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EXPERIMENTAL ARTICLES

An Oligonucleotide Primer System for Amplification of the Ribulose-1,5-Bisphosphate Carboxylase/Oxygenase Genes of Bacteria of Various Taxonomic Groups

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Abstract—Based on the analysis of GenBank nucleotide sequences of the *cbbL* and *cbbM* genes, coding for the large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBPC), the key enzyme of the Calvin cycle, a primer system was designed that allows fragments of these genes about 800 bp long to be PCR-amplified for various photo- and chemotrophic bacteria. The efficiency of the designed primer system in detection of RuBPC genes was demonstrated in PCR with DNA of taxonomically diverse bacteria possessing RuBPC genes with a known primary structure. Nucleotide sequences of RuBPC gene fragments of bacteria belonging to the genera *Acidithiobacillus, Ectothiorhodospira, Magnetospirillum, Methylocapsa, Thioalkalispira, Rhodobacter*, and *Rhodospirillum* were determined to be deposited with GenBank and to be translated into amino acid sequences and subjected to phylogenetic analysis.

Key words: ribulose-1,5-bisphosphate carboxylase/oxygenase, *cbbL, cbbM*, PCR, oligonucleotide primers, phylogenetic analysis.

Ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBPC) is the key enzyme of autotrophic CO_2 fixation in the Calvin cycle. Although the properties of RuBPC have been thoroughly studied by classical biochemical methods [1], data on the origin and evolution of this enzyme are scarce [2]. It is known that autotrophic organisms that assimilate CO_2 via the Calvin cycle belong to different and fairly remote evolutionary lineages. Therefore, the question of whether the origin of RuBPC is mono- or polyphyletic remains open. To investigate the origin and evolution of RuBPC, it is necessary to use molecular biological methods of comparative analysis of nucleotide sequences of the structural genes encoding this protein in different groups of prokaryotes. Comparison of phylogenetic trees constructed based on the analysis of the genes of 16S rRNA and RuBPC makes it possible to speculate on the peculiarities of evolution and functioning of RuBPC in different prokaryotes.

Four RuBPC forms have been distinguished [2–4]. Form I consists of eight large (L) and eight small (S) subunits (*cbbL* and *cbbS* genes, respectively). The large subunits fulfill a catalytic function; the role of the small subunits has not been completely understood. They possess no catalytic activity, but their presence increases considerably the activity of the enzyme, likely due to the stabilization of the hexadecameric form and to conformational shifts in the active center of the enzyme.

RuBPC form I is subdivided into two types, "green" and "red." These two enzyme types differ in the amino acid composition of the large subunits. The "green"-like RuBPC occurs in chloroplasts of terrestrial plants; "green" algae; cyanobacteria; and representatives of α-, β-, and γ-proteobacteria. The "red"-like RuBPC occurs in nongreen algae and in some representatives of α- and β-proteobacteria. In *Rhodobacter azotoformans*, both "red"- and "green"-like enzymes have been found [5].

RuBPC form II (*cbbM* gene) consists of large subunits (L_n) only; their number varies from two to eight depending on the organism. The catalytic subunits of form I and form II enzymes differ considerably and exhibit only 25–30% homology of their amino acid sequences [2, 3]. Many autotrophic bacteria, including certain purple nonsulfur bacteria and thiobacilli, simultaneously possess both form I and form II RuBPC. Despite the great degree of divergence of the amino acid sequences of the enzyme forms I and II, they con-

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tain highly conserved segments pertaining to the active site. The conservation of the amino acid residues important for catalysis provides for the uniformity of the active centers and three-dimensional structures of the large subunits of all bacterial RuBPC.

Genes encoding RuBPC form III were detected in certain archaea [6]. It was shown that, at least in *Methanococcus janaschii*, this gene is expressed during autotrophic growth and its product exhibits RuBPC activity [7].

Finally, a gene encoding a RuBPC-like protein (RuBPC type IV) was identified during sequencing of the genome of the "green" sulfur bacterium *Chlorobium tepidum* [4]. An analogous gene was isolated and sequenced from *C. limicola* forma *thiosulfatophilum.* Supposedly, the product of this gene plays an important role in the sulfur metabolism and in the response to oxidative stress.

Oligonucleotide primers for amplification of RuBPC gene fragments and determination of their nucleotide sequences have already been suggested. However, an important defect of the earlier suggested primers is that they were designed for the amplification of RuBPC genes of narrow groups of prokaryotes or even of individual microbial species [4, 5, 8–10]. This defect is mainly due to the limited amount of data on the primary structure of prokaryotic RuBPC genes (as compared to the amount of data available on the 16S rRNA genes and RuBPC genes of plants).

The aim of this work was to design and test a fairly universal primer system that would allow amplification of RuBPC gene fragments suitable for phylogenetic analysis from a bacterial spectrum as wide as possible.

MATERIALS AND METHODS

Subjects of study. Microbial strains were from the collections of the Department of Microbiology, Moscow State University, and various laboratories of the Institute of Microbiology, Russian Academy of Sciences (RAS), Moscow; the Institute of Biochemistry and Physiology of Microorganisms, RAS, Pushchino; Astrakhan State Technical University; and the Bioengineering Center, RAS, Moscow (Table 1).

Isolation of DNA. DNA isolation was performed by an earlier developed method [11]. The DNA concentration in the preparations obtained was 10–60 μg/ml. RNA was present in the preparations in trace amounts (less than 1% according to electrophoretic data).

