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EXPERIMENTAL ARTICLES

Molecular Phylogeny and Taxonomy of Colorless, Filamentous Sulfur Bacteria of the Genus *Thiothrix*

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Abstract—Comprehensive investigation combining molecular genetic techniques and comparative studies of morphological and physiological properties made it possible to resolve the disputed issue of the taxonomic status of the groups *"T. nivea"* and "Eikelboom type 021N" of the genus *Thiothrix*. The phylogenetic trees constructed on the basis of 16S rRNA and *gyrB* gene sequences demonstrated that members of the genus *Thiothrix* formed a cluster within the order *Thiotrichales*. According to the "ribosomal" tree, the cluster of the genus *Thiothrix* was divided into two main groups, I and II, corresponding to the groups *"T. nivea"* and "Eikelboom type 021N". The levels of similarity between the 16S rRNA gene sequences of *Thiothrix* species reached 88.9–100%. On the contrary, in the "gyrase" tree, these species were not divided into *"T. nivea"* and "Eikelboom type 021N" groups. The levels of similarity between the amino acid sequences of the *gyrB* gene fragments of *Thiothrix* species varied from 74.5 to 99.2%. Importantly, members of the groups *"T. nivea"* and "Eikelboom type 021N" formed very similar 16S rRNA secondary structures in the variable region V3, where a 30-nucleotide deletion characteristic of all *Thiothrix* species was detected. Phenotypic analysis of the studied bacteria revealed some morphological and physiological properties shared by the groups *"T. nivea"* and "Eikelboom type 021N". The data obtained indicate that members of the groups *"T. nivea"* and "Eikelboom type 021N" are phenotypically and genetically heterogeneous species within the single monophyletic genus *Thiothrix*..

Keywords: *gyrB, Thiothrix*, 16S rRNA, colorless sulfur bacteria.

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Filamentous sulfur-oxidizing bacteria of the genus *Thiothrix* are enclosed in distinct slimy sheaths, form rosettes, and reproduce by means of gonidia. In the presence of oxidized sulfur compounds, elemental sulfur is accumulated inside the cells [1].

Recent comparative investigations of the phenotypic and genotypic properties of *Thiothrix* species cast doubt on the phylogenetic integrity of this taxon [2, 3]. The genus *Thiothrix* consists of nine species traditionally divided by many researchers into two separate groups, *"T. nivea"* (*T. nivea, T. fructosivorans*, and *T. unzii*) and "Eikelboom type 021N" (*T. eikelboomii, T. disciformis*, and *T. flexilis*), according to their geno- and phenotypic properties [2]. Five *Thiothrix* strains were recently isolated from sulfide-rich environments and described as representatives of the novel species *T. caldifontis* ($G1^T$, G2, P, and K2) and *T. lacustris* (BL^T); according to their pheno- and genotypic properties, they are considered members of the *"T. nivea"* group [4]. Another species, *T. defluvii*, is phylogenetically closest to *T. flexilis*; however, determination of the species status of *T. defluvii* is based only on the results of the 16S rRNA gene analysis [2].

For decades, the taxonomic position of the "Eikelboom type 021N" group within the genus *Thiothrix* has been a matter of argument [2, 3, 6]. On the one hand, since its members differ from those of the *"T. nivea"* group not only in their 16S rRNA gene sequences (the level of 16S rDNA similarity between members of these groups does not exceed 92%), but also in the DNA G+C base content, as well as in some morphological and physiological properties, they were proposed to be reclassified into a new genus. Moreover, based on the high level of 16S rDNA polymorphism (90.6–94.5% similarity) within the group "Eikelboom type 021N", Wagner [6], Kanagawa [3], and Aruga [2] suggested to elevate the species *T. eikelboomii, T. disciformis*, and *T. flexilis* to the rank of

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independent genera. On the other hand, the results of multiphase studies conducted by Howarth [8] indicated the unity of the two groups of sulfur bacteria, *"T. nivea"* and "Eikelboom type 021N", at the genus level. First, in the "ribosomal" tree, bacteria of the genus *Thiothrix* form a single deep monophyletic cluster within the family *Thiotrichaceae*. Second, the species *T. nivea, T. unzii, T. fructosivorans, T. defluvii, T. disciformis,* and *T. eikelboomii* share common deletions in the region between nucleotides 455 and 472 of the 16S rRNA secondary structure, which have not been detected in the closest taxa. Third, the possibilities for reliable taxonomic identification of members of the groups *"T. nivea"* and "Eikelboom type 021N" are limited, since the morphological and physiological properties of these bacteria are variable, being, to a large extent, determined by the cultivation conditions and the ability of microorganisms to adapt to ambient conditions. According to Howarth, variability of the phenotypic properties was also detected in filamentous bacteria of the genus *Leucothrix*, the taxon most closely related to *Thiothrix*, according the results of the 16S rRNA gene analysis.

