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EXPERIMENTAL ARTICLES

Use of Genes of Carbon Metabolism Enzymes as Molecular Markers of *Chlorobi* Phylum Representatives

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Abstract—This work examined the feasibility of using certain genes of carbon metabolism enzymes as molecular markers adequate for studying phylogeny and ecology of green sulfur bacteria (GSB) of the *Chlorobi* phylum. Primers designed to amplify the genes of ATP citrate lyase (*aclB*) and citrate synthase (*gltA*) revealed the respective genes in the genomes of all of the newly studied GSB strains. The phylogenetic trees constructed based on nucleotide sequences of these genes and amino acid sequences of the conceptually translated proteins were on the whole congruent with the 16S rRNA gene tree, with the single exception of GltA of *Chloroherpeton thalassium*, which formed a separate branch beyond the cluster comprised by other representatives of the *Chlorobi* phylum. Thus, the *aclB* genes but not *gltA* genes proved to be suitable for the design of primers specific to all *Chlorobi* representatives. Therefore, it was the *aclB* gene that was further used as a molecular marker to detect GSB in enrichment cultures and environmental samples. AclB phylotypes of GSB were revealed in all of the samples studied, with the exception of environmental samples from soda lakes. The identification of the revealed phylotypes was in agreement with the identification based on the FMO protein gene (*fmo*), which is a well-known *Chlorobi*-specific molecular marker.

Keywords: green sulfur bacteria (GSB), *Chlorobi*, reductive TCA cycle (rTCA cycle), ATP citrate lyase, citrate synthase, phylogeny

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Green sulfur bacteria (GSB) are physiologically homogeneous group (obligate anaerobic anoxygenic phototrophs) that forms a separate evolutionary lineage (*Chlorobi* phylum) distinct from other bacterial lineages. Until recently, GSB systematics was based on cell morphology, pigment composition, and physiological and biochemical properties [1, 2]. All of the known GSB contain antenna structures (chlorosomes). Formation of gas vacuoles was considered important for differentiation of GSB genera. For species differentiation, much significance was given to culture coloration (green/brown), which is determined by the presence of specific carotenoids (chlorobactene or isorenieratene) and bacteriochlorophylls *c*, *d*, or *e*. However, comparative sequence analysis of two molecular markers, the 16S rRNA genes and the *fmo* genes, which encode the *Chlorobi*-specific FMO-protein (Fenna–Matthews–Olson protein) of the photosynthetic reaction center, resulted in considerable reorganization of the GSB group [3]. In several cases, strains earlier assigned to one species were distributed among different species and even genera. Apart from species whose type strains were lacking in collections, the only species not affected by reclassification was *Chloroherpeton thalassium* [4], which forms a distinct branch within the GSB cluster in both 16S rRNA and FMO-protein trees and exhibits pronounced phenotypic distinctions from other *Chlorobi* representatives. Comparative analysis of GSB genome sequences also showed distinct position of the genus *Chloroherpeton* [5]. Therefore, it was recently proposed to assign bacteria that are close to *Chloroherpeton* representatives to a separate family *Chloroherpetonaceae* within the order *Chlorobiales* [6, 7].

Most representatives of the *Chlorobi* phylum are capable of autotrophic assimilation of carbon dioxide, which they perform via the reductive tricarboxylic acid cycle (rTCA cycle) [8–10]. This cycle is in fact a reversed tricarboxylic acid cycle (TCA cycle, or Krebs cycle) in which the enzymes catalyzing irreversible reactions (citrate synthase, NAD-dependent 2-oxoglutarate dehydrogenase, and succinate dehydrogenase) are substituted for by enzymes that can catalyze reactions of the opposite direction (ATP citrate lyase, ferredoxin-dependent 2-oxoglutarate synthase, and fumarate reductase, respectively). The key enzyme of the rTCA cycle, ATP citrate lyase, which catalyzes the key reaction of ATP-dependent conversion of citrate to acetyl-CoA and oxalacetate, is encoded by two genes, *aclA* and *aclB*, only found in the genomes of

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Table 1. GSB pure cultures studied in this work

Strains	Phylogenetic affiliation	Sequenced fragment length		Collection	Reference
		aclB gene	*gltA* gene		
L	*Chlorobaculum parvum*	863	826	Department of Microbiology, Faculty of Biology, Moscow State University	[27]
X	*Chlorobaculum limnaeum*	860	826		
C	*Chlorobaculum limnaeum*	950	826		
M	'Chlorobaculum macestae'	936	826		[22]
PhvPS1	*Chlorobium phaeovibrioides*	971	ND	Institute of Microbiology, RAS	[23]
PrPS10	*Chlorobium phaeovibrioides*	971	ND		
ChlvPS10	*Chlorobium phaeovibrioides*	971	ND		

"ND" means that PCR was not run.