Primer design. The search for nucleotide sequences of RuBPC genes of various taxonomic groups was performed in the GenBank database. The nucleotide sequences were aligned using the CLUSTALW v 1.75 program. The consensus sequence was deduced and the conserved sites in it were sought with the use of original software developed by A.I. Marusina (Bioengineering Center, RAS, unpublished data).

Optimization of PCR conditions for amplification of RuBPC gene fragments. To decrease the number of nonspecific fragments and to increase the concentration of the required fragment, the PCR conditions were optimized according to the Taguchi procedure [12].

Amplification of RuBPC gene fragments. The amplification was performed on a Cetus 480 (Perkin Elmer, Sweden) thermal cycler using the primers designed and the thermostable BioTaq DNA polymerase (Dialat LTD, Russia) according to the manufacturer's recommendations. The temperature profiles of PCR are presented in Table 2. Analysis of PCR products was performed by electrophoresis in 1% agarose gel. For the documentation of the electrophoretic separation of PCR products, the BioDocII system (Biometra, Germany) was used. Isolation and purification of PCR products were performed using low-gelling-point agarose and a Wizard DNA Purification System kit (Promega, USA) according to the manufacturer's recommendations.

Sequencing was performed by the Sanger method using a Silver Sequencing kit (Promega, USA) according to the manufacturer's recommendations with minor modifications. Electrophoresis was run on Macrophore (Pharmacia, Sweden) and SQ3 Sequencer (Hoefer, USA) devices; the thickness of the polyacrylamide gel was 0.19 mm. For sequencing in both directions, the forward and reverse primers designed were used.

Phylogenetic analysis of the sequences studied. Primary comparison of the de novo obtained sequences with the sequences available in GenBank was performed using the NCBI BLAST software (http://www.ncbi.nlm.nih.gov/blast). Editing of the sequences was performed using the BioEdit program (http://jwbrown.mbio.ncsu.edu/BioEdit/bioedit.html). The sequences were aligned with the corresponding sequences of the closest bacterial species using the CLUSTALW v 1.75 program. Unrooted phylogenetic trees of the bacteria studied were constructed by the methods implemented in the TREECONW software package.

Deposition of nucleotide sequences. The nucleotide sequences of *cbbL* and *cbbM* gene fragments determined in the course of this work were deposited with GenBank (accession numbers, AY450586–AY450592).

Determination of RuBPC activity. The RuBPC activity was determined in *Ectothiorhodospira shaposhnikovii* no. 1, *Rhodospirillum rubrum* 1R, and *Rhodobacter sphaeroides* 2R. *R. rubrum* and *Rh. sphaeroides* were grown photoheterotrophically on the Ormerod medium with acetate (0.1%) and $NaHCO_3$ (0.2%) [13]; *E. shaposhnikovii* was grown photoautotrophically on Larsen medium with $NaHCO_3$ (0.3%), $Na_2S_2O_3$ (0.1%), and $Na_2S \cdot 9H_2O$ (0.1%) [14]. Exponential-phase cells were harvested by centrifugation (20000 *g*, 20 min, 4°C). To obtain cell extracts, bacterial biomass washed with 50 mM Tris–HCl buffer

Table 1. Organisms studied and the forms of RuBPC genes revealed

Taxon	Strain	*cbbL* gene of "green"-like form I RuBPC	*cbbL* gene of "red"-like form I RuBPC	*cbbM* gene of form II RuBPC	Source
Acidithiobacillus ferrooxidans	TFY	+	–	+	INMI RAS
Rhodobacter capsulatus	B-10	+	–	+	DM MSU
Rhodobacter sphaeroides	2R	–	+	+	DM MSU
Rhodospirillum rubrum	1R	–	–	+	DM MSU
Phaeospirillum fulvum	5K	+	–	+	DM MSU
Chloroflexus aurantiacus	B3	–	–	–	DM MSU
Chloroflexus aurantiacus	OK-70	–	–	–	DM MSU
Chloroflexus sp.	Andorra	–	–	–	DM MSU
Ectothiorhodospira shaposhnikovii	1	+	–	+	DM MSU
Ectothiorhodospira mobilis	8115	+	–	+	DM MSU
Heliobacterium sulfidophilum	BR-4	–	–	–	INMI RAS
Heliorestis baculata	OS-H1	–	–	–	INMI RAS
Methylobacter modestohalophilus	10S	–	–	–	IBPM RAS
Methylobacter alcaliphilus	20Z	–	–	–	IBPM RAS
Methylocystis puriformis	62	–	–	–	INMI RAS
Methylocystis puriformis	79	–	–	–	INMI RAS
Methylocapsa acidiphila	B2	–	+	–	INMI RAS
Methylocella palustris	K2a	–	–	–	INMI RAS
Methylosinus trichosporium	5	–	–	–	INMI RAS
Methylocystis echinoides	2	–	–	–	INMI RAS
Beggiatoa sp.	D402	–	+	ND	INMI RAS
Beggiatoa sp.	D405	–	–	–	INMI RAS
Beggiatoa alba	DSM 1416	–	–	–	INMI RAS
Leucothrix sp.	1WS	–	–	–	INMI RAS
Leucothrix thiophila	2WS	–	–	–	INMI RAS
Leucothrix sp.	3WS	–	–	–	INMI RAS
Leucothrix sp.	5WS	–	–	–	INMI RAS
Leucothrix thiophila	6WS	+	–	–	INMI RAS
Leucothrix mucor	DSM 2157	–	–	–	INMI RAS
Thiobacillus thioparus	ATCC 8158	ND	ND	+	INMI RAS
Thiothrix arctophila	1N	–	–	+	INMI RAS
Spirillum kriegii	D430	–	–	–	INMI RAS
Spirillum winogradskii	D427	–	–	–	INMI RAS
Thioalkalispira microaerophila	ALEN 1	+	–	ND	INMI RAS
Thioalkalivibrio thiocyanoxidans	ARH2	+	–	+	INMI RAS
Thiomicrospira sp.	HL5	+	–	ND	INMI RAS
Thialkalimicrobium sibiricum	AL7	+	–	ND	INMI RAS
Magnetospirillum magnetotacticum	7	ND	ND	+	ASTU
Escherichia coli	DHL 5α	–	–	–	Bioeng. Center, RAS

