains are sulfide-containing, flowing waters, in which the filaments can produce one of the most conspicuous microbial aggregations seen in nature (13). These organisms also occur regularly in activated sludge (26) and in irrigation systems (7), where they are associated with bulking and clogging of drip holes, respectively. In marine environments, *Thiothrix*-like bacteria are found in microbial mats at hydrothermal vents (27) and as gut symbionts of invertebrates (25).

Historically, observations of filamentous sulfur bacteria belonging to the genera *Thiothrix* and *Beggiatoa* were an important stage in Winogradsky's work which eventually led to the concept of bacterial chemosynthesis (29). However, it was only recently that workers confirmed Winogradsky's assumption that *Thiothrix* strains may be capable of autotrophic growth. It was found that *Thiothrix ramosa*, an isolate obtained from a spring in Latvia, grows by using either thiosulfate or carbon disulfide as a sole energy source (16). Since the claim of Keil that he had grown a *Thiothrix* strain autotrophically was later repudiated (21), *Thiothrix ramosa* is the first representative of this genus that was cultivated in an inorganic medium. All other officially recognized *Thiothrix* isolates grow only heterotrophically or chemolithoheterotrophically (12).

The members of the genus *Thiothrix* apparently have versatile metabolic capabilities, and the defining characteristics of this genus are based largely on morphology (12, 13). Recently, monoclonal antibodies that recognize a variety of *Thiothrix* strains have been developed, suggesting that these bacteria may be related (4). However, the reliability of such criteria for phylogenetic definition of the genus is not clear. Furthermore, we wanted to see whether the species that are able to grow autotrophically are related to species which have never been grown autotrophically. In this study we investigated the relationship of *Thiothrix ramosa* to *Thiothrix nivea* and other mem-

bers of the *Proteobacteria* by performing a 16S rRNA phylogenetic analysis. *Thiothrix nivea* has been shown to grow chemolithoheterotrophically and has been placed in the gamma subdivision of the *Proteobacteria* in previous analyses (11, 20).

MATERIALS AND METHODS

Culture methods and media. *Thiothrix ramosa* was obtained from the Institute of Microbiology, Russian Academy of Sciences, Moscow, Russia. The growth medium used contained (per liter) 0.5 g of $(\text{NH}_4)_2\text{SO}_4$, 0.1 g of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.05 g of $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.11 g of K_2HPO_4 , 0.085 g of KH_2PO_4 , 1.0 ml of a vitamin B mixture, and 1.0 ml of a trace metal solution. The vitamin solution (2) and the trace metal solution (3) were filter sterilized separately and were added to the salt solution, which had been autoclaved. Sodium thiosulfate and sodium bicarbonate solutions were added to final concentrations of 4 and 5 mM, respectively.

The cells were grown in a chemostat by using a 300-ml jacketed vessel connected to a thermocirculator (Haake, Paramus, N.J.), which was used to maintain the temperature at 23°C. The culture was stirred continuously and bubbled with sterile air at a flow rate of 110 ml min⁻¹. The pH was maintained at a neutral value by automatic titration with a 1 M Na_2CO_3 stock solution by using a Fisher Accumet pH meter controller. The chemostat was inoculated (10%, vol/vol) with a thiosulfate-grown batch culture. Cells were harvested from a steady-state culture by centrifugation, frozen, and stored at -80°C.

Nucleic acid manipulation. Nucleic acids were extracted and purified by the method of Jarrel et al. (9), with slight modifications. Briefly, cells previously stored at -80°C were ground in liquid nitrogen. TE buffer (5 ml) was added to the frozen cell slurry, and the mixture was allowed to thaw on ice. Then sodium dodecyl sulfate and proteinase K were added to final concentrations of 1% and 50 µg/ml, respectively. After the mixture had been incubated at 60°C for 1 h, NaCl was added to a concentration of 0.5 M, and the preparation was incubated on ice for an additional 1 h. The lysate was cleared by centrifugation at 25,000 × g for 15 min. The supernatant was transferred, and the nucleic acids were precipitated with an equal volume of cold isopropanol. The precipitate was collected and dissolved in TE buffer to which RNase A was added. The final extraction was performed by using standard phenol-chloroform and chloroform treatment methods followed by ethanol precipitation.