prokaryotes that employ the rTCA cycle for autotrophic fixation of carbon dioxide. Apart from GSB, these are *Epsilonproteobacteria* [11–13], some representatives of hyperthermophilic hydrogen-oxidizing bacteria of the phylum *Aquificae* [14], and "*Candidatus* Nitrospira defluvii" from the phylum *Nitrospirae* [15]. ATP citrate lyase activity was also shown for the sulfate-reducing deltaproteobacterium *Desulfobacter hydrogenophilus* [16]; however, no genomic data are available for this organism. In certain *Aquificae* and *Nitrospirae* representatives, the key reaction of the rTCA cycle is catalyzed by two other enzymes, citryl-CoA synthetase and citryl-CoA lyase [17, 18]. Based on genomic data, it is supposed that rTCA cycle is also operative in some representatives of alphaproteobacteria (*Magnetococcus* sp. MC-1) [19] and gammaproteobacteria (autotrophic symbionts of mollusks) [20]. Interestingly, the mollusk symbiont rTCA cycle most likely operates in parallel to the Calvin cycle, which is a unique situation. In the genomes of these organisms, the *alcA* and *alcB* genes have been found, which encode active ATP citrate lyase; it belongs, however, to a presumably new type (type II).

Based on the analysis of the sequences of genes encoding rTCA cycle key enzymes, a hypothesis was advanced about their evolutionary relations with each other and with succinyl-CoA synthetase [14, 17].

Until recently, it was thought that citrate synthase, the enzyme that catalyzes a reaction reverse to citrate cleavage, is not involved in autotrophic assimilation of CO_2 via the rTCA cycle. Moreover, it seemed obvious that simultaneous presence of both enzymes in the cell would lead to cyclic synthesis and cleavage of citrate with a meaningless waste of the ATP energy. However, in the course of investigation of rTCA cycle enzymes in GSB, citrate synthase activity has been found along with the activity of ATP-dependent citrate lyase [21 and Ivanovsky, unpublished data]. Moreover, genes encoding this enzyme (*gltA*) were found in all sequenced genomes of *Chlorobi* representatives and in genomes of some representatives of other phyla capable of carbon dioxide fixation via rTCA cycle. To explain these findings, hypotheses have been advanced postulating operation in these organisms of either an incomplete TCA cycle [21] or of additional reactions in the rTCA cycle [Ivanovsky, unpublished data]; these hypotheses are currently under discussion.

The aim of the present work was to design oligonucleotide primers for the detection and sequencing of *aclB* and *gltA* genes in the genomes of GSB of the *Chlorobi* phylum, as well as estimation of the feasibility of using the data obtained with the help of these primers in phylogenetic and molecular biological studies.

MATERIALS AND METHODS

The subjects of this study were GSB strains from the culture collection of the Microbiology Department of the Moscow State University Biological Faculty and recent GSB (*Chlorobi* phylum) isolates from the culture collection of the Institute of Microbiology RAS, as well as enrichment cultures and environmental samples of various origins (Tables 1, 2).

Isolation and cultivation of pure cultures and obtaining of enrichments was carried out as described earlier [22, 23].

Isolation of total DNA was performed by a modified Birnboim–Doly alkaline extraction procedure [24] and Wizard technology (Promega, United States).

Design of primers. Nucleotide sequences of the genes encoding beta subunit of ATP citrate lyase (*aclB*) and citrate synthase (*gltA*) in representatives of various taxonomic groups, including GSB of the *Chlorobi* phylum, were retrieved from the from the GenBank/EMBL/DDBJ database (http://www.ncbi.nlm.

Table 2. GSB enrichments and environmental samples studied in this work

Sample	Sequenced fragment length		Source
	fmo gene	*aclB* gene	
Enrichment cultures			
enrBlSea1	833	934	A brown-colored GSB culture from water sampled at the Black Sea continental slope near town of Gelendzhik from a hydrogen sulfide zone (depth of 150–180 m)
enrMV2	830	692	A green-colored GSB culture from surface layer of sediments of a small fresh thermal lake located in a weakly illuminated cave Proval in town of Pyatigorsk (pH 7.2–7.4, water temperature of 33°C, sulfide content of 8–10 mg L^{-1})
enr2078	416	959	A green-colored GSB culture from a sample of cyanobacterial mat on the surface layer of sediments and decaying algae in a brook estuarine and at the White Sea shore (Gulf of Rugozerskaya); water temperature of 15–20°C, pH 7.0–7.5
enrKonanEr	775	692	A green-colored GSB culture from the chemocline zone at 11-m depth in the freshwater low-sulfide karst Lake Konan-Er in the south of the Marii El Republic at the spurs of Klenovogorskaya hill. pH 7.3, water temperature of about 40°C; earlier, symbiotic forms of GSB were detected in this lake [35]
enrM	827	692	A green-colored GSB culture from a sulfide spring at Golovin Volcano caldera, Lake Kipyashchee shore, Kunashir Island, Southern Kurils. Temperature of about 30°C, pH 4–6
enrBF	647	888	A green-colored GSB culture from a mat on the surface of near-shore bottom sediments of South China Sea at Vietnam coast
Environmental samples			
probeK5	672	749	Water samples from sulfide springs on the shore of Lake Kipyashchee (Kurils, Kunashir Island, Golovin Volcano caldera). pH 4.5–6.0, temperature of 20–25°C, sulfide content of 20–35 mg/L. Massive development of GSB was observed. Samples were taken from one spring: K-5, near the outlet; K-6, further downstream
probeK6	640	749	
probe05-2	–	–	Integral environmental samples (mixture of 0- to 10-cm sediments) from small hypersaline soda lakes of Kulunda Steppe, Altai; salinity of 30–520 g/L, pH 9.45–11.05
probe05-3	–	–	
probeT5-07	–	–	
probeS-06	–	–	
Probe05-1	–	–	