The above shows that our current knowledge about the phylogeny of sulfur bacteria of the genus *Thiothrix* is limited. The goal of the present work was to make up for this lack of knowledge. Based on analysis of the published data and the results of our studies, we carried out a taxonomic revision of *Thiothrix* species and studied the phylogenetic relationships within this genus. Unlike previous investigations, comparison of the genotypic properties of *Thiothrix* species was carried out not only by traditional analysis of the primary structure of the 16S rRNA genes, but also by phylogenetic analysis of amino acid sequences of the *gyrB* gene fragments. In addition, analysis of the secondary structure of the 16S rRNA genes of *Thiothrix* species was performed, and special attention was given to the construction and comparative investigation of the models of the variable regions V1–V8.

MATERIALS AND METHODS

Subjects of study. Bacterial strains isolated and described by us (*Thiothrix* sp. AS, *T. lacustris* BL^T = DSM 21227^T, *T caldifontis* G1^T = DSM 21228^T), as well as the type strains of *Thiothrix* species obtained from the strain collections DSMZ (*Thiothrix* sp. CT3 DSM 12730, *T. nivea* DSM 5205^T, *T disciformis* B3-1^T DSM 14473, and *T. flexilis* EJ2M-B^T DSM 14609) and ATCC (*T. fructosivorans* I ATCC 49749, *T. unzii* A1^T ATCC 49747, *T. eikelboomii* AP3^T ATCC 49788, and *Leucothrix mucor* ATCC 25107^T), were the subjects of this study.

DNA extraction. DNA extraction from the pure cultures of filamentous sulfur bacteria was carried out by the modified method described in [8].

Amplification and sequencing of the 16S rRNA and gyrB gene fragments of the studied strains were carried out as described in [4].

Phylogenetic analysis. The obtained nucleotide and deduced amino acid sequences of *Thiothrix* strains were aligned with the relevant sequences of bacteria of the order *Thiotrichales* using the CLUSTAL W v. 1.75 software package. The phylogenetic trees were constructed using the neighbor-joining method implemented in the MEGA5 software package [10].

To predict the RNA secondary structures, the RNA structure 5.2 [11] and Silva (http://www.arb-silva.de) software packages were used.

The DNA G+C base content was determined according to the thermal denaturation curves in a Pye Unicum SP 1800 spectrophotometer and calculated by the method of Owen et al. [12].

The fatty acid analysis was performed on a Sherlock gas chromatograph according to [13].

RESULTS AND DISCUSSION

Phenotypic analysis. The division of filamentous sulfur bacteria of the genus *Thiothrix* into two phylogenetic groups, *"T. nivea"* (*T. nivea, T. fructosivorans, T. lacustris, T. caldifontis*, and *T. unzii*) and "Eikelboom type 021N" (*T. eikelboomii, T. disciformis*, and *T. flexilis*) was confirmed in many respects by the pattern of phenotypic differences between these groups (Table 1). Slow growth of *T. defluvii* on nutrient media made it impossible to investigate its physiological and biochemical properties in detail.

Strains of the *"T. nivea"* are characterized primarily by distinct mucous sheaths enclosing their filaments, as well as by some common physiological properties. In particular, strains of this group are capable of accumulating elemental sulfur inside their cells and are characterized by the relatively high DNA G+C base content (49.3–52%). Strains of the "Eikelboom type 021N" group have large cells, their DNA G+C base content is lower (43.9–46.1%), and they are capable of utilizing various sugars, including glucose, mannose, maltose, and trehalose. It should be noted that this group shares a number of physiological properties (e.g., carbon sources and catalase activity) with *L. mucor*, the type species of another genus, *Leucothrix*.