Note: ND stands for "not determined." INMI RAS: Institute of Microbiology, Russian Academy of Sciences, Moscow; DM MSU: Department of Microbiology, Moscow State University; IBPM RAS: Institute of Biochemistry and Physiology of Microorganisms, Russian Academy of Sciences, Pushchino; ASTU: Astrakhan State Technical University, Astrakhan; Bioeng. Center RAS: Bioengineering Center, Russian Academy of Sciences.

Table 2. Protocols for the amplification of RuBPC gene fragments

Amplification with the use of	Composition of the reaction mixture	Temperature profile of PCR
primers RubIgF–RubIgR, specific to the *cbbL* genes of "green"-like form I RuBPC	In 25 μl: buffer (17 mM $(NH_4)_2SO_4$; 6 mM Tris–HCl, pH 8.8; 4 mM $MgCl_2$; 1 mM dithiothreitol), 6.25 nmol of dNTP, 25 ng of template DNA, 7.8 pmol of each primer, and 1.25 U of BioTaq DNA polymerase.	first cycle: 94°C for 3 min, 58°C for 1 min, and 72°C for 1 min; 7 subsequent cycles: 94°C for 30 s, 58°C for 20 s, and 72°C for 45 s; last 28 cycles: 94°C for 30 s, 45°C for 30 s, and 72°C for 30 s; final extension, 72°C for 7 min.
primers RubIrF–RubIrR, specific to the *cbbL* genes of "red"-like form I RuBPC	In 25 μl: buffer (17 mM $(NH_4)_2SO_4$; 6 mM Tris–HCl, pH 8.8; 4 mM $MgCl_2$), 6.25 nmol of dNTP, 17.5 ng of template DNA, 3.9 pmol of each primer, and 1.25 U of BioTaq DNA polymerase.	first cycle: 94°C for 3 min, 66°C for 1 min, and 72°C for 1 min; 7 subsequent cycles: 94°C for 30 s, 66°C for 20 s, and 72°C for 45 s; last 28 cycles: 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s; final extension, 72°C for 7 min.
primers RuIIF1–RuIIR3, specific to the *cbbM* genes of form II RuBPC	In 20 μl: buffer (17 mM $(NH_4)_2SO_4$; 6 mM Tris–HCl, pH 8.8; 6 mM $MgCl_2$), 6.25 nmol of dNTP, 40 ng of template DNA, 7.2 pmol of each primer, and 1.25 U of BioTaq DNA polymerase.	first cycle: 94°C for 3 min, 66°C for 30 s, and 72°C for 30 s; 35 subsequent cycles: 94°C for 30 s, 66°C for 30 s, and 72°C for 30 s; final extension, 72°C for 7 min.
nested PCR with primers RuIIF1–RuIIR3/RuIIF2–RuIIR2, specific to the *cbbM* genes of form II RuBPC	In 20 μl: buffer (17 mM $(NH_4)_2SO_4$; 6 mM Tris–HCl, pH 8.8; 6 mM $MgCl_2$), 6.25 nmol of dNTP, template DNA (for primary PCR, 40 ng; for secondary PCR, 0.2 μl of the reaction mixture after the primary PCR), 7.2 pmol of each primer, and 1.25 U of BioTaq DNA polymerase.	Primary PCR with primers RuIIF1–RuIIR3: first cycle: 94°C for 3 min, 60°C for 30 s, and 72°C for 1 min; 30 subsequent cycles: 94°C for 30 s, 60°C for 30 s, and 72°C for 1 min; final extension, 72°C for 7 min. Secondary PCR with primers RuIIF2–RuIIR2: first cycle: 94°C for 3 min, 60°C for 30 s, and 72°C for 30 s; 27 subsequent cycles: 94°C for 30 s, 60°C for 30 s, and 72°C for 30 s; final extension, 72°C for 7 min.

(pH 7.8) was resuspended in 5 ml of the same buffer supplemented with 5 mM dithiothreitol and disrupted ultrasonically (22 kHz, 3 min, 4°C). Nondisrupted cells and large cell fragments were sedimented by centrifugation (40000 *g*, 20 min, 4°C); the supernatant was used in further experiments. The RuBPC activity was determined radiochemically from the ribulose-1,5-bisphosphate-dependent fixation of $NaH^{14}CO_3$ in a reaction mixture of the following composition (final volume, 0.1 ml): 50 mM Tris–HCl (pH 7.5), 5 mM dithiothreitol, 5 mM $MgCl_2$, 15 mM $NaH^{14}CO_3$ (0.04 MBq), and cell extract (0.5–1.0 mg of protein). The reaction was initiated by the addition of 1 mM ribulose-1,5-bisphosphate after preincubation of the mixture for 15 min. Protein was determined by the Lowry method using bovine serum albumin as a standard.

