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EXPERIMENTAL ARTICLES

Phylogeny of Ribulose-1,5-Bisphosphate Carboxylase/Oxygenase Genes in Haloalkaliphilic Obligately Autotrophic Sulfur-Oxidizing Bacteria of the Genus *Thioalkalivibrio*

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Abstract—Fragments of genes of the "green-like" form I ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) of eight species of haloalkaliphilic obligately autotrophic sulfur-oxidizing bacteria of the genus *Thioalkalivibrio* have been revealed and sequenced using previously developed oligonucleotide primers. The data obtained are used for the construction of phylogenetic trees on the basis of nucleotide sequences of RuBisCO genes and their conceptual translations into amino acid sequences. Comparative analysis of the 16S rRNA and RuBisCO gene trees reveals discrepancies between their topologies. According to a RuBisCO gene analysis, the genus *Thioalkalivibrio* is not monophyletic, and its inner divergence conforms to the significant morphological differences observed between the species. Presumably, horizontal (interspecies) gene transfer was involved in the evolution of the genus *Thioalkalivibrio.*

Key words: haloalkaliphilic sulfur-oxidizing bacteria, *Thioalkalivibrio*, ribulose-1,5-bisphosphate carboxylase/oxygenase, RuBisCO, phylogenetic analysis.

Until recently, only acidophilic and neutrophilic species were known among obligately and facultatively autotrophic sulfur-oxidizing bacteria (SOB). However, as a result of intensive microbiological research on soda lakes, a new group of haloalkaliphilic obligately autotrophic SOB has been found. These bacteria are distinguished by their ability to grow optimally in saline alkaline media at pH values from 9 to 10.6 [1]. Currently, this group includes three genera of gammaproteobacteria: *Thioalkalimicrobium, Thioalkalivibrio*, and *Thioalkalispira* [2, 3]. The genus *Thioalkalivibrio* is the most widespread in soda lakes, in particular, hypersaline soda lakes. It is characterized by low growth rates, high resistance to sodium salts, and significant metabolic and species diversity. Currently, this genus incorporates nine species of haloalkaliphilic obligately chemolithoautotrophic SOB. According to data from 16S rRNA sequencing, the genus *Thioalkalivibrio* forms a single phylogenetic cluster. However, the species of this genus significantly differ, both morphologically and metabolically [2, 4–8].

Currently, in phylogenetic studies, alternative molecular markers that are not connected to the mechanisms of protein synthesis are widely used. The selected markers are usually the functional genes responsible for metabolic properties specific to the microorganism group under study.

Most autotrophic organisms assimilate CO_2 via the Calvin cycle. The key enzyme of this cycle is ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO). It has been shown that the *Thioalkalivibrio* species have RuBisCO activity and, thus, fix CO_2 via the Calvin cycle [1]; however, the type and structure of their RuBisCO, as well as their RuBisCO gene sequences, remain unknown.

In eubacterial cells, RuBisCO occurs in two major forms. Form I is the most widespread and is present in the cells of most autotrophic bacteria, algae, and terrestrial plants. It consists of eight large (L) and eight small (S) subunits, which are encoded by *cbbL* and *cbbS* genes, respectively. Only the large subunits have a catalytic function. Form I RuBisCO is subdivided into two types, "green-like" and "red-like," which differ in the amino acid composition of their large subunits. The green-like RuBisCOs are present in the chloroplasts of terrestrial plants; green algae; cyanobacteria; and representatives of alpha, beta-, and gamma-proteobacteria. The red-like RuBisCOs are found in most nongreen

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algae and some representatives of α- and β-proteobacteria. Both the green-like and red-like enzymes have been found in *Rhodobacter azotoformans* [9]. Form II RuBisCOs (*cbbM* gene) occur much more rarely and only in bacteria. Form II consists of only large subunits (L_n), from two to eight, depending on the organism.

In our previous work, we developed and tested a maximally universal system of oligonucleotide primers allowing amplification of the genes of large subunits of RuBisCOs from different taxonomic groups of bacteria [10]. In addition, we sequenced the *cbbL* gene of green-like form I RuBisCO from *Thioalkalispira microaerophila*, the only species of another genus of obligately autotrophic SOB.