"–" Means that fragments were not detected.

nih.gov). Alignment of nucleotide sequences, calculation of the consensus sequence, and search of conserved sites in it were performed with the use of the BioEdit software package with built-in CLUSTALW (http://jwbrown.mbio.ncsu.edu/BioEdit/bioedit.html). For the amplification of *fmo* gene fragments, primers earlier designed by other researchers [25] were used.

Amplification and sequencing of fragments of the studied genes. The amplification mixture (25 μL) was of the following composition: 1× buffer for BioTaq DNA polymerase (17 mM $(NH_4)_2SO_4$; 67 mM Tris-HCl, pH 8.8; 4 mM $MgCl_2$); 6.25 nmol of each dNTP; 25 ng of template DNA; 20 pmol of each of the two appropriate primers; and 1.5 units of BioTaq DNA polymerase (Dialat Ltd., Russia).

The PCR temperature profile was as follows: 94°C for 9 min; 30 cycles of 94°C for 30 s, 56°C for 40 s, and 72°C for 1 min; 72°C for 7 min; and cooling to 4°C. The PCR products were purified from admixtures and nonspecific products by electrophoresis in low melting temperature agarose using Wizard PCR Preps System (Promega). PCR products were analyzed by electrophoresis in 1% agarose gel and sequenced on an ABI 3700 automatic sequencer (Applied Biosystems) using the Big Dye Terminator version 3.1 kit and appropriate primers.

Table 3. Oligonucleotide primer systems for amplification of the genes encoding FMO protein (*fmo*), ATP citrate lyase (*aclB*) and citrate synthase (*gltA*)*

Primer	Primer sequence, 5'–3'	Position in the gene**	Reference
F-Start-fmo	ATGGCTCTTTTYGG	1–14	[25]
R-889-fmo	CCGACCATNCCGTGRTG	905–889	
AclB-48F	GGGCATTCCYGTGCCKAATTATG	48–70	This work
AclB-gsb-1063R	GAAGCCGAGCARCARATYATGG	1063–1083	
Cs-gsb-232F	GGBTAYCCGATCGAKCAGCT	232–251	This work
Cs-gsb-1147R	GCRAAVAGVACVGGRAACAT	1147–1166	

* Degenerate positions in the primers are designated as follows: Y = T, C; R = A, G; M = A, C; K = T, G; W = A, T; S = G, C; B = T, G, C; V = A, G, C; D = A, T, G; H = A, T, C; N = G, A, T, C.

** Primer positions are indicated as positions in relevant genes of *Chlorobium tepidum* ATCC 49652^T.

Cloning was performed with the use of the CloneJet PCR cloning kit (Fermentas) with the pJET1.2/blunt plasmid according to the manufacturer's protocol. From each clone library, 20 clones were randomly chosen and checked for the presence of the insert by PCR with specific primers and further electrophoresis in 1.0% agarose gel stained with ethidium bromide (0.5 μg/mL). Clones containing the target insert were used for further analysis.

Conceptual translation and phylogenetic analysis of nucleotide and amino acid sequences. Conceptual translation of the newly determined nucleotide sequences was performed with the BioEdit software package (http://jwbrown.mbio.ncsu.edu/BioEdit/bioedit.html). The sequences were aligned with relevant sequences of reference bacterial strains using the CLUSTALW program built in BioEdit. Phylogenetic trees were constructed with the use of the neighbor-joining algorithm implemented in the TREECONW software package [26].

Deposition of nucleotide sequences. The newly determined nucleotide sequences of the ATP citrate lyase and citrate synthase genes have been deposited in GenBank with accession nos. KF220611– KF220636.

RESULTS AND DISCUSSION

Detection of ATP citrate lyase and citrate synthase genes in pure cultures of *Chlorobi* phylum bacteria. The first stage of our work involved in silico analysis of nucleotide sequences of the *aclB* (type I) and *gltA* genes present in the publicly available genome sequences of bacteria that employ rTCA cycle. This analysis was aimed at revealing conserved sites selectively specific to *Chlorobi* phylum and designing relevant oligonucleotide primers.