At the same time, it is significant that some *Thiothrix* species do not possess the characteristic properties of their phenotypic groups. For instance, similar to members of the group "Eikelboom type 021N", chains of *T. unzii* cells are not enclosed in sheaths; *T. lacustris* is capable of utilizing aspartate and glutamate, while *T. caldifontis* is capable of utilizing only aspartate; *T. fructosivorans* utilizes fructose and sucrose as substrates. Similarly, along with members of the group *"T. nivea", T. eikelboomii* and *T. disciformis* accumulate elemental sulfur inside their cells (the lat-

Table 1. Phenotypic and chemotaxonomic properties of members of the genera *Thiothrix* and *Leucothrix*

Properties	"*T. nivea*" group					"Eikelboom type 021N" group			*Leucothrix mucor*
	T. lacustris	*T. caldifontis*	*T. fructosivorans*	*T. unzii*	*T. nivea*	*T. flexilis*	*T. disciformis*	*T. eikelboomii*	
Polysaccharide sheath	+	+	+	–	+	–	–	–	–
Cell size, μm	0.9–2.3 × 4.4–6.3	0.9–2.2 × 3.2–6.5	1.2–2.5 × 1.2–2.5	0.7–1.5 × 0.7–3.0	1.0–1.5	1.0–4.0 × 0.5–5.5	1.2–3.0 × 0.5–3.0	1.0–8.0 × 0.4–8.0	0.8–2.5 × 0.5–1.0
Resistance to NaCl (%)	ND	ND	ND	ND	ND	2	0.5	0.5	1.5
Temperature optimum, °C	24	25	25–27	ND	ND	20–30	25–30	25–30	25
Nitrate reduction	+	+	+	+	+	+	–	+	–
Catalase activity	–	–	±	–	–	+	+	+	+
Utilization of reduced sulfur compounds as electron donors	+	+	+	+	+	–	±‡	+**	±†
Intracellular accumulation of elemental sulfur	+	+	+	+	+	–	±‡	+	±†
Carbon sources:									
D-fructose	–	–	+	–	–	+	+	+	+
sucrose	–	–	+	–	–	+	+	+	+
D-glucose	–	–	–	–	–	+	+	+	+
trehalose	–	–	–	–	–	+	+	+	+
maltose	–	–	–	–	–	+	+	+	+
mannose	–	–	–	–	–	+	+	+	–
glycerol	–	–	–	–	–	–	+	+	–
mannitol	–	–	–	–	–	+	+	+	+
succinate	+	+	+	+	–	+	+	+	ND
butyrate	ND	ND	–	–	ND	–	+	+	ND

Table 1. (Contd.)

Properties	"*T. nivea*" group					"Eikelboom type 021N" group			*Leucothrix mucor*
	T. lacustris	*T. caldifontis*	*T. fructosivorans*	*T. unzii*	*T. nivea*	*T. flexilis*	*T. disciformis*	*T. eikelboomii*	
hydroxybutyrate	ND	ND	–	–	ND	+	+	+	+
citrate	ND	ND	–	–	–	–	+	–	ND
aspartate	+	+	–	–	–	+	+	ND	ND
glutamate	+	–	–	–	–	+	+	+	+
Hydrolysis of polymeric compounds:									
gelatin	–**	–**	+	+	+	+	+	+	–
casein	–	–	–	+	+	–	–	–	–
starch	–	–	–	–	–	–**	–**	–	–**
Fatty acid composition, %			*	**	***				**
C16:0	64.5	26.7	22.5	40.4	21.4	26	18	33	14.0
C16:1	10.9	38.7	39.3	41.4	45.5	30	13	13	44.9
C18:1	24.6	27.3	28.1	11.3	26.9	44	68	52	38.3
DNA G+C base content, mol %	51.4	52	51.5*	49.3**	52	44.0–44.4	43.9–44.7	44.1–46.1	47–49

Note: The data presented were obtained from [5, 7] (*T. unzii* and *T. fructosivorans*), [16] (*T. nivea*), [2] ("Eikelboom type 021N"), [4] (*T. lacustris* and *T. caldifontis*), and [17] (*L. mucor*). "+" stands for detected; "–" for not detected; "±" indicates weak growth; ND stands for "no data".

* [4].

** Our unpublished data.

*** Data obtained from DSMZ.

£ Limits of the DNA G+C base content (%) of the species, including the type and non-type strains [2].

† Lithotrophic growth in the presence of reduced sulfur compounds and intracellular accumulation of elemental sulfur are possible only at low concentrations of organic matter [18].