RESULTS AND DISCUSSION

Primer design. At the first stage of this work, we performed computer analysis of the data available in modern databanks on the nucleotide sequences of RuBPC genes of bacteria of various phylogenetic groups (Table 3); the aim was to reveal the most rigorously conserved sites and to design universal primers corresponding to these sites. As a result of this analysis, we found several sites with a degree of conservation of no less than 60% in the consensus nucleotide sequences of genes of the large subunit of "green"- and "red"-like form I RuBPC (the *cbbL* genes) and genes of form II RuBPC (*cbbM* genes) (Fig. 1). Based on the consensus nucleotide sequence, pairs of oligonucleotide primers were designed that allow amplification of RuBPC gene fragments about 800 bp long. These primers, as well as additional internal primers used for sequencing or for nested PCR, are shown in Table 4.

Testing of the primer system designed with autotrophic bacteria with a known primary structure of RuBPC genes. Testing of the primer system designed was performed with DNA preparations from the following bacterial strains: *Acidithiobacillus ferrooxidans* TFY, *Rhodobacter sphaeroides* 2R, and *Rhodospirillum rubrum* 1R. Identification of strains TFY and 2R as representatives of the above-mentioned species was confirmed by DNA–DNA hybridization and by sequencing and analysis of 16S rRNA genes [15, 16]. All representatives of these species are classical autotrophs, and their RuBPC are well studied at the genetic and molecular level, as well as in terms of the structure and functioning of the enzymes [3, 17]. GenBank contains sequences of *cbbL* genes of form I RuBPC from three strains of the species *A. ferrooxidans* (including the type strain) and strain HR of

Table 3. Taxonomic diversity of microorganisms used for the design of primers specific to RuBPC genes

RuBPC form	Phylogenetic group	Genera	Number of sequences used for multiple alignment
"Green"-like form I	Cyanobacteria	*Synechococcus*, *Anabaena*, *Prochlorothrix*, *Prochlorococcus*, *Arthrospira*	40
	α-Proteobacteria	*Rhodobacter*, *Nitrobacter*	
	β-Proteobacteria	*Hydrogenophaga*, *Hydrogenophilus*, *Nitrosomonas*, *Thiomonas*, *Thiobacillus*, *Ralstonia*	
	γ-Proteobacteria	*Allochromatium*, *Hydrogenovibrio*, *Methylococcus*, *Nitrosococcus*, *Acidithiobacillus*, *Halothiobacillus*	
"Red"-like form I	Gram-positive bacteria	*Sulfobacillus*	15
	α-Proteobacteria	*Rhodobacter*, *Bradyrhizobium*, *Sinorhizobium*, *Xanthobacter*	
	β-Proteobacteria	*Nitrosospira*, *Ralstonia*	
Form II	α-Proteobacteria	*Magnetospirillum*, *Rhodobacter*, *Rhodopseudomonas*, *Rhodospirillum*	29
	β-Proteobacteria	*Thiomonas*, *Thiobacillus*	
	γ-Proteobacteria	*Halothiobacillus*, *Acidithiobacillus*, *Hydrogenovibrio*	

Rh. sphaeroides and the sequence of the *cbbM* gene of form II RuBPC from strain K01999 of *R. rubrum*.

Electrophoresis of PCR products (Figs. 2, 3, and 4 for "green"- and "red"-like form I RuBPC and form II RuBPC, respectively) showed that, for *A. ferrooxidans* TFY, *Rh. sphaeroides* 2R, and *R. rubrum* 1R, fragments of the expected size were obtained. To prove that the obtained amplicons were actually fragments of RuBPC genes, we performed their sequencing in both directions with the use of the amplifying and sequencing primers of the system tested. Comparative analysis showed a nucleotide sequence homology of no less than 69% between the sequences determined in this work and the sequences of the corresponding genes available from GenBank. Strains of the corresponding species had the closest sequences of the RuBPC genes (no less than 90% homology of nucleotide sequences). The level of distinctions revealed did not exceed that