The aim of the present work was to apply the above-mentioned system of primers to the detection, amplification, and sequencing of RuBisCO genes in the type strains of *Thioalkalivibrio* species and to carry out phylogenetic analysis of the results obtained.

MATERIALS AND METHODS

The subjects of this study were the type strains of eight species of the genus *Thioalkalivibrio* from the collection of the Winogradsky Institute of Microbiology, Russian Academy of Sciences.

The isolation of DNA and amplification and sequencing of fragments of RuBisCO genes were conducted as described in [10].

Phylogenetic analysis of the sequences. Editing of the obtained sequences was carried out using the BioEdit software package (http://jwbrown.mbio.ncsu.edu/ BioEdit/bioedit.html). Primary comparison of the de novo determined sequences with sequences within the GenBank database was performed using NCBI BLAST software (http://www.ncbi.nlm.nih.gov/blast). A further comparative analysis was conducted using GenBank sequences of the *cbbL* and 16S rRNA genes of autotrophic bacteria harboring green-like form I RuBisCOs.

The GenBank *cbbL* gene sequences used were those of *Wautersia metallidurans* CH34, AAAI02000032; *Hydrogenophaga pseudoflava* DSM 1083, U55037; *Acidithiobacillus ferrooxidans* ATCC 23270^T *cbbL*-1, AF129925; *Acidithiobacillus ferrooxidans* ATCC 23270^T *cbbL*-2, AF307091; *Thiobacillus denitrificans* ATCC 25259^T, L42940; *Allochromatium vinosum* ATCC 17899^T *cbbL*-1, M26396; *Allochromatium vinosum* ATCC 17899^T *cbbL*-2, D90204; *Thiomonas intermedia* K12, AF046933; *Nitrobacter winogradskyi* ATCC 14123^T, AF109914; *Nitrobacter vulgaris* T3, L22885; *Ectothiorhodospira shaposhnikovii* DSM 243^T, AY450587; *Methylococcus capsulatus* Bath, AB082933; arsenite-oxidizing bacterium MLHE-1, AF483208; *Hydrogenophilus thermoluteolus* TH-1^T, D30764; *Rhodobacter capsulatus*, L82000; *Synechococcus* sp. WH7803, U46156; *Halothiobacillus neapolitanus* ATCC 23641^T, AF038430; *Thioalkalispira microaerophila* ALEN 1^T, AY450586; *Hydrogenovibrio marinus* MH-110, *cbbL*-1, D43621; *Hydrogenovibrio marinus* MH-110, *cbbL*-2, D43622; *Prochlorothrix hollandica*, X57359; *Synechococcus* sp. PCC 6301, X03220; *Synechococcus* sp. PCC 7002, D13971; and *Anabaena* sp. PCC 7120, J01540.

The GenBank 16S rRNA gene sequences used were those of *Thioalkalivibrio jannaschii* ALM 2^T, AF329083; *Thioalkalivibrio versutus* Al 2^T, AF126546; *Thioalkalivibrio nitratis* ALJ 12^T, AF126547; *Thioalkalivibrio thiocyanoxidans* ARH 2^T, AF 302081; *Thioalkalivibrio paradoxus* ARH 1^T, AF 151432; *Thioalkalivibrio nitratireducens* ALEN 2^T, AY079010; *Thioalkalivibrio thiocyanodenitrificans* ArhD^T, AY360060; *Thioalkalivibrio denitrificans* ALJD^T, AF 126545; *Ectothiorhodospira shaposhnikovii* DSM 243^T, M59151; arsenite-oxidizing bacterium MLHE-1, AF406554; *Allochromatium vinosum* ATCC 17899^T, M26629; *Methylococcus capsulatus* ACM 3302^T, X72771; *Acidithiobacillus ferrooxidans* ATCC23270^T, AJ278718; *Hydrogenophilus thermoluteolus* TH-1^T, AB009828; *Thiobacillus denitrificans* NCIMB 9548^T, AJ243144; *Thiomonas intermedia* ATCC 5466^T, AY455809; *Wautersia metallidurans* CH34, Y10824; *Hydrogenophaga pseudoflava* ATCC 33668^T, AF078770; *Halothiobacillus neapolitanus* DSM 581^T, AF173169; *Hydrogenovibrio marinus* JCM 7688^T, D86374; *Rhodobacter capsulatus* ATCC11166^T, D13474; *Nitrobacter winogradskyi* ATCC 14123^T, AY055796; *Synechococcus* sp. PCC 7002, AJ000716; *Anabaena* sp. PCC 7120, X59559; *Prochlorococcus marinus* GP2, AF001472; *Prochlorothrix hollandica*, AJ007907; and *Synechococcus* sp. PCC 6301, X03538.