‡ Lithotrophic growth in the presence of reduced sulfur compounds and intracellular accumulation of elemental sulfur are possible only at low concentrations of organic matter (our unpublished data).

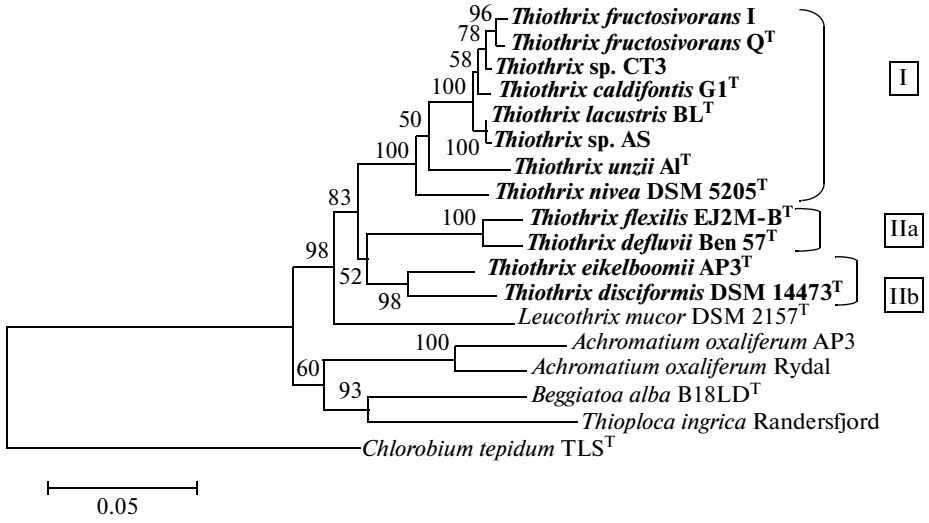

Fig. 1. Phylogenetic tree based on comparative analysis of the 16S rRNA gene sequences of members of the family *Thiotrichaceae* closely related to the genus *Thiothrix*.

ter only at low concentrations of organic matter, 50–100 mg/L), while *T. flexilis* is incapable of utilizing glycerol as the growth substrate. Also, the predominance of the fatty acids $C_{16:1}$, $C_{16:0}$, and $C_{18:1}$ is a characteristic trait of these phenotypically heterogeneous groups of the genus *Thiothrix*.

Phylogenetic analysis of the 16S rRNA gene sequences. In the phylogenetic tree of the family *Thiotrichaceae, Thiothrix* strains formed a single cluster divided into two main groups (I and II) (Fig. 1) corresponding to the groups *"T. nivea"* and "Eikelboom type 021N", respectively. The level of similarity between the 16S rRNA gene sequences of these groups did not exceed 91.9% (Table 2).

The majority of members of the phylogenetic group I (*T. fructosivorans, T. caldifontis*, and *T. lacustris* strains, as well as *Thiothrix* sp. CT3) were found to be closely related. The level of similarity between the 16S rRNA gene sequences of these strains varied from 98.7 to 100%. At the same time, the type strains of two other species of this group, *T. nivea* and *T. unzii*, formed separate branches, rather distant from other representatives of group I. The level of similarity between their 16S rRNA gene sequences, as well as the similarity level between these strains and other representatives of group I, was 93.9–95.6%. The results of the 16S rRNA gene analysis indicated that group I was not genetically heterogeneous. The revealed genetic heterogeneity contradicts the results of phenotypic analysis, including the results of the detailed studies of sulfur metabolism, which did not show any fundamental differences between strains within this group, except for *T. unzii*, whose filaments were not enclosed in polysaccharide sheaths.

The phylogenetic group II of sulfur bacteria of the genus *Thiothrix* split into two separate subgroups, IIa and IIb. The level of similarity between the 16S rRNA gene sequences of these subgroups was only 90.6–91.6%. Subgroup IIa included *T. defluvii* and *T. flexilis* (Fig. 1) with a high level of 16S rDNA similarity (97.2%). The extent of phylogenetic divergence of members of subgroup IIb (*T. eikelboomii* and *T. disciformis*) was found to be higher (94.5% similarity). However, representatives of subgroups IIa and IIb, despite the phylogenetic distance between them, shared a wide range of phenotypic properties, according to which these strains were combined into one group, "Eikelboom type 021N".