A PCR was used to generate 16S ribosomal DNA for direct sequencing. The PCR mixture contained approximately 100 ng of template DNA, universal *Bacteria* primers 27f and 1492r (10) (100 pmol each), and 2.5 mM MgCl_2 . Depending on the intended direction of sequencing, one of the two amplification primers was biotinylated. The DNA was denatured at 94°C for 2 min, and this was followed by 35 cycles consisting of 1 min at 94°C, 60 s at 50°C, and 2 min at 72°C.

The PCR product was sequenced directly by the method of Hultman et al. (8), in which avidin-coated magnetic beads are used. The biotinylated PCR product was bound to these beads and then denatured and washed while it was on the beads. The standard Sequenase (U.S. Biochemicals) protocol was used for sequencing reactions. The sequencing primers used were primers 1492r, 1392r, 1101r, 907r, 690r, 519r, and 342r for the rRNA-like strand and primers 1115f, 704f, 357f, and 27f for the opposite strand (10).

Phylogenetic analysis. The *Thiothrix ramosa* 16S rRNA secondary structure was reconstructed manually by using templates published by the Ribosomal Database Project (14) to help identify homologous sequence positions. Sequences were aligned manually in the Genetic Data Environment (19). All of the reference sequences and the basic alignment to which the *Thiothrix ramosa*

* Corresponding author.

† Contribution no. 8993 of the Woods Hole Oceanographic Institution.

‡ Present address: Institute of Microbiology, Russian Academy of Sciences, Moscow, Russia.

TABLE 1. Evolutionary distance matrix for *Thiothrix ramosa* and representative members of the *Proteobacteria*^a

Organism	% Evolutionary distance to:																		
	<i>Thiothrix ramosa</i>	<i>Thiothrix nivea</i>	<i>Bathymodiolus thermophilus</i> symbiont	<i>Calymene magnifica</i> symbiont	<i>Riftia pachyptila</i> symbiont	<i>Solemya reidi</i> symbiont	<i>Thyasira flexuosa</i> symbiont	<i>Thiomicrospira</i> sp. strain L12	<i>Thiomicrospira thyasiris</i>	<i>Dichelobacter nodosus</i>	<i>Pseudomonas aeruginosa</i>	<i>Chromatium tepidum</i>	<i>Escherichia coli</i>	<i>Vibrio marinus</i>	Clone FL5	<i>Piscirickettsia salmonis</i>	<i>Thiobacillus hydrothermalis</i>		
<i>Thiothrix nivea</i>	93.6																		
<i>Bathymodiolus thermophilus</i> symbiont ^b	83.6	84.1																	
<i>Calymene magnifica</i> symbiont ^b	83.8	84.0	92.6																
<i>Riftia pachyptila</i> symbiont ^b	86.4	86.0	88.2	85.5															
<i>Solemya reidi</i> symbiont ^b	83.3	84.3	83.6	82.5	89.9														
<i>Thyasira flexuosa</i> symbiont ^b	85.2	86.1	85.6	84.9	94.1	88.2													
<i>Thiomicrospira</i> sp. strain L12	80.1	79.3	81.6	80.9	85.3	83.7	84.4												
<i>Thiomicrospira thyasiris</i>	80.1	79.9	83.0	82.3	84.3	83.6	84.2	90.6											
<i>Dichelobacter nodosus</i>	81.8	81.0	80.8	80.0	83.6	83.4	82.8	78.7	80.1										
<i>Pseudomonas aeruginosa</i>	82.4	83.4	83.1	83.3	87.7	87.6	86.1	81.7	83.9	83.2									
<i>Chromatium tepidum</i>	83.5	83.3	78.4	78.9	86.1	85.2	84.0	79.0	79.6	81.8	83.5								
<i>Escherichia coli</i>	79.4	79.6	80.4	78.9	81.8	82.0	83.0	78.5	79.1	80.7	84.2	80.7							
<i>Vibrio marinus</i>	80.2	80.9	81.2	80.7	82.5	83.2	82.7	80.5	82.2	80.0	84.7	80.3	86.6						
Clone FL5	82.9	82.7	84.5	82.1	85.1	85.2	86.2	81.9	82.9	82.9	84.1	79.0	82.5	80.8					
<i>Piscirickettsia salmonis</i>	83.0	84.3	80.9	82.4	84.2	85.2	84.4	83.1	81.8	83.5	84.8	82.1	83.2	82.7	82.8				
<i>Thiobacillus hydrothermalis</i>	82.4	82.5	82.3	82.1	84.9	86.6	85.1	78.6	81.4	83.8	84.8	83.8	82.6	79.3	82.1	83.9			
<i>Neisseria gonorrhoeae</i>	78.8	79.9	76.7	75.9	81.4	80.1	80.3	75.7	75.9	81.3	81.3	78.6	79.2	76.0	80.3	78.5	81.8		