The analyzed sequences were aligned using the CLUSTALW v 1.75 software package. Phylogenetic trees were constructed by the different methods implemented in the TREECONW [11], PHYLIP [12], and GENEBEE [13] software packages.

Deposition of nucleotide sequences. The nucleotide sequences of fragments of RuBisCO genes determined in this work were deposited with the GenBank database (accession numbers, AY914800–AY914807).

RESULTS

Detection and sequencing of RuBisCO genes in different species of the genus *Thioalkalivibrio*. For the detection and sequencing of RuBisCO genes from the type strains of *Thioalkalivibrio* species, we used our previously developed system of oligonucleotide primers [10]. In all of the strains studied, amplification with the corresponding primers generated PCR products analogous to fragments of the green-like form I RuBisCO obtained in positive control reactions. The use of primers specific to the genes of red-like form I and form II RuBisCOs did not yield the corresponding PCR products in any of the species.

As a result of the sequencing of the PCR products obtained, we determined sequences (about 750-bp) of

the DNA fragments for all of the strains studied. Preliminary screening in the GenBank database revealed a high level of similarity between the nucleotide sequences determined de novo and the *cbbL* gene sequences of other bacteria (84–89%), confirming their affiliation to one family of genes.

Phylogenetic analysis of *cbbL* genes. We aligned the obtained nucleotide sequences of RuBisCO gene fragments, as well as the deduced amino acid sequences of the corresponding proteins, with analogous sequences of green-like form I RuBisCOs available from the GenBank database and compared 741 positions for nucleotides and 247 positions for amino acid residues. The topologies of the phylogenetic trees constructed on the basis of these alignments were identical in all of the methods used: the neighbor-joining (Figs. 1b, 1c), maximum parsimony, Fitch–Margoliash, and the maximum topological similarity methods (data not shown).

Generally, the topology of the obtained phylogenetic trees was similar to that of the trees for green-like form I RuBisCO genes presented in [14, 15]. Moreover, analogously to these earlier results, the obtained topologies did not coincide with those of the 16S rRNA gene trees. This was true for *Thioalkalivibrio* strains in particular. According to an analysis of the 16S rRNA gene sequences, all *Thioalkalivibrio* species form a monophyletic cluster within a phylogenetic subdivision of the family *Ectothiorhodospiraceae* (Fig. 1a); however, the cluster is divided into three phylogenetic groups. At the same time, the green-like form I RuBisCO genes of the genus *Thioalkalivibrio* are not monophyletic, although their division into independent clusters partly correlates with the phylogenetic divergence revealed by the 16S rRNA gene analysis.