All *aclB* genes of the 12 strains of GSB of the *Chlorobi* phylum whose genome sequences are available in GenBank were at least 70% identical, whereas their identity value with the relevant genes of representatives of other phyla, capable of carbon dioxide fixation via rTCA cycle, was no higher than 45% (these were genes from genome sequences of eight epsilonproteobacteria, six phylum *Aquificae* representatives, and one phylum *Nitrospira* representative, "*Candidatus* N. defluvii"). This was in agreement with available data [14] according to which *aclB* genes of epsilonproteobacteria and phylum *Aquificae* representatives are closer to each other and differ significantly from relevant genes of *Chlorobi* and eukaryotes. It is evidently due to this fact that the earlier proposed primer systems [11] proved inefficient in *alcB* gene amplification on template DNA from seven newly studied pure GSB cultures, identified earlier based on comparative analysis of 16S rRNA and *fmo* genes [22, 23, 27]. Therefore, we undertook an analysis of the conservation pattern of *aclB* genes from genome sequences of 12 *Chlorobi* phylum representatives, and it proved possible to design a new primer pair (Table 3) that was specific to GSB and allowed for amplification of *alcB* gene fragments about 1000 bp in length. PCR with these primers yielded fragments of the expected length for all of the seven GSB pure cultures studied (Table 1). The identity level between the newly determined nucleotide sequences and relevant GSB sequences available in GenBank was above 70%, indicating that the newly determined sequences belonged to *aclB* gene fragments. Thus, presence of genes encoding the key rTCA cycle enzyme was revealed in all GSB strains that we studied, in accordance with earlier obtained data on the operation of rTCA cycle and activity of relevant enzymes (including ATP citrate lyase) in strains *Ch. limnaeum* C and *Ch. parvum* L [28 and Ivanovsky, unpublished data].

The *gltA* genes, which encode citrate synthase, were found in all of the 12 GSB genome sequences; however, they were also present in the genome sequences of some epsilonproteobacteria and phylum *Aquificae* representatives. Like *aclB* genes, the *gltA* genes of most of the GSB were no less than 70% identical to each other, whereas their identity level with the relevant genes of representatives of other phyla (genes from genome sequences of six epsilonproteobacteria, three phylum *Aquificae* representatives, and one phylum *Nitrospirae* representative, "*Candidatus* N. defluvii") was no higher than 50%. At the same time, the identity level of the *gltA* gene of the GSB *Chp. thalassium* was no higher than 55% both with other *Chlorobi* phylum representatives and with representatives of other phyla used in the analysis. Because of this high degree of divergence, we failed to find in the *gltA* gene consensus sequence conserved sites selectively specific to GSB. The conserved sites that we did find were also present in the *gltA* genes of representatives of other phyla; therefore, oligonucleotide primers targeting them (Table 1) should not be selectively specific to *Chlorobi*. Nevertheless, using these newly designed primers, we managed to amplify *gltA* gene fragments about 900 bp in length on the template genomic DNA of four GSB strains (Table 1). The identity level of the nucleotide sequences of these gene fragments with relevant GSB gene sequences available in GenBank was above 70%, indicating that the fragments belonged to the *gltA* genes. Thus, the presence of genes encoding citrate synthase, the enzyme that catalyzes the reaction reverse to citrate cleavage in rTCA cycle, was revealed in all GSB strains that we studied, including strain *Ch. limnaeum* C, for which the activity of this enzyme had earlier been demonstrated [Ivanovsky, unpublished data].

Phylogenetic analysis of the ATP citrate lyase genes *aclB*. Six of the GSB strains studied in this work were earlier identified, based on the analysis of 16S rRNA and *fmo* genes, as representatives of the species *Ch. limnaeum* (strains C and X), *Ch. parvum* (strain L), and *Chlorobium phaeovibrioides* (strains PhvPC1, PrPC10, and ChlvPC10), and the seventh strain, strain M, was identified as a representative of *"Chlorobaculum macestae"*, a species that has not yet been validated [22, 24, 27]. Our comparative analysis used nucleotide and conceptually translated amino acid sequences of *aclB* genes of the newly studied strains and relevant sequences retrieved from all publicly available genome sequences of representatives of *Chlorobi* and other phyla capable of CO_2 fixation via rTCA cycle and possessing type I ATP citrate lyase. After alignment of the sequences, comparative analysis used 1080 and 360 positions for nucleotide and amino acid sequences, respectively.

The topologies of trees constructed based on nucleotide (not shown) and amino acid sequences (Fig. 1) did not exhibit essential differences. According to these trees, all *aclB* gene sequences of the strains under study belonged to a common cluster of the *Chlorobi* phylum. The results did not contradict to those obtained by phylogenetic analysis based on comparison of 16S rRNA genes (the latter analysis, in addition to 16S rRNA genes retrieved from genome sequences, also involved the gene of *Ch. limnaeum*, for which genome sequence is not available). The *aclB* gene sequence of *Ch. parvum* L was virtually identical to that of the type strain of *Ch. parvum* (99.9% nucleotide sequence identity and 99.7% amino acid sequence identity). The *aclB* genes of strains C and X were 100% identical, in terms of both amino acid and nucleotide sequences. In the cluster comprised by *Chlorobaculum* species, they formed a separate branch (these two strains, according to 16S rRNA gene analysis, belong to *Ch. limnaeum*, whose *aclB* genes were not represented in GenBank). The *aclB* genes of strains PhvPC1, PrPC10, and ChlvPC10 were also virtually identical to each other (99.6–100% identity for nucleotide sequences and 100% for amino acid sequences) and to the relevant sequences of the *C. phaeovibrioides* type species (98.9–99.3% and 99.4–100% identity for nucleotide and amino acid sequences). The *aclB* gene sequence of *"Ch. macestae"* strain M, like the 16S rRNA gene sequence of this strain, formed a separate branch within the genus *Chlorobaculum*. The data that we obtained extend the database of *aclB* gene sequences of GSB of the *Chlorobi* phylum.