^a Evolutionary distance values were calculated from the alignment used in the phylogenetic analyses by using the correction of Jukes and Cantor.

^b A sulfur-oxidizing, chemoautotrophic symbiont.

sequence was added were obtained from the Ribosomal Database Project. Sequences were chosen from previously published work and on the basis of high levels of similarity to the *Thiothrix ramosa* sequence by using the BLAST program (1). Initially, a larger set of sequences was used, and outgroups were removed one at a time; remaining nucleotide positions were kept if the topology of the tree did not change. The final data set included 845 nucleotide positions.

Phylogenetic analyses were performed by using the distance, parsimony, and maximum-likelihood methods. Distance and bootstrap analyses were performed by using the program DNADIST with the Jukes-Cantor correction, the program SEQBOOT with 100 replicates, and the program FITCH with input randomization and global rearrangement. All of these programs are contained in the PHYLIP 3.4 package (6) and were implemented through the Genetic Data Environment (19). The parsimony analysis, including the bootstrap analysis, was performed by using PAUP 3.0 (22). For the maximum-likelihood analysis we used the fastDNAml program available from the Ribosomal Database Project. Trees were constructed by allowing jumbled addition of taxa and global rearrangement of the branches. Bootstrap values are shown if they were greater than 50%.

Nucleotide sequence accession numbers and strain designations. The 16S ribosomal DNA sequence of *Thiothrix ramosa* has been deposited in the GenBank database under accession number U32940. The other organisms, and/or GenBank accession numbers of the other sequences used in our analysis are as follows: *Bathymodiolus thermophilus* symbiont, M99445; *Calymene magnifica* symbiont, M99446; clone FL5, L10936; *Chromatium tepidum* MC^T (= ATCC 43061^T) (T = type strain), M59150; *Dichelobacter nodosus* 198A (= ATCC 27521) (reference strain), M35016; *Escherichia coli*, J01695; *Neisseria gonorrhoeae* B5025^T (= ATCC 43061^T), X07714; *Pseudomonas aeruginosa* NIH18 (= ATCC 25330), X60783; *Pseudorickettsia salmonis* LF-89^T (= ATCC VR 1361^T), X60783; *Riftia pachyptila* symbiont, M99451; *Solemya reidi* symbiont, L07864; *Thiomicrospira* sp. strain L12, L01576; *Thiobacillus hydrothermalis* r3^T (= DSM 7121^T), M90662; *Thyasira flexuosa* symbiont, L01575; *Thiothrix nivea* JP2^T (= ATCC 35100^T [neotype strain]), M79435, M79436, and M79437; *Thiomicrospira thyasiris* TG2^T (= DSM 5322^T), L01479; and *Vibrio marinus* MP1 (= ATCC 15381) (source, C. R. Woese).

RESULTS AND DISCUSSION

A single product was obtained from PCR amplification of *Thiothrix ramosa* DNA. Direct sequencing of this product yielded 1,430 nucleotides of uninterrupted sequence in the region between nucleotide position 12 and nucleotide position 1441 (*E. coli* numbering). The secondary structure of the *Thio-*

thrix ramosa sequence was very similar to the secondary structure of the *Thiothrix nivea* sequence, as determined by manual reconstruction. One noteworthy characteristic was the absence of the stem corresponding to positions 455 to 477, which is also missing in *Thiothrix nivea* (11). This is unusual in members of the gamma subdivision of the *Proteobacteria* and appears to be a unique feature of the genus *Thiothrix*. A similarity matrix constructed with the Jukes-Cantor correction and aligned sequences obtained from the Ribosomal Database Project showed that the level of similarity between the two *Thiothrix* sequences was 93.6%, while the levels of similarity to the sequences of other members of the gamma subdivision of the *Proteobacteria* were approximately 10% lower (Table 1).