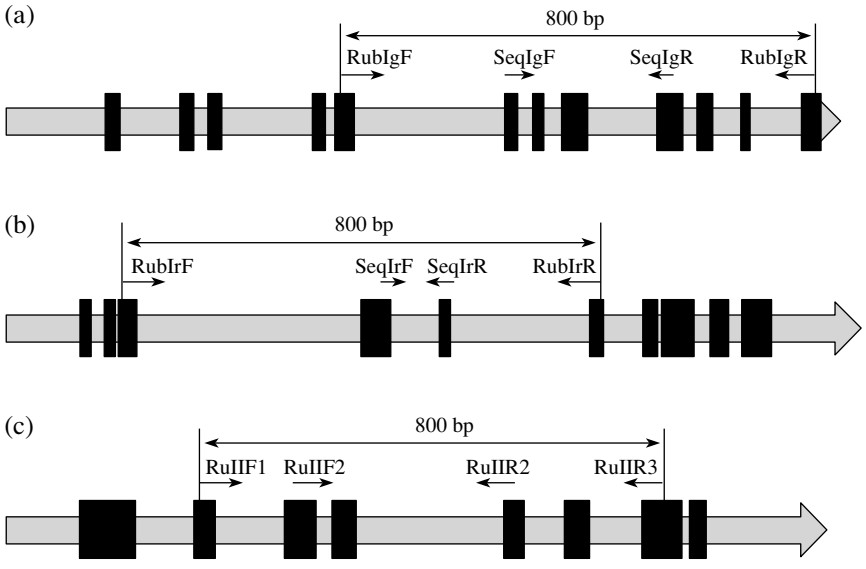

Fig. 1. Location of the conserved sites used to design the primers for fragment amplification in genes of different forms and types of RuBPC. Conserved sites are shown by black rectangles in (a) *cbbL* gene of "green"-like form I RuBPC (at least 60% conserved) of *A. ferrooxidans* ATCC 19859, (b) *cbbL* gene of "red"-like form I RuBPC (at least 75% conserved) of *Rh. sphaeroides* HR, and (c) *cbbM* gene of form II RuBPC (at least 55% conserved) of *R. rubrum* K01999.

Table 4. System of oligonucleotide primers for different forms of RuBPC genes

Gene form	Primer designation	Primer sequence*	Target position in the corresponding gene**
cbbL gene of "green"-like form I RuBPC	RubIgF	5'-GAYTTCACCAARGAYGAYGA-3'	571–590
	RubIgR	5'-TCRAACTTGATYTCYTTCCA-3'	1363–1382
	SeqIgF	5'-TGCAYATYCAYCGHGCHAT-3'	851–869
	SeqIgR	5'-GGCATRTGCCAIACRTGGAT-3'	1123–1142
cbbL gene of "red"-like form I RuBPC	RubIrF	5'-GCVACCTGGACSGTSGTVTGG-3'	196–216
	RubIrR	5'-TCGCCYTCSAGCTTGCCSAC-3'	997–1016
	SeqIrF	5'-CAGCCCTTYATGCAYTGGCGCGA-3'	631–653
	SeqIrR	5'-GTACATBTCYTCCATSGTRCC-3'	739–759
cbbM gene of form II RuBPC	RuIIF1	5'-GGHAACAACCARGGYATGGGYGA-3'	328–350
	RuIIF2	5'-GGIACVATCATCAARCCVAA-3'	484–503
	RuIIR2	5'-TGRCCIGCICGRTGRTARTGCA-3'	851–872
	RuIIR3	5'-CGHAGIGCGTTCATGCCRCC-3'	1105–1124

* Designations for degenerated positions are as follows: Y = T, C; R = A, G; S = G, C; B = T, G, C; V = A, G, C; H = A, T, C.
** The primer positions are given in terms of the sequences of the corresponding genes of *A. ferrooxidans* ATCC 19859 (the *cbbL* gene of "green"-like form I RuBPC), *Rh. sphaeroides* HR (the *cbbL* gene of "red"-like form I RuBPC), and *R. rubrum* K01999 (the *cbbM* gene of form II RuBPC).

typical of strains of one and the same species (for the three strains of *A. ferroxidans,* the distinction level is 1.1–26.4%). Such a distinction level can be explained by intraspecies divergence of the RuBPC gene nucleotide sequences. Thus, strains *A. ferrooxidans* TFY, *Rh. sphaeroides* 2R, and *R. rubrum* 1R are suitable as positive controls for PCR detection of the *cbbL* genes of "green"- and "red"-like from I RuBPC and the *cbbM* gene of from II RuBPC, respectively.

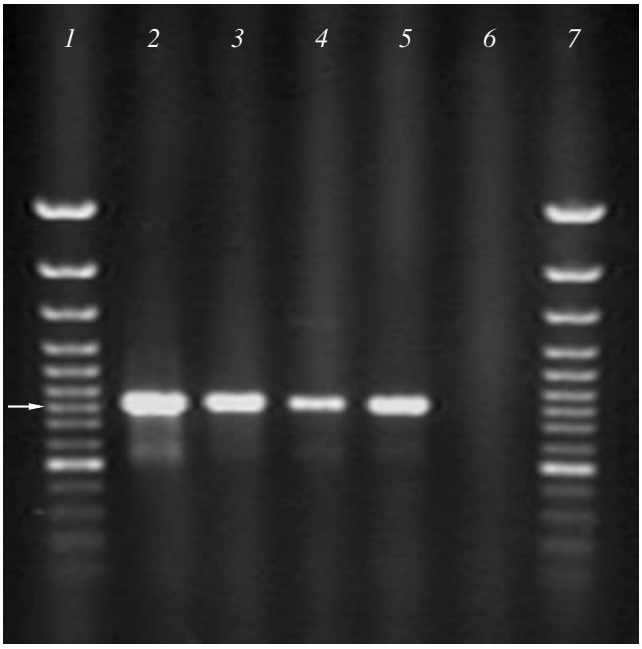

Fig. 2. Electrophoretic analysis of PCR products obtained with template DNA from various bacteria with primers RubIgF–RubIgR. The arrow shows the PCR fragment of the gene of "green"-like form I RuBPC. Lanes: (*1, 7*) 100-bp DNA molecular weight marker (Fermentas); (*2*) *A. ferrooxidans* TFY; (*3*) *Thioalkalivibrio thiocyanoxidans* ARH2; (*4*) *Thiomicrospira* sp. HL5; (*5*) *Thioalkalimicrobium sibiricum* AL7; (*6*) control without template DNA.