In addition, the phylogenetic relations both within and between these clusters are different for RuBisCO trees constructed on the basis of nucleotide and amino acid sequences (Figs. 1b, 1c). The largest cluster, cluster 1, including the species *Thioalkalivibrio versutus, T. jannaschii, T. nitratis*, and *T. thiocyanoxidans* and characterized by high sequence similarities (91.5–96.6% for nucleotides and 93.2–98.0% for amino acids) and strong bootstrap support (80 to 100% depending on the method of tree construction), was present in both types of RuBisCO trees and corresponded to 16S rDNA phylogenetic group 1. The position of the branching point of this cluster was unstable in both types of RuBisCO trees, with bootstrap support being no higher than 30% for all of the methods of tree construction. Cluster 2, including the species *T. nitratireducens* and *T. paradoxus* and characterized by high sequence similarities (91.9% for nucleotides and 96.3% for amino acids) and absolute bootstrap support (100%), was also present in both types of RuBisCO trees and corresponded to 16S rDNA phylogenetic group 2. In all of the RuBisCO trees, this cluster was grouped, with a bootstrap support of 70 to 96%, with one of the two *cbbL* genes of the purple sulfur bacterium *Allochromatium vinosum.* However, the sequence similarities were relatively low (82.3–82.6% for nucleotides and 86.2–86.6% for amino acids). Cluster 3, including species *T. denitrificans* and *T. thiocyanodenitrificans* and corresponding to 16S rDNA phylogenetic group 3, was present only in the tree constructed on the basis of nucleotide sequences. This cluster was characterized by a relatively high similarity of its sequences (90.0%) but a lower, in comparison with the two other clusters, bootstrap support (33-82%), as well as a more unstable position of its branching point (with bootstrap support of no higher than 23%). In addition, in the tree constructed on the basis of amino acid sequences, this cluster collapsed into two independent branches, which were both characterized by unstable phylogenetic positions (with bootstrap support of no higher than 50%).

DISCUSSION

The genera *Thioalkalimicrobium* and *Thioalkalispira* and the genus *Thioalkalivibrio*, studied in this work, are the first discovered representatives of obligately haloalkaliphilic chemolithoautotrophic bacteria. Comparative phylogenetic analysis of the 16S rRNA gene, a traditional molecular marker, and the gene encoding the large subunit of RuBisCO, the key enzyme of CO_2 autotrophic fixation, may reveal data providing an insight into the evolution and taxonomy of these bacteria. In particular, the analysis of the 16S rRNA genes revealed their phylogenetic affiliation to purple sulfur bacteria of the family *Ectothiorhodospiraceae*, which inhabit the same haloalkaline ecosystems. This allows us to assume that these bacterial groups have a common origin.

The RuBisCO gene analysis did not reveal any close phylogenetic relatedness between the only species of the genus *Thioalkalispira* and any of the species of the genus *Thioalkalivibrio* (Fig. 1), which justifies the separation of these two types of haloalkaliphilic SOB into two different genera on the basis of phenotypic and genotypic data.

A basic discrepancy in the phylogenetic structure of the genus *Thioalkalivibrio* was revealed by a comparison of the phylogeny of genes of different types. The polyphyletic nature of the RuBisCO genes did not agree with the established earlier monophyletic nature of the 16S rRNA genes. At the same time, the haloalkaliphilic SOB species under consideration were united into a single genus mainly based on the results of the 16S rRNA analysis suggesting their monophyletic origin. However, a considerable degree of correlation does exist between the two types of phylogenetic trees.

Comparative analysis of the phylogenies of both types showed that the species of the largest group, *T. versutus, T. jannaschii, T. nitratis*, and *T. thiocyanoxidans*, are undoubtedly related and have a common origin. Although, on the basis of their physiologic and

