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EXPERIMENTAL ARTICLES

Phylogeny of Anoxygenic Filamentous Phototrophic Bacteria of the Family *Oscillochloridaceae* as Inferred from Comparative Analyses of the *rrs*, *cbbL*, and *nifH* Genes

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Abstract—Phylogeny of anoxygenic filamentous phototrophic bacteria (AFPB) of the family *Oscillochloridaceae* (*Oscillochloris trichoides* DG6^T and the recently isolated strains *Oscillochloris* sp. R and C6) was studied based on comparative analyses of the genes coding for 16S rRNA (*rrs*), ribulose-1,5-bisphosphate carboxylase/oxygenase (*cbbL*), and nitrogenase (*nifH*). The sequences of the genes studied proved to be identical in the three strains, which is in agreement with data obtained earlier that showed a lack of differentiating phenotypic distinctions between these strains; therefore, it is proposed that the new strains should be identified as representatives of the species *O. trichoides.* Using an earlier designed system of oligonucleotide primers and a specially designed additional primer, fragments of the *cbbL* genes of the "red-like" form I RuBisCO were amplified and sequenced for all of the *O. trichoides* strains. Analysis of the *cbbL* genes suggested a separate position of the bacteria studied in the phylogenetic tree, where *O. trichoides* strains formed an independent branch, which, apart from this species, also included the only studied species of gram-positive facultatively chemoautotrophic bacteria, *Sulfobacillus acidophilus.* In the phylogenetic tree inferred from the analysis of *nifH* genes, the bacteria under study also formed a new separate branch, deviating near the root, which indicated a lack of relatedness between them and other phototrophic bacteria. The data obtained support the conclusion that AFPB has an ancient origin and their allocation as one of the main evolutionary lineages of eubacteria, which was made based on the analysis of ribosomal genes.

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Key words: anoxygenic filamentous phototrophic bacteria, AFPB, 16S rRNA, *rrs*, ribulose-1,5-bisphosphate carboxylase/oxygenase, RuBisCO, *cbbL*, nitrogenase, *nifH*, phylogenetic analysis.

The group of anoxygenic filamentous phototrophic bacteria (AFPB) includes representatives of the genera *Chloroflexus, Oscillochloris, Chloronema, Heliothrix*, and *Roseiflexus* [1, 2]. These microorganisms, which form multicellular trichomes, proved to be phylogenetically distinct from other lineages of phototrophs but, at the same time related to a number of chemotrophic bacteria assigned to the phylum *Chloroflexi* [2]. Not long ago, all AFPB were included in the family *Chloroflexaceae*. However, recently, based on the investigation of their phenotypic and genotypic properties, representatives of the genus *Oscillochloris* were assigned to a new family, *Oscillochloridaceae* [3]. A phylogenetic study of an AFPB enrichment culture identified as *"Chloronema giganteum"* showed that the nucleotide sequence of the 16S rRNA gene of this bacterium falls in the same cluster as the genes of *Oscillochloridaceae* representatives [4]. The recently described *Candidatus* "Chlorothrix halophila," isolated from a microbial mat in a hypersaline environment, phylogenetically belongs to a lineage that includes *Chloroflexus* and *Oscillochloris* [5]. *Roseiflexus* is phylogenetically remote from other representatives of the group and forms a separate branch of AFPB [1].

Among AFPB, only representatives of the genera *Chloroflexus, Oscillochloris*, and *Roseiflexus* have been isolated in pure cultures. The capacity for autotrophic growth has been demonstrated in representatives of

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Oscillochloris and *Chloroflexus* and also in *Candidatus* "Chlorothrix halophila". It was shown that in *C. aurantiacus* new pathways of autotrophic CO_2 fixation operate, the reductive dicarboxylic acid cycle [6, 7] or the 3-hydroxypyruvate cycle [8]. Despite its autotrophic capacity, *C. aurantiacus* prefers photoheterotrophic conditions and can utilize a wide spectrum of organic compounds as electron donors and carbon sources, both in the light and dark, aerobically and anaerobically.

As far as the carbon metabolism of *Candidatus* "Chlorothrix halophila" is concerned, it is only known that the 3-hydroxypropionate cycle is most probably not involved in autotrophic assimilation of CO_2, since the activity of its key enzyme, propionyl-CoA synthase, could not be detected [5].

As distinct from members of the family *Chloroflexaceae*, bacteria of the family *Oscillochloridaceae*, represented by a single genus and species, *Oscillochloris trichoides*, are obligate photolithoautotrophs that can only grow anaerobically in the presence of sulfide. These bacteria are incapable of assimilatory sulfate reduction; they phototrophically use sulfide as the electron donor, oxidizing it to elemental sulfur, which is deposited outside the cells. As in purple bacteria and cyanobacteria, autotrophic assimilation of carbon dioxide occurs in *O. trichoides* via the Calvin–Benson–Bassham cycle [9].