All of the *Chlorobi* phylum representatives, including both the newly studied strains and strains with available genome sequences, formed a separate monophyletic cluster in the tree of conceptually translated *aclB* genes. The same was true for representatives of other higher taxa studied in this work: phyla *Aquificae* and *Nitrospirae* and class *Epsilonproteobacteria*; this clustering pattern was in accordance with the results of 16S rRNA gene analysis (Fig. 1). Thus, we have shown that the evolution of ATP-dependent citrate lyase, the key enzyme of autotrophic CO_2 fixation via rTCA cycle, proceeded in parallel with the evolution of ribosomal genes. Our results also demonstrate the possibility of using *aclB* genes for selective detection and identification of GSB in molecular-ecological studies of natural autotrophic communities in various habitats.

Phylogenetic analysis of the citrate synthase genes *gltA*. Phylogenetic analysis of *gltA* genes involved four GSB strains belonging to the species *Ch. limnaeum* (strains C and X), *Ch. parvum* (strain L), and *"Ch. macestae"* (strain M). Additionally, the analysis used all *Chlorobi* phylum representatives for which genome sequences are available, and representatives of other phyla whose genome sequences harbor both *aclB* and *gltA* genes. After alignment of the sequences, the comparative analysis used 870 and 290 positions for nucleotide and amino acid sequences, respectively.

Phylogenetic trees of *gltA* genes constructed based on analysis of nucleotide sequences (not shown) and their conceptual amino acid translations (Fig. 1) did not exhibit essential differences. The sequences of cit-