The presently accepted threshold value of 16S rRNA gene sequence similarity used for description of potentially new genera of prokaryotes is 93–94%. According to this statement, the phylogenetic groups I, IIa, and IIb exhibiting 16S rRNA similarity levels of 88.9–91.9% may be formally described as new genera. However, the similarity criterion corresponding to the intergeneric level of 16S rRNA similarity is based primarily on the results of study of existing taxa. At the same time, none of the existing bacterial taxa is fully studied, and the extent of phylogenetic divergence even of the well-characterized taxa differs considerably. Moreover, high phylogenetic heterogeneity of *T. fructosivorans, Thiothrix* sp. CT3, *T. caldifontis, T. lacustris, Thiothrix* sp. AS, *T. nivea*, and *T. unzii* within group I did not manifest itself at the phenotypic level. The distribution of *T. flexilis, T. eikelboomii*, and *T. disciformis* between the phylogenetic subgroups IIa and IIb did not correlate with the results of the phenotypic analysis.

Hence, the discrepancy between the results of phenotypic and phylogenetic analyses indicated that further taxonomic revision of the genus *Thiothrix* based on analysis of other phylogenetic markers was required.

Table 2. Similarity level (%) based on comparative analysis of the 16S rRNA gene sequences of *Thiothrix* strains and reference strains of the order *Thiotrichales*

Thiothrix lacustris BL^T (EU642572)	100														
Thiothrix sp. AS (HQ236427)	100	100													
Thiothrix caldifontis G1^T (EU642573)	98.8	98.8	100												
Thiothrix sp. CT3 (AF148516)	99.1	99.1	99.0	100											
Thiothrix fructosivorans I (L79963)	98.8	98.8	98.7	99.2	100										
Thiothrix fructosivorans Q^T (L79962)	98.8	98.8	98.9	99.4	99.4	100									
Thiothrix unzii A1^T (HQ897926)	95.0	95.0	95.1	95.6	93.9	93.9	100								
Thiothrix nivea DSM 5205^T (HQ823668)	95.0	95.0	95.1	94.7	93.6	93.9	94.6	100							
Thiothrix defluvii Ben57^T (AF127020)	90.8	90.8	90.6	90.7	90.3	89.6	89.6	90.0	100						
Thiothrix flexilis EJ2M-B^T (AB042545)	91.1	91.1	91.2	91.2	90.1	90.1	88.9	89.7	97.2	100					
Thiothrix eikelboomii AP3^T (AB042819)	91.9	91.9	91.7	91.9	91.6	91.4	91.6	91.5	91.6	90.6	100				
Thiothrix disciformis DSM 14473^T (HQ823669)	91.1	91.1	91.1	91.0	90.5	90.4	90.5	90.3	91.6	90.6	94.5	100			
Leucothrix mucor ATCC 25107^T (HQ897925)	89.5	89.5	89.5	89.5	88.8	89.1	88.0	88.9	88.6	88.7	87.9	89.0	100		
Thioploca ingrica (L40998)	85.4	85.4	85.9	85.7	84.6	84.6	85.2	85.6	84.7	85.6	85.8	85.0	83.7	100	
Achromatium oxaliferum (L42543)	85.7	85.7	85.2	85.6	84.9	84.9	84.7	86.5	85.7	85.1	86.1	86.6	85.0	86.4	100

Phylogenetic analysis of amino acid sequences of the *gyrB* gene fragments. Analysis of protein-coding genes is presently widely used for phylogenetic studies, especially in cases when the results of traditional 16S rRNA gene analyses may be ambiguous. Comparative analysis of the genes belonging to the group of the so-called housekeeping genes may be the most promising approach. As phylogenetic markers, these genes have the same advantages as 16S rRNA genes: universality, evolutionary conservatism, and vertical inheritance. At the same time, at different taxonomic levels, phylogenetic analysis of the housekeeping gene sequences can yield better results, since (1) different genes have different levels of conservatism, and interspecific differences in some gene sequences can be more distinct than in the case of 16S rRNA genes, and (2) in the genome, these genes are present in a single copy. The *gyrB* gene encoding the B subunit of DNA gyrase is such a phylogenetic marker frequently used for taxonomic studies [9].