The key enzyme in this cycle is ribulose-1,5-bisphosphate carboxylase (RuBisCO). In eubacteria, RuBisCO occurs in two main forms. Form I is more widespread, occurring not only in the majority of autotrophic bacteria but also in algae and terrestrial plants. Form I consists of eight large (L) and eight small (S) subunits, which are coded by the *cbbL* and *cbbS* genes, respectively; the catalytic function is performed by large subunits. In turn, form I RuBisCO is subdivided into two types, "green-like" and "red-like," which differ in the amino acid composition of the large subunits. The "green-like" RuBisCO is more widespread; it occurs in the chloroplasts of terrestrial plants and green algae, as well as in cyanobacteria and representatives of α-, β-, and γ-proteobacteria. The "red-like" RuBisCO has been found in a narrower range of organisms: in chloroplasts of many nongreen algae and in some representatives of α- and β-proteobacteria. In *Rhodobacter azotoformans*, both "red-like" and "green-like" enzymes have been revealed [10]. Form II RuBisCO (*cbbM* gene) occurs much more rarely, and mainly in bacteria; it consists of only large subunits (L_n), their number varying from two to eight in different organisms. Many autotrophic bacteria, including some nonsulfur purple bacteria and thiobacilli, possess both form I and form II RuBisCO. The presence of an active RuBisCO enzyme has been demonstrated in various *Oscillochloris* strains [11]; however, its type and the nucleotide sequence of the *cbb* genes encoding it remained unknown before the present work.

One more remarkable feature of the metabolism of *Oscillochloridaceae* representatives, which distinguishes them from representatives of *Chloroflexaceae*, is their capacity for dinitrogen fixation [12]. The nitrogenase enzyme complex, which is the key one in this process, consists of two subunits: a FeMo protein, encoded by the *nifD* and *nifK* genes, and a Fe protein, encoded by the *nifH* gene; before the present work, these genes were not studied in *Oscillochloris* representatives.

Currently, analysis of molecular markers alternative to the ribosomal genes is widely used in phylogenetic studies; functional genes responsible for the metabolic peculiarities of the microbial group studied are usually used as such markers.

The aim of the present study was detection, sequencing, and phylogenetic analysis of the genes encoding RuBisCO (*cbb*) and nitrogenase (*nifH*) in the type strain *O. trichoides* DG-6 and in two more *Oscillochloris* isolates from natural freshwater habitats, with a comparison of the results of this analysis with the results of the analysis of the 16S rRNA genes (*rrs*) of the same strains.

MATERIALS AND METHODS

Microorganisms and cultivation conditions. Three mesophilic AFPB strains isolated from different freshwater habitats and belonging to the family *Oscillochloridaceae* were the subjects of this study. The type strain *O. trichoides* DG-6 was isolated from a hydrogen sulfide spring in the Caucasus, while strain *Oscillochloris* sp. R was from a water body near Moscow, and strain C6 was an isolate from a small body of water in Siberia. The *Oscillochloris* cultures were cultivated as described earlier [13].

Isolation of DNA and amplification and sequencing of the genes under study. For the isolation and purification of DNA, an earlier described procedure [14] was used.

For the amplification and sequencing of the 16S rRNA genes (*rrs*), universal bacterial primers [15] were used; for amplification and sequencing of the *cbb* and *nifH* genes, earlier developed primer systems were used [16, 17].

Sequencing of amplification products was performed according to Sanger, using the Big Dye Terminator v.3.1 kit on an automatic ABI 3730 sequencer (Applied Biosystems Inc., United States), according to the instructions of the manufacturer.

Phylogenetic analysis of nucleotide and amino acid sequences. Editing of the sequences was performed using the BioEdit Software Package [http://jwbrown.mbio.ncsu.edu/BioEdit/bioedit.html]. Primary comparison of the de novo obtained sequences with sequences available from the GenBank database was performed with the use of the NCBI BLAST software package [http://www.ncbi.nlm.nih.gov/blast].