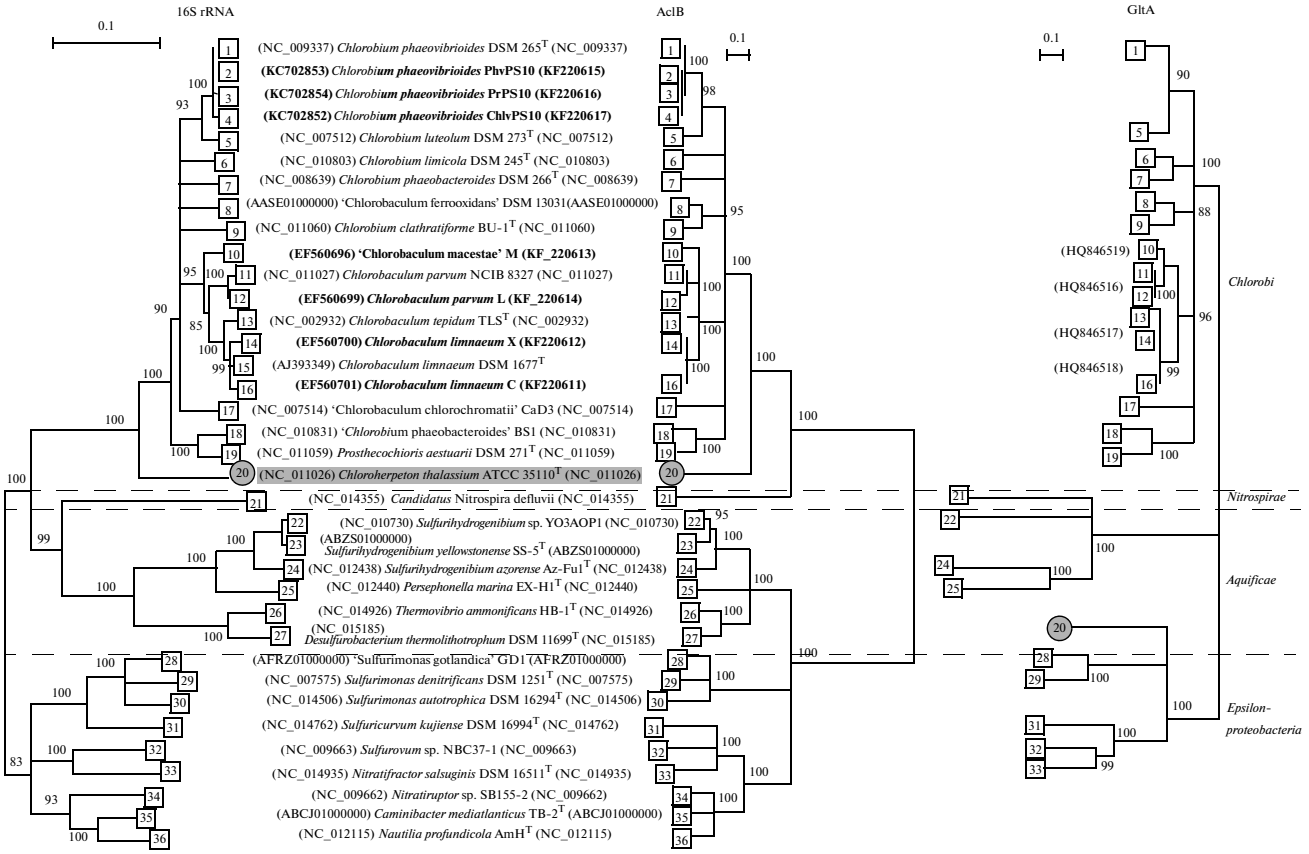

Fig. 1. Phylogenetic trees for representatives of *Chlorobi*, *Epsilonproteobacteria*, *Aquificae*, and *Nitrospirae*, constructed based on analysis, with the use of neighbor-joining algorithm, of nucleotide sequences of 16S rRNA genes and conceptual amino acid translations of *aclB* and *gltA* genes. The names of the GSB pure cultures for which *aclB* and *gltA* gene sequences were determined in this work are set in bold. The sequences belonging to the type strain of the species *Chp. thalassium* are shaded gray. The scale bar corresponds to an evolutionary distance of 10 nucleotide/amino acid substitutions per 100 residues. Numbers at the nodes indicate statistical significance of the branching order as determined by bootstrap analysis of 1000 alternative trees. Only values above 80% were considered significant; branching points with lower bootstrap support were collapsed.

rate synthase genes were virtually identical for strains *Ch. limnaeum* X and C (99.8 and 100% identity for nucleotide and amino acid sequences, respectively). These strains formed a separate branch in the tree. The *gltA* gene sequences of strain *Ch. parvum* L were 100% identical with relevant sequences of the type strain of this species, and also formed, together with these sequences, separate branches. The *gltA* sequences of *"Ch. macestae"* strain M formed another distinct branch with identity levels not higher than 88.3% for nucleotide sequences and 94.4% for amino acid sequences; this position corresponded to the status of *"Ch. macestae"* as a separate species. Thus, the positions of the newly studied strains on the citrate synthase tree were analogous to their phylogenetic positions determined based on analysis of 16S rRNA and *aclB* genes.

On the whole, the topologies of trees constructed for GSB based on the analysis of genes encoding citrate synthase (*gltA*), ATP citrate lyase (*aclB*), and 16S rRNA were congruent. The only exception was the distinct and uncertain position of *Chp. thalassium* in the citrate synthase tree, due to which *Chlorobi* representatives did not form a monophyletic cluster in this tree (Fig. 1). Two hypotheses can be proposed to explain this phenomenon: horizontal gene transfer or different rates of evolutionary change in different lineages. The latter hypothesis seems more plausible because *Chp. thalassium* has unusual phenotypic properties and occupies a distinct position also in trees based on other molecular markers (albeit in those trees its position is within the *Chlorobi* cluster).

Although analysis of 16S rRNA genes is the primary approach to study prokaryote phylogeny, genes encoding various metabolic functions are also considered as alternative molecular markers. The most promising in this respect is the comparative analysis of the nucleotide sequences of genes belonging to the so-called housekeeping genes, i.e., genes determining essential metabolic processes [29] that are vitally important for all living organisms or for their particular groups. In particular, the *fmo* gene, encoding the FMO protein (Fenna–Matthews–Olson protein) [25], is among such genes for the *Chlorobi* phylum, since

possession of the *fmo* gene is believed to be indispensable for GSB, and because its phylogeny for GSB (including strains studied in the present work) is in good agreement with the "ribosomal phylogeny" [22, 27].

The *gltA* gene, encoding citrate synthase, the key enzyme of the TCA cycle, is usually viewed as a housekeeping gene in pro- and eukaryotes that employ this cycle, and it is used as such a gene in phylogenetic analysis of various groups of bacteria employing TCA cycle [30–32], as well as in studies that use multilocus sequence typing (MLST) [33]. Data from analysis of genome sequences, as well as our data obtained with specific primers, demonstrate the presence of the citrate synthase gene in all GSB studied. At the same time, both analysis of genome sequences and data obtained with specific primers demonstrate that in other phyla, only some of the representatives that fix CO_2 via the rTCA cycle possess citrate synthase genes. Most of citrate synthase genes present in representatives of the *Chlorobi* phylum are of common evolutionary origin, and their phylogeny corresponds to the 16S rRNA gene-based taxonomic structure of the phylum; therefore, their evolution, as well as the evolution of *aclB* genes, proceeded in parallel with the evolution of 16S rRNA genes, which is typical of housekeeping genes. Thus, our phylogenetic analysis confirms the vital importance of citrate synthase genes for GSB, as well as for some other bacteria employing the rTCA cycle for autotrophic fixation of CO_2, albeit the operation mode of this enzyme remains debatable.

Detection and phylogenetic analysis of *aclB* genes of GSB in enrichment cultures and environmental samples. Since we succeeded in designing a oligonucleotide primer pair selectively specific to *aclB* genes of *Chlorobi* phylum representatives, our next task was to test the applicability of this primer pair to detection and analysis of these genes in enrichment cultures and environmental samples. In parallel control experiments, we performed PCR amplification of fragments of the *fmo* gene, which is considered to be a specific molecular marker for representatives of the *Chlorobi* phylum [25].