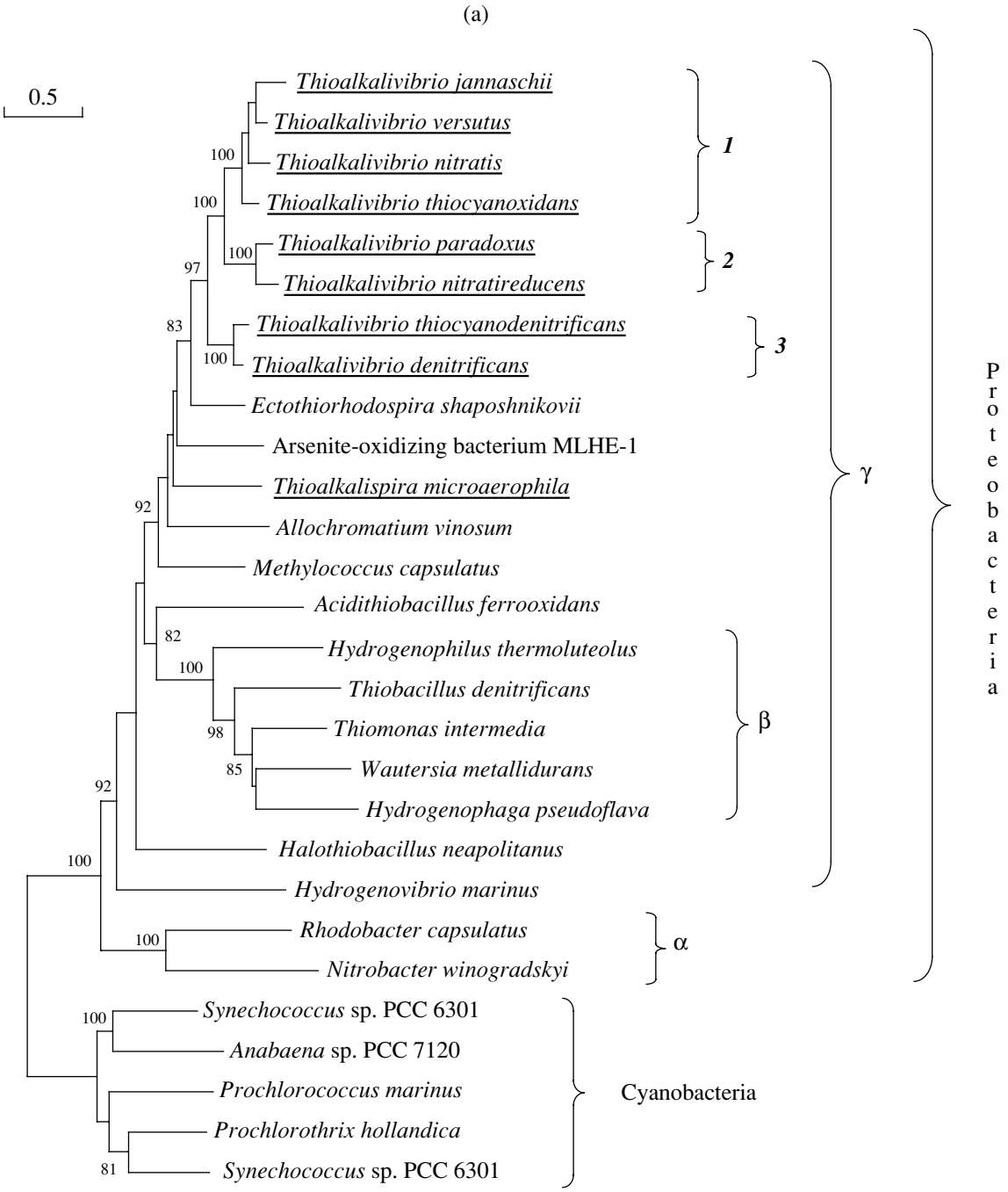

Fig. 1. Unrooted phylogenetic trees of the autotrophic bacteria under study (possessing green-like form I RuBisCO) constructed by the neighbor-joining method. The numerals at the branching points show the significance of the branching order as determined by bootstrap analysis of 100 alternative trees. Only values above 70% were considered significant. (a) Tree constructed on the basis of an analysis of nucleotide sequences of 16S rRNA genes. The underlined sequences refer to obligately haloalkaliphilic chemolithoautotrophic bacteria of the genera *Thioalkalivibrio* and *Thioalkalispira.* The bar shows evolutionary distance, corresponding to 5 substitutions per 100 nucleotides. (b) Tree constructed on the basis of an analysis of nucleotide sequences of *cbbL* genes. The sequences in bold were determined in this work. The sequence belonging to *Thioalkalispira microaerophila* is underlined. The bar shows evolutionary distance, corresponding to 10 substitutions per 100 nucleotides. (c) Tree constructed on the basis of an analysis of the deduced amino acid sequences of RuBisCO. The sequences in bold were determined in this work. The sequence belonging to *Thioalkalispira microaerophila* is underlined. The bar shows evolutionary distance, corresponding to 5 substitutions per 100 amino acid residues.