Our phylogenetic analysis revealed that the two *Thiothrix* species clearly form a distinct group belonging to the gamma subdivision of the *Proteobacteria* (Fig. 1). These organisms fell into a single clade with all three analysis methods used; the bootstrap values were 100% in both the distance and parsimony analyses, and the branch was significantly positive at $P < 0.01\%$ in the maximum-likelihood analysis. The overall position of the genus *Thiothrix* was near the group containing endosymbiotic, chemoautotrophic symbionts, as reported previously (11, 17). However, this node did not bootstrap significantly and changed position slightly with the different methods which we used, while the overall topology of the tree was unchanged. While the genus *Thiothrix* does belong to the gamma subdivision of the *Proteobacteria*, its closest relatives in this systematic unit cannot be determined with certainty yet.

Thiothrix strains have often been distinguished solely on the basis of morphology. However, experience has shown that morphological criteria are generally unreliable as tools in bacterial phylogenetic systematics (28). Our results indicate that the genus *Thiothrix* may be one of the few genera that can be identified by appearance. Among the filamentous sulfur bacteria, holdfast formation and growth in rosettes are unique to

FIG. 1. Unrooted evolutionary distance tree based on the 16S rRNA sequences of *Thiothrix ramosa* and representative members of the *Proteobacteria*. All of the bacteria shown on the tree except *N. gonorrhoeae* belong to the gamma subdivision of the *Proteobacteria*; *N. gonorrhoeae* is a member of the beta subdivision of the *Proteobacteria*. Bootstrap values greater than 50% are shown at the nodes; the upper values are values obtained in the distance analysis, and the lower values are values obtained in the parsimony analysis. Bar = 5% nucleotide difference per sequence position.

the genus *Thiothrix* (23). The only other organism that a *Thiothrix* strain may currently be mistaken for is *Leucothrix mucor*. On the one hand, these bacteria are also filamentous, grow in rosettes, and form gonidia; on the other hand, they are purely heterotrophic, never accumulate sulfur globules, and do not have a sheath (5). So far, *Leucothrix* strains have been isolated only from marine environments, and pure cultures of *Thiothrix* strains have been obtained only from freshwater sources. However, some *Thiothrix*-like organisms observed at marine hydrothermal vents do not have a sheath and vary in cell length and width (15a). At this time the genus *Thiothrix* is distinguished from the genus *Leucothrix* on the basis of the presence of a sheath and sulfur accumulation, but it is not clear whether these are distinguishing features that reflect the natural relationship of these organisms. This issue can only be resolved by a phylogenetic analysis of *Leucothrix* strains and filamentous bacteria isolated from natural samples.

Autotrophy may be one of the defining characteristics of the genus *Thiothrix*. Since the isolation of *Thiothrix ramosa*, at least two additional strains that are able to grow autotrophically on reduced sulfur compounds have been described (23, 24). Like the study of *Beggiatoa* strains, which took over 100 years to be grown autotrophically (15), the study of *Thiothrix* strains was hampered for a long time by difficulties in obtaining pure cultures. *Thiothrix nivea* was the first species to be isolated and characterized in detail (12). This organism appears to require a source of reduced sulfur for growth, and it has been suggested that *Thiothrix nivea* is an obligate chemolithoheterotroph (13). Attempts to grow *Thiothrix nivea* autotrophically

and to measure the activity of the CO₂-fixing enzyme of the Calvin-Benson cycle, ribulose-1,5-bisphosphate carboxylase-oxygenase, have failed so far. However, this species may possess the genetic potential to grow autotrophically since genes encoding the large subunit of ribulose-1,5-bisphosphate carboxylase-oxygenase have been detected by DNA hybridization (18). Other *Thiothrix* strains have only been grown heterotrophically, and in many cases it is not clear whether these organisms have been tested for lithotrophy. Thus, a reexamination of these strains may be valuable for determining if growth in inorganic medium is one of the defining features of the genus *Thiothrix*.

Overall, our results should give confidence to workers who identify *Thiothrix* strains in environmental samples on the basis of morphological characteristics. Future tests may reveal how widely distributed autotrophy is in this genus.

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