Detection and sequencing of RuBPC genes in representatives of various taxa of prokaryotes. The primer system designed was used to reveal RuBPC

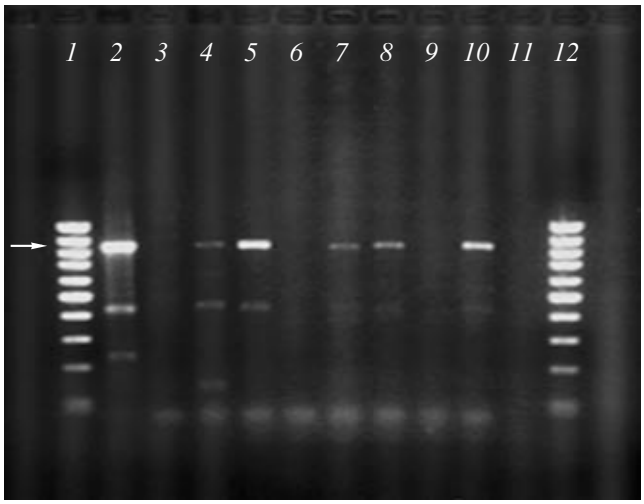

Fig. 3. Electrophoretic analysis of products of three-stage PCR on template DNA from various bacteria with primers RubIgF–RubIgR. The arrow shows the PCR fragment of the gene of "red"-like form I RuBPC. Lanes: (*1, 12*) 100-bp DNA molecular weight marker (Fermentas); (*2*) *Rh. sphaeroides*; (*3*) *Escherichia coli*; (*4*) *Methylocaldum* sp. 108; (*5*) *Methylocapsa acidiphila*; (*6*) *Methylocella palustris*; (*7*) *M. echinoides*; (*8*) *Beggiatoa* sp. D402; (*9*) *Leucothrix* sp. 3WS; (*10*) *L. thiophila* 4WS; (*11*) control without template DNA.

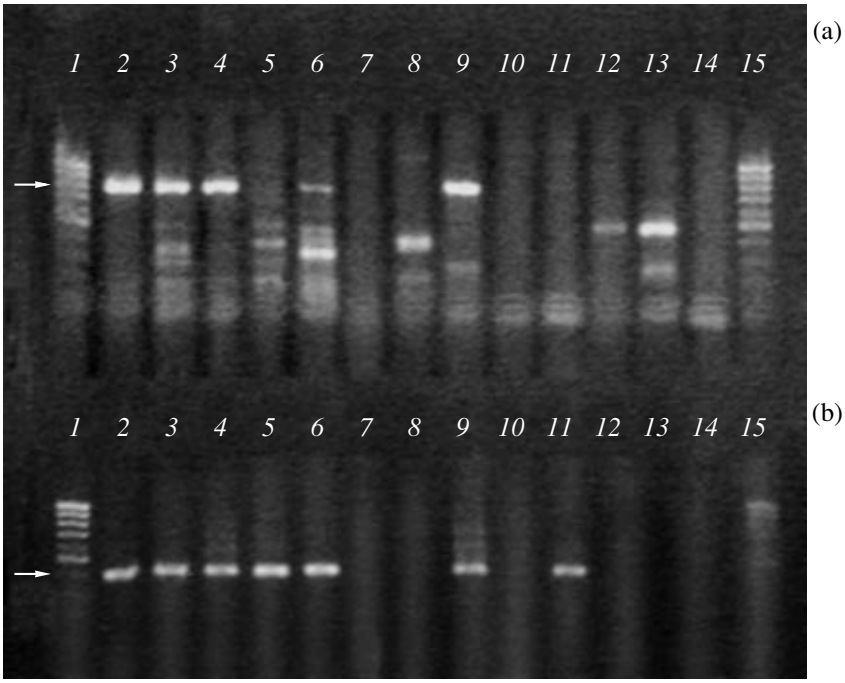

Fig. 4. Electrophoretic analysis of PCR products obtained on template DNA from various bacteria (a) with primers RuIIF1–RuIIR3 and (b) after nested PCR with primers RuIIF1–RuIIR3 at the first stage and primers RuIIF2–RuIIR2 at the second stage. The arrow shows the PCR fragment of the gene of form II RuBPC. Lanes: (*1, 15*) 100-bp DNA molecular weight marker (Fermentas); (*2*) *R. rubrum*; (*3*) *Rh. sphaeroides*; (*4*) *Rh. capsulatus*; (*5*) *Phaeospirillum fulvum; (6) E. mobilis*; (*7*) *Ch. aurantiacus* OK-70fl; (*8*) *Escherichia coli*; (*9*) *Thiobacillus thioparus*; (*10*) *Heliorestis baculata*; (*11*) *Thiothrix arctophila*; (*12*) *L. thiophila* 4WS; (*13*) *Leucothrix* sp. 3WS; (*14*) control without template DNA.

genes in various prokaryotes not studied earlier by analogous methods. Genes of form I and form II RuBPC were detected in a number of photo- and chemotrophic bacteria (Table 1). Thus, taken together, the primer pairs designed make up a universal system efficient in the detection of RuBPC genes of different forms. This system was also used for sequencing of amplified fragments of the genes of "green"-like form I RuBPC that we revealed in the type strain of the purple sulfur bacterium *E. shaposhnikovii* no. 1 and the type strain of a recently described new genus and species, *Thioalkalispira microaerophila* ALEN 1 [18]; of "red"-like form I RuBPC that we found in the type strain of the new genus and species of acidophilic methanotrophs *Methylocapsa acidiphila* B2 [19]; and of the form II RuBPC of strain 7, which, based on its phenotypic properties and 16S rRNA gene sequence, was tentatively identified as *Magnetospirillum magnetotacticum* [20].