PCR with the specific primers yielded products of expected length (about 1000 bp for *aclB* genes and 900 bp for *fmo* genes) in all of the enrichment cultures we studied and in two of the investigated environmental samples (Table 2). Each of the clonal libraries constructed (about 20 clones in each library) represented a GSB monoculture in terms of both of the genes analyzed (one phylotype at an OTU level of 97% nucleotide sequence identity). In different libraries, the phylotypes could be either taxonomically the same or different; in any case, all of them represented three genera of the family *Chlorobiaceae: Chlorobium, Chlorobaculum*, and *Prosthecochloris*.

The sequences of fragments of both marker genes (*aclB* and *fmo*) from enrichment culture enrM proved to be close to relevant sequences of strains PhvPC1, PrPC10, and ChlvPC10 (99.2–99.4 and 98.9–99.4% for nucleotide and 98.6–100 and 99.3–100% for amino acid sequences, respectively). Among the above-mentioned strains, only PhvPC1 culture had a chocolate-brown coloration, whereas cells of the other cultures were green-colored. Earlier, based on the analysis of the 16S rRNA genes, strains PhvPC1, PrPC10, and ChlvPC10 were identified as representatives of the species *C. phaeovibrioides* [23], with which they form a common cluster in all of the trees constructed (Figs. 1, 2), in spite of significant differences in morphology and pigment composition. Therefore, the enrM phylotype may also be identified as a representative of this species. It should be mentioned that the enrM enrichment culture is also green-colored, whereas the type strain of *C. phaeovibrioides* has a chocolate-brown coloration. In addition, the cluster of *Chlorobium* species included the phylotype from the enrKonanEr enrichment culture, which, in the AclB and FMO trees, formed a separate branch and presumably represents a new species of this genus (Fig. 2).

The sequences of fragments of both marker genes (*aclB* and *fmo*) from enrichment culture enrMV2 were identical to relevant sequences of *Ch. limnaeum* strains C and X (99.7 and 99.4–99.7% for nucleotide and 100 and 99.7–100% for amino acid sequences, respectively). Since there are no data on the sequence of the *aclB* gene for the type strain of *Ch. limnaeum*, strains C and X and phylotype enrMV2, which can also be identified as a representative of this species, formed a separate branch in the AclB tree (Fig. 2). Additionally, a separate branch within the cluster of *Chlorobaculum* species was formed by the nearly identical sequences of both markers from enrichment enr2078 and environmental samples probeK5 and probeK6 (*aclB* and *fmo*, 99.9 and 99.5% for nucleotide and 99.6 and 99.5% for amino acid sequences); presumably, these phylotypes represent a new species within this genus.

Sequences of fragments of both marker genes (*aclB* and *fmo*) from the marine enrichment culture enrBF formed a separate branch within the cluster of *Prosthecochloris* species; thus, this phylotype is presumably a representative of a new species of this genus. At the same time, the sequences of both these markers from one more member of *Prosthecochloris* species cluster, the phylotype from the enrichment culture enrBlSea1, were 100% identical (at the nucleotide level as well) with relevant sequences from the complete genome of strain BS1, also isolated from the chemocline zone of the Black Sea [34]. This strain was initially identified as a representative of the species *C. phaeobacteroides*, but, phylogenetically, it belongs to the cluster of *Prosthecochloris* species and evidently needs reclassification.

Thus, among the phylotypes identified in this work, there were both representatives of known GSB species and presumable representatives of new species.