A phylogenetic tree (Fig. 2) with the topology differing considerably from that of the "ribosomal" tree (Fig. 1) was constructed on the basis of the deduced amino acid sequences of the *gyrB* gene of filamentous bacteria of the genus *Thiothrix*. In the phylogenetic tree, the *gyrB* genes of the genus *Thiothrix* are clustered together, similar to the 16S rRNA genes; however, the strains inside the cluster occupied different positions, and division into the groups *"T. nivea"* and "Eikelboom type 021N" was not observed. In the "gyrase" tree, strains of the species *T. caldifontis, T. lacustris, T. fructosivorans*, as well as *Thiothrix* sp. AS and *Thiothrix* sp. CT3, were clustered in a single group of closely related microorganisms similar to that in the "ribosomal" tree; however, this group included *T. unzii*, which, in the "ribosomal" tree, was separated from other strains within group I and occupied an intermediate position between these closely related species and *T. nivea*. The levels of similarity between the amino acid sequences of the *gyrB* gene fragments

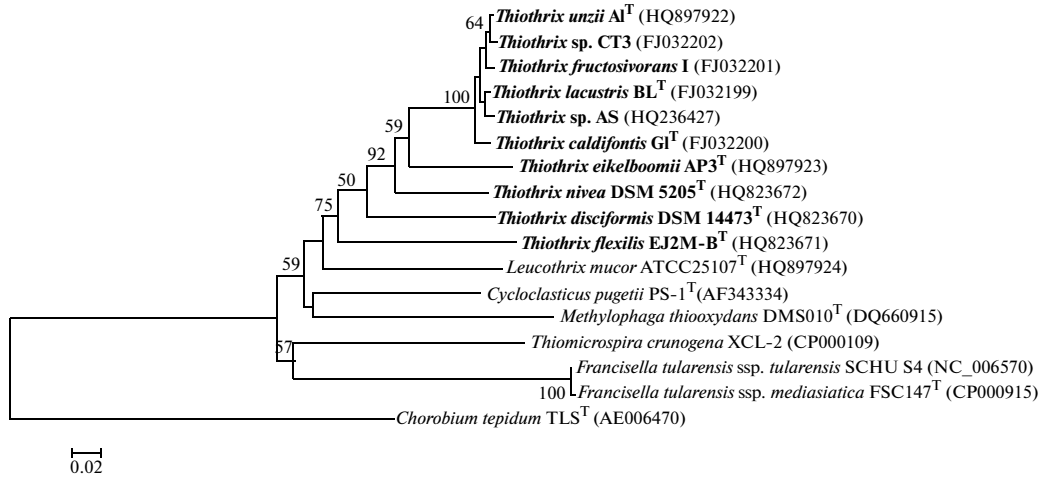

Fig. 2. Phylogenetic tree based on comparative analysis of deduced amino acid sequences of the *gyrB* gene fragments demonstrates the taxonomic position of *Thiothrix* strains within the order *Thiotrichales*.

within this group of *Thiothrix* species varied from 96.9 to 99.2% (Table 3). Other species, *T. flexilis, T. disciformis, T. eikelboomii*, and *T. nivea*, occupied separate phylogenetic branches at an equal distance both from each other and from the group of the closely related species *T. unzii, T. caldifontis, T. lacustris*, and *T. fructosivorans* (74.5–88.2% similarity) (Table 3).

Hence, the topologies of the phylogenetic trees based on two types of data (*gyrB* and 16S rDNA) differed significantly. At the same time, *gyrB* gene analysis confirmed the existence of the common universal cluster of *Thiothrix* species, previously revealed by 16S rRNA gene analysis.

Analysis of 16S rRNA secondary structures. Determination of the 16S rRNA secondary structure modes is used as an additional criterion confirming the common origin or divergence of bacterial taxa. The 16S rRNA secondary structure shows the highest stability during evolution. The primary structure (the nucleotide sequence of the gene encoding 16S rRNA) may be altered by the forces of natural selection or accidental events. For closely related organisms, the configuration of loops and helixes ("hairpins"), the elements of the RNA secondary structure is specific and facilitates the determination of the phylogenetic unity of the group.