Blast searches in GenBank performed for the nucleotide sequences of the gene fragments that we PCR-amplified and sequenced showed the high degree of their similarity (65–73%) with analogous sequences from the genomes of other bacteria, confirming their affiliation with the RuBPC gene family. These data do not strictly prove the assumption that the genes detected encode a functionally active enzyme that allows the microorganisms studied to fix CO_2 via the Calvin cycle; nevertheless, they provide a profound basis for further studies of this possibility.

Analysis of amino acid sequences of translated fragments of RuBPC genes. Conceptual translation of the determined sequences of RuBPC gene fragments showed that the level of their similarity with the closest analogous sequences was 81.5–98.0%. The amino acid sequences of the fragments translated contained the GXDFXKXDE structural motif (positions 196–204 in *Synechococcus* sp. PCC 6301 numbering), in which lysine-201 is responsible for the formation of carbamate during RuBPC activation [21]. In addition, the fragments translated contained all the amino acid residues necessary for the formation of the active center. For "green"-like form I enzyme, these were histidine-294, arginine-295, histidine-327, lysine-334, leucine-335, serine-379, glycine-381, 403, and 404 (*Synechococcus* sp. PCC 6301 numbering); for "green"-like form I enzyme, asparagine-123, lysine-175, 177, histidine-294, and arginine-295 (*Synechococcus* sp. PCC 6301 numbering); for form II enzyme, asparagine-111, lysine-166, 168, histidine-287, arginine-288, histidine-321, lysine-329, and methionine-330 (*R. rubrum* K01999 numbering). Thus, the fragments obtained contain the amino acid residues necessary for the formation of the active center and may be assumed to be fragments of genes that code for a functionally active enzyme. This assumption is supported by measure-