(b)

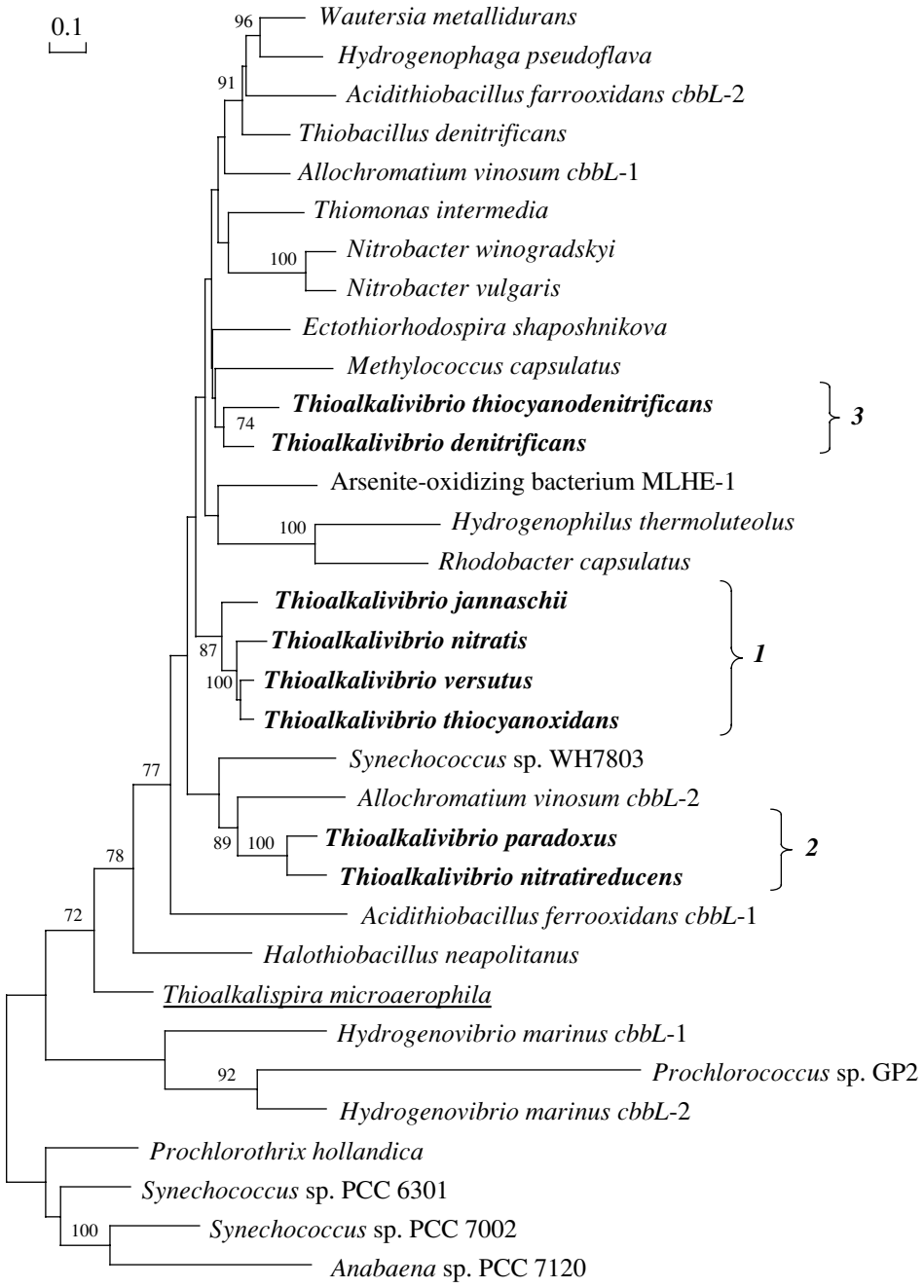

Fig. 1. (Contd.)

metabolic differences, the representatives of this group can be divided into independent species, all of them are of a typical curved shape and are motile by means of a single polar flagellum, and most of them are capable of growth in hypersaline environments and produce a yellow pigment. The slightly halophilic denitrifying rods *T. denitrificans* and *T. thiocyanodenitrificans* form a separate phylogenetic group, which, according to the 16S rRNA sequence analysis, is the closest to the branching point of the whole cluster of the genus *Thioalkalivibrio* (Fig. 1a). Their greatest degree of genetic divergence relative to the other representatives of the genus *Thioalkalivibrio* is also confirmed by a low (not exceeding 20%) level of DNA–DNA hybridization [2, 8]. According to the phylogenetic analysis of the RuBisCO genes, this group has also significantly diverged from the major