The secondary structure models of eight variable 16S rRNA regions (V1: positions 61–106), (V2: 136–227), (V3: 437–497; Fig. 3), (V4: 588–651), (V5: 821–879), (V6: 997–1044), (V7: 1118–1155), and (V8: 1241–1296) were analyzed. In region V3 of all members of the genus *Thiothrix*, a common deletion was detected between *E. coli* positions 455 and 472. The most plausible secondary structure models of the region V3 of all *Thiothrix* species were found to be very similar and differed from those of members of other genera of the order *Thiotrichales*, in particular, from the models of the phylogenetically close genus *Leucothrix* (Fig. 3). The common deletion revealed in *Thiothrix* species resulted in the formation of the secondary structure in the form of a helix shortened compared to the closest *Leucothrix* species. The secondary structures of region V3 of *Thiothrix* differed insignificantly by some nucleotides of their loop hairpins, internal loops, and helixes. No evolutionary significant events (insertions or deletions) that would allow unambiguous division of the genus *Thiothrix* into phylogenetically separate groups were detected in the variable V1, V2, and V4–V8 regions. In these regions, only nucleotide replacements were detected, which have a lower evolutionary weight than nucleotide insertions and deletions [14]. Moreover, each of these regions (V1, V2, and V4–V8) represented its own unique group of species which did not reflect the expected phenotypic or genotypic diversity of *Thiothrix* species.

The similarity in the configuration of the evolutionary significant region V3 of the 16S rRNA secondary structures demonstrates the common origin and phylogenetic unity of *Thiothrix* species. The absence of taxonomic correlations between the species composition of bacterial groups obtained by sequence analysis of less significant 16S rRNA regions (V1, V2, V4–V8) indicates the intrageneric heterogeneity of the studied genus *Thiothrix*. Variations of some nucleotides which form loops and helixes in the secondary structure of region V3 in all representatives of this genus confirm this assertion.

Taxonomic conclusion. The obtained comprehensive phenotypic and phylogenetic data demonstrate the common origin and phylogenetic unity of members of the groups *"T. nivea"* and "Eikelboom type 021N" within the genus *Thiothrix*. This conclusion was derived from the following facts:

(1) Variability of the morphological and physiological properties of members of the groups *"T. nivea"* and "Eikelboom type 021N". Importantly, despite the

Table 3. Similarity level (%) based on comparative analysis of amino acid sequences of the *gyrB* gene fragments of *Thiothrix* strains and reference strains of the order *Thiotrichales*

Thiothrix lacustris BL^T (FJ032199)	100													
Thiothrix sp. AS (HQ236428)	99.2	100												
Thiothrix caldifontis G1^T (FJ032200)	97.7	98.0	100											
Thiothrix sp. CT3 (FJ032202)	98.9	98.0	97.5	100										
Thiothrix fructosivorans I (FJ032201)	98.3	98.0	97.5	98.9	100									
Thiothrix unzii A1^T (HQ897922)	98.9	98.6	96.9	99.4	98.9	100								
Thiothrix nivea DSM 5205^T (HQ823672)	87.6	88.2	87.9	87.0	87.6	87.3	100							
Thiothrix flexilis EJ2M-B^T (HQ823671)	79.2	79.2	79.2	79.2	79.2	79.3	80.8	100						
Thiothrix eikelboomii AP3^T (HQ897923)	88.2	88.2	87.0	88.2	74.5	87.9	86.8	80.6	100					
Thiothrix disciformis DSM14473^T (HQ823670)	83.7	84.2	84.3	83.4	83.9	83.7	83.7	82.0	84.5	100				
Leucothrix mucor ATCC25107^T (HQ897924)	78.5	78.5	78.7	78.5	77.6	78.2	78.5	77.3	76.5	76.5	100			
Francisella tularensis SCHU S4 (NC_006570)	70.1	69.6	70.1	70.4	70.1	70.7	70.6	72.0	69.3	69.9	69.3	100		
Thiomicrospira crunogena XCL-2 (CP000109)	72.1	72.4	71.8	71.3	71.5	71.5	74.9	73.7	73.5	74.4	73.8	70.7	100	
Methylophaga thiooxidans DMS010^T (DQ660915)	74.1	74.1	74.4	74.1	73.5	73.5	72.6	69.2	71.3	73.0	72.4	69.3	72.4	100

morphological and physiological isolation of the groups *"T. nivea"* and "Eikelboom type 021N", some species exhibited certain uncharacteristic phenotypic properties shared by both groups. The content of the G+C base pairs in the DNA of members of the groups *"T. nivea"* and "Eikelboom type 021N" is 49–52% and 44–46%, respectively, which agrees with generally accepted views on the fluctuation limits of the molar G+C content in the DNA of species belonging to the same genus. Numerous studies conducted to find the allowable level of differences in the DNA G+C content of bacterial taxa showed that the differences between members of the same species in their G+C content should not exceed 5%, whereas the differences between members of the same genus should not exceed 10%. For instance, the content of the G+C base pairs in the DNA of *Staphylococcus* species varies from 30 to 39% [15].