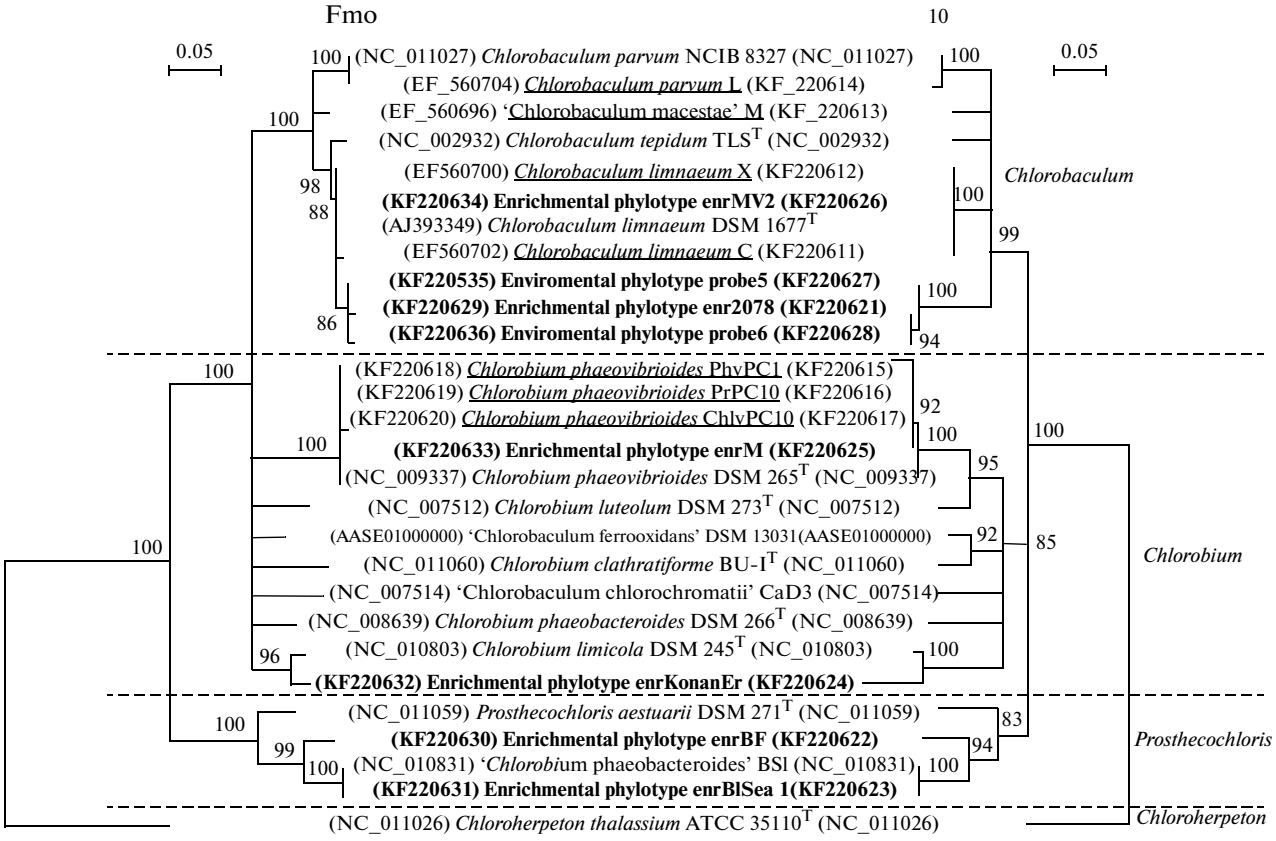

Fig. 2. Phylogenetic trees for representatives of the phylum *Chlorobi* from enrichment cultures and environmental samples of various origins. The trees were constructed based on analysis, with the use of neighbor-joining algorithm, of conceptual amino acid translations of *aclB* and *fmo* genes. The names of the GSB phylotypes for which *aclB* and *fmo* gene sequences were determined in this work are set in bold. The names of the GSB pure cultures for which *aclB* gene sequences were determined in this work are underlined. The scale bar corresponds to an evolutionary distance of 5 amino acid substitutions per 100 residues. Numbers at the nodes indicate statistical significance of the branching order as determined by bootstrap analysis of 1000 alternative trees. Only values above 80% were considered significant; branching points with lower bootstrap support were collapsed.

Our results demonstrate minimal GSB diversity in the investigated enrichment cultures and environmental samples: in each of the samples studied a single GSB species was represented. Analogous results were obtained in a 16S rRNA gene-based study of the GSB diversity in the Black Sea chemocline, where a single phylotype, identical to strain BS1, was invariably revealed over several years [34]. The identity of this microorganism to the phylotype from our Black Sea enrichment culture enrBlSea1 additionally demonstrates its specificity to the ecological conditions of the Black Sea. Another marine GSB, represented by phylotype enrBF and probably belonging to a yet unknown *Prosthecochloris* species, also proved to be specific to a particular marine ecosystem, that of the South China Sea near Vietnam coast.

The GSB originating from freshwater environments were less specific to particular habitats. Thus, in the Kislo-Sladkoe stratified brackish lake at the White Sea shore and in mats in the mesophilic zone of hydrothermal vents on Kunashir Island, identical strains and phylotypes of *C. phaeovibrioides* were found (PhvPC1, PrPC10, and ChlvPC10 and enrM). *Chlorobaculum* spp. were found both in sulfide springs of Kuril Islands and Caucasus (probeK5, probeK6, and enrMV2) and in a White Sea estuarine (enr2078). However, phylotype enrKonanEr, originating from a thermal karst lake, proved to be unique, lacking close relatives either among described species or among new GSB isolates and phylotypes. It can be assumed that this species is a component of the symbiotic consortia "Pelochromatium roseum" and/or "Pelochromatium roseo-viride", which occur in this lake [35].

Soda lakes turned out to be the natural environments where were we failed to reveal either of the *Chlorobi* molecular markers searched for. Lack of positive results of PCR with GSB-specific primers is in agreement with published microbiological data: GSB have never been isolated from soda lakes. The situation with epsilonproteobacteria should however be noted: despite the lack of reports on their isolation from soda lakes, our application of *aclB*-specific primers [11] to soda lake sediment samples revealed novel phylotypes belonging to this taxon [36]. Therefore, the question as

to whether GSB phylotypes adapted to soda lake conditions do exist remains open.

It may be concluded that the results obtained in the present work confirm the feasibility of using the *aclB* genes for the analysis of phylogenetic diversity of *Chlorobi* phylum representatives in natural ecosystems as a molecular marker additional to the earlier proposed ones. Moreover, obtaining new information on the primary structure of the genes determining carbon metabolism enzymes and the use of this information in phylogenetic analysis will advance the understanding of the functional peculiarities of these enzymes, as well as the understanding of the evolution both of the enzymes per se and of the diverse autotrophic microorganisms employing them.

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