(c)

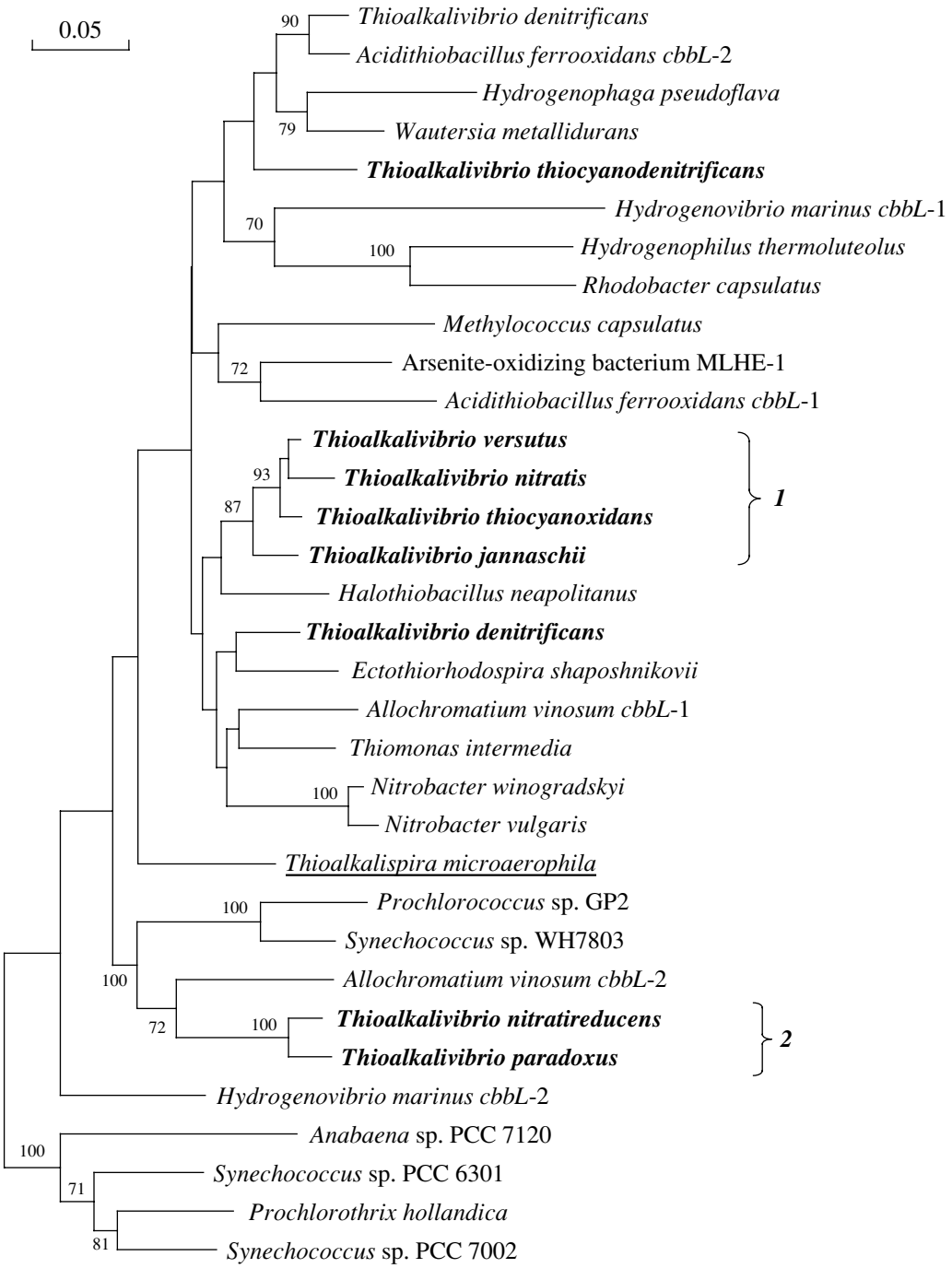

Fig. 1. (Contd.)