(2) In the phylogenetic trees constructed on the basis of 16S rRNA gene sequences and the deduced amino acid sequences of the *gyrB* gene fragments, members of the groups *"T. nivea"* and "Eikelboom type 021N" form a single deep monophyletic cluster within the family *Thiotrichaceae* and the order *Thiotrichales*, respectively. However, when comparing the dendrograms constructed on the basis of analysis of both types of data, discrepancies in the positions of some species within this cluster were detected, which indicates the internal genetic heterogeneity of the genus *Thiothrix*.

(3) The common origin and phylogenetic unity of *Thiothrix* species are confirmed by the results of comparative analysis of 16S rRNA secondary structures. Members of the groups *"T. nivea"* and "Eikelboom type 021N" formed very similar V3 secondary structures of the variable 16S rRNA region.

Hence, the diversity of the groups *"T. nivea"* and "Eikelboom type 021N" at the phenotypic and genotypic levels is probably due to the intrageneric variability at this stage of the evolution of the genus *Thiothrix,* and not due to some macroevolutionary processes resulting in the formation of new genera. Therefore, *"T. nivea"* and "Eikelboom type 021N" consist of different species of the same genus, *Thiothrix*. Within the

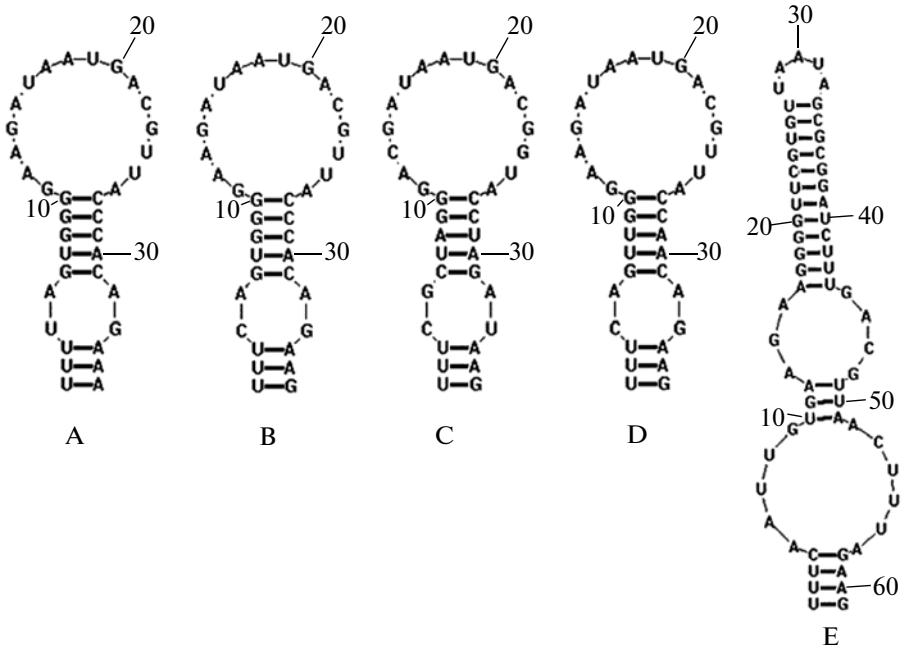

Fig. 3. Presumable secondary structures of the variable region V3 (*E. coli* positions 437–497) of *Thiothrix* species (A, B, C, D) and *Leucothrix mucor* (E). Secondary structures: *T. eikelboomii* (A); *T. nivea* (B)*; T. defluvii* and *T. flexilis* (C); *T. unzii, T. fructosivorans, T. caldifontis, T. lacustris,* and *Thiothrix* sp. CT3 (D).

group "Eikelboom type 021N", the discrepancy between the results of phenotypic and genotypic analyses was revealed. The pronounced genetic heterogeneity of the species with similar phenotypes, *T. eikelboomii, T. flexilis*, and *T. disciformis*, may indicate that, during the evolution of these species, neutral changes were accumulated in their nucleotide sequences, which can be detected with molecular techniques; however, they did not result in any phenotypic changes. The fate of these changes depends, to a great extent, on stochastic processes, and any genetic heterogeneity within a population is the intermediate stage between the fixation and loss of genetic characters [14]. In theory, this process may result in the formation of new taxa during evolution.

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