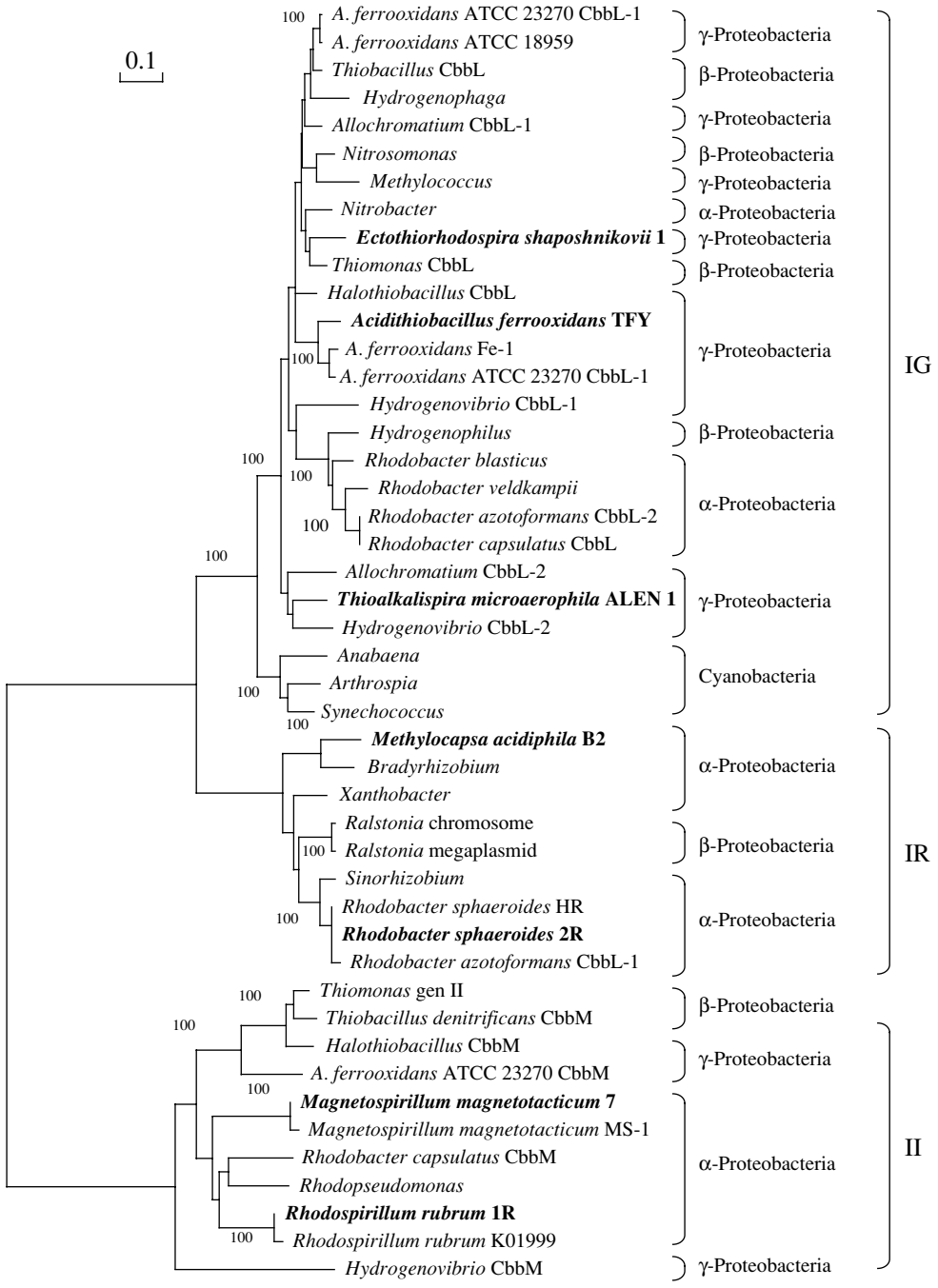

Fig. 5. Unrooted phylogenetic tree constructed based on the comparison of amino acid sequences of RuBPC (form II and large subunits of form I). Sequences determined in the present work are set in boldface. Scale bar shows the evolutionary distance corresponding to 10 substitutions per 100 amino acids. Numerals at the branching point show the significance of the branching order, determined by bootstrap analysis of 100 alternative trees (values above 70 are considered significant). Taxon names at brackets on the right show the correspondence of the topology of the RuBPC tree with data from phylogenetic analysis of 16S rRNA genes.

ments of RuBPC activity that we performed for some of the bacteria studied: *E. shaposhnikovii* no. 1 (29 nmol/(min mg protein)), *R. rubrum* (18 nmol/(min mg protein)), and *Rh. sphaeroides* 2R (27 nmol/(min mg protein)). Thus, at least in these bacteria, the RuBPC genes are expressed, which should allow them to fix CO_2 via the Calvin cycle.

Phylogenetic analysis. For the phylogenetic analysis of the conceptual translations of the de novo determined RuBPC gene sequences, we retrieved from GenBank analogous sequences for strains of cultivated bacteria belonging to various phylogenetic groups. The phylogenetic tree was constructed based on an alignment containing 342 amino acid residues. The

sequences compared split into three major clusters in accordance with their affiliation to different forms of RuBPC genes (Fig. 5). The cluster of the "green"-like form I RuBPC united α-, β-, and γ-proteobacteria, as well as cyanobacteria. Within this cluster, the GenBank RuBPC sequences of *A. ferrooxidans* strains were distributed among two subclusters; strain TFY studied in the present work belonged, at a maximum possible significance level (100), to the cluster that included the *cbbL*-1 genes of the type strain ATCC 23270 and strain Fe1; this fact confirms the correctness of the strain TFY identification. The RuBPC amino acid sequence of the alkaliphilic obligate chemolithotroph *Thioalkalispira microaerophila* ALEN 1 had as its closest relatives RuBPC of γ-proteobacteria (at least 81% homology); however, all of them were approximately equidistant from the *Thioalkalispira microaerophila* RuBPC, analogously to the earlier studied nucleotide sequences of 16S rRNA genes [18].

The results of comparative analysis of amino acid sequences of the "green"-like RuBPC of *E. shaposhnikovii* no. 1 were in contradiction with the results of phylogenetic analysis based on 16S rRNA gene comparison: the sequence closest to *E. shaposhnikovii* no. 1 proved to be a β-proteobacteria representative, *Thiomonas intermedia* K12 (89.7% homology).

The cluster of the "red"-like form I RuBPC combined α- and β-proteobacteria; the diversity of organisms possessing this RuBPC type proved to be considerably lower than the diversity of organisms possessing the "green"-like enzyme. The strain *Rh. sphaeroides* 2R studied in this work belonged, at a maximum possible value of bootstrap analysis (100), to the cluster that united the closely related species of α-proteobacteria *Rh. sphaeroides* HR and *Rh. azotoformans*; this confirms the correctness of the strain 2R identification.

The detection of the RuBPC gene in *Methylocapsa acidiphila* B2, which phylogenetically belongs to α-proteobacteria (the *Rhizobium–Agrobacterium* group) was quite unexpected since operation of the serine pathway of C_1 compound assimilation had been shown in this organism and no RuBPC activity had been detected [19]. In the RuBPC amino acid sequences, the organism closest to *Methylocapsa acidiphila* B2 was the α-proteobacteria representative *Bradyrhizobium japonicum* (88.4% homology); this correlates with data of 16S rRNA gene analysis.

The cluster of form II RuBPC included α-, β-, and γ-proteobacteria; the diversity of organisms possessing this RuBPC form was not great compared to the "green"-like form I RuBPC cluster. The de novo determined RuBPC fragment sequences of *R. rubrum* 1R and *M. magnetotacticum* no. 7 proved to be virtually identical (98.2–99.6% homology) to the analogous GenBank sequences of other strains of these species.

It should be noted that the topology of the phylogenetic tree constructed based on the analysis of RuBPC sequences exhibits considerable distinctions with the tree for the same organisms constructed proceeding from data of analysis of 16S rRNA genes (Fig. 5). This conclusion agrees with the earlier conclusion of Watson and Tabita [2] and is most probably explained by peculiarities of the evolution of the RuBPC genes. However, it cannot be excluded that, upon extension of the number of known RuBPC sequences, the topology of the RuBPC tree will change.

It may be concluded that the obtaining of new information on the primary structure of RuBPC molecules and its use in phylogenetic analysis will improve our understanding of the evolution of this enzyme, as well as of the evolution of autotrophic microorganisms. The most important task in the nearest future is the analysis of the RuBPC genes in organisms for which the pathways of CO_2 assimilation are debatable. Among such organisms are "green" sulfur and nonsulfur bacteria, which are the subject of our current research.

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