group of the obligately aerobic species. Interspecific relations within this group are traceable only by comparison on the nucleotide level, which is characterized by a higher resolving power than that on the amino acid level. However, in our opinion, at this stage of the investigation, it is not possible to conclude whether the RuBisCO genes of these two groups of the genus *Thioalkalivibrio* are monophyletic or polyphyletic, especially since the positions of their branching points in the corresponding phylogenetic trees are not stable. It is possible that, given such a significant degree of genetic divergence, the resolving power of the analysis of these genes may not be high enough to reveal phylogenetic relations. Furthermore, the size of the data sampling on

the primary structure of RuBisCO genes is significantly less than that of the data sampling on ribosomal genes, and it is possible that the topology of the corresponding trees may change with an increase in the number of compared organisms from different phylogenetic divisions. This suggestion is particularly relevant for the representatives of the family *Ectothiorhodospiraceae*, which, according to the 16S rRNA gene analysis, includes the genus *Thioalkalivibrio.*,

The phylogeny of the species *T. nitratireducens* and *T. paradoxus* is of particular interest. According to the 16S rRNA gene analysis, these species comprise a sole phylogenetic group within the studied genus. Their common origin and phylogenetic separation from the other representatives of the genus *Thioalkalivibrio* is justified by their high level of DNA–DNA homology in comparison with the very low level of DNA–DNA homology in other groups and by their very unusual, for *Thioalkalivibrio* species, cell morphology (the cells of both species are large immotile cocci with sulfur inclusions) [4]. The analysis of both the nucleotide and amino acid sequences showed that the RuBisCO genes of this group also have a common origin and differ significantly from the genes of the other species of the genus *Thioalkalivibrio.* However, unlike the species of the two other groups, these two species cluster with the purple sulfur bacterium *Allochromatium vinosum*, which belongs to another family of gamma-proteobacteria, *Chromatiaceae*. This clustering suggests there is a high probability of a common origin of the RuBisCO genes in these microorganisms, which differ in their phylogenetic positions and physiologic and metabolic characteristics. One of the possible evolutionary mechanisms that could have taken place in this case is horizontal gene transfer, which, presumably, played a significant role in the evolution of the family of RuBisCO genes [14, 15]. In particular, horizontal gene transfer may have taken place in the evolution of RuBisCO genes in the genera *Rhodobacter* [9] and *Nitrosomonas* [16]. In our case, this process may have taken place at the level of a common ancestor of both *Thioalkalivibrio* species. Taking into consideration the relatively low sequence similarity of the RuBisCOs of these species and *A. vinosum*, the transfer may have occurred between the aforementioned ancestor and another, as yet unknown, species of the family *Chromatiaceae* or, more likely, the ancestor of the family *Chromatiaceae*.

It is noteworthy that both of these *Thioalkalivibrio* species are morphologically very similar to *Allochromatium* while, at the same time, being so different from the other *Thioalkalivibrio* species that one of them was named *T. paradoxus.* This case is just one of the numerous examples of the inconsistency between the taxonomic position determined on the basis of 16S rRNA sequences and other characteristics of the organism in question. Although 16S rRNA is held as the predominant criterion, such an approach, in our opinion, is not fully justified. In the case of *T. paradoxus* and *T. nitratireducens*, it would seem more appropriate to reclassify them in an independent genus. At the same time, taking into consideration the striking morphological similarity between the photoautotroph *Allochromatium* and the chemoautotroph *Thioalkalivibrio*, two different types of sulfur-dependent organisms, it might be speculated that gene transfer between these phylogenetically distant organisms would have to involve not only individual genes but also genetic blocks. This assumption, if accepted, may complicate the creation of a natural system of bacteria and require new taxonomic approaches to be developed. Practical recommendations on the use of phylogenetic analysis of molecular markers alternative to 16S rRNA genes in bacterial taxonomy have not yet been developed. However, we believe that using such additional phylogenetic data (in particular, concerning genes vital for the group in question) might help to clear up equivocal evolutionary and taxonomic cases.

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