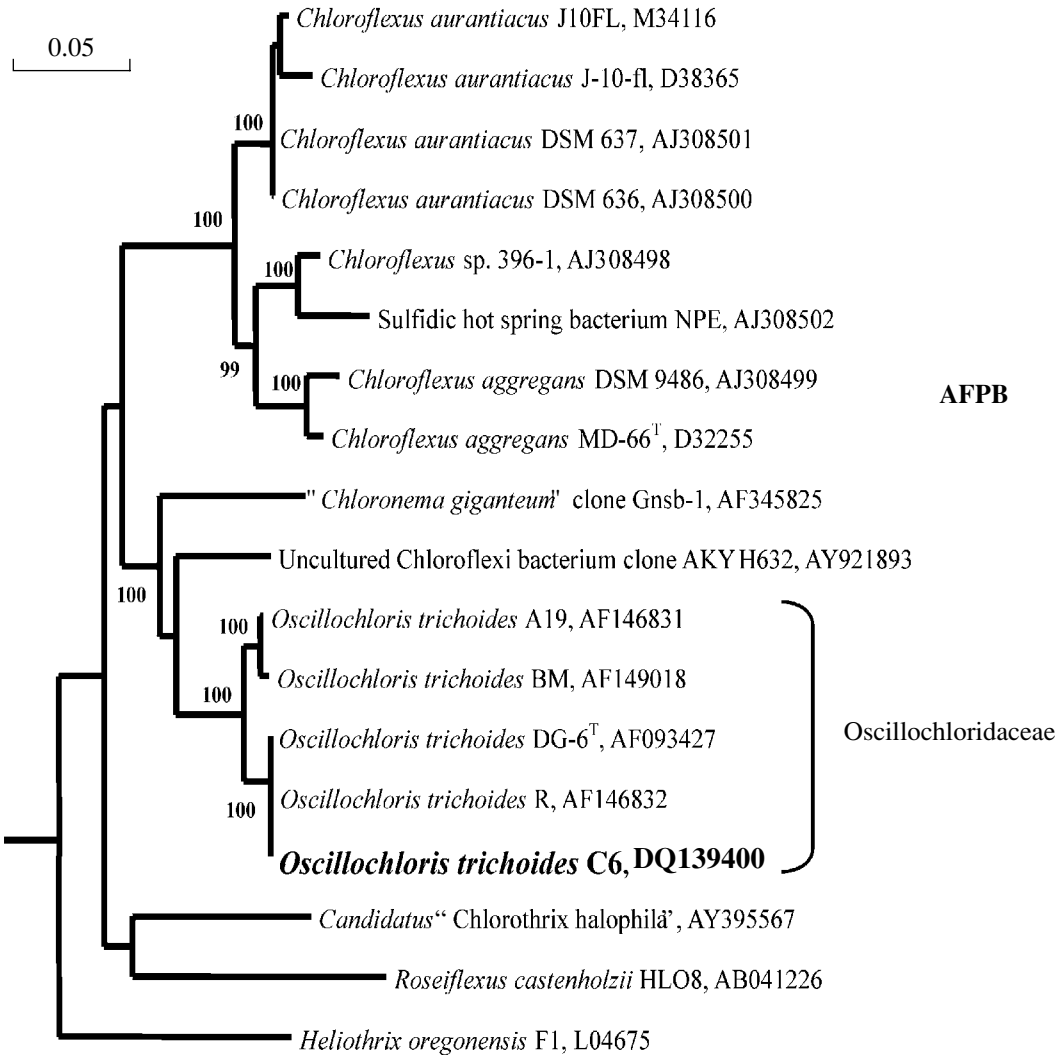

Fig. 1. Phylogenetic tree of AFPB inferred from the analysis of nucleotide sequences of 16S rRNA genes (*rrs*). The tree was constructed using the neighbor-joining algorithm with the *E. coli* sequence taken as an outgroup. The sequence determined in the present study is set in bold. Numerals show the significance of the branching order, as determined by bootstrap analysis (values above 95 were considered significant). Scale bar shows evolutionary distance corresponding to 5 substitutions per 100 nucleotides.

Further comparative analysis used sequences of the *rrs, ccbL*, and *nifH* genes retrieved from GenBank. Available nucleotide sequences and conceptual translations of the genes of interest were aligned using the CLUSTALW v 1.75 program. Phylogenetic trees were constructed using the methods implemented in the software packages TREECONW [http://bioc-www.uia.ac.be/u/yvdp/treeconw.html] and PHYLIP [http://evolution.genetics.washington.edu/phylip.html]. The significance of the branching order was determined (in %) based on bootstrap analysis of 1000 alternative trees.

Deposition of the nucleotide sequences. The nucleotide sequences of the 16S rRNA, (*rrs*), *cbbL*, and *nifH* gene fragments were deposited in GenBank under accession numbers DQ139400–DQ139407.

RESULTS

Sequencing and analysis of the 16S rRNA genes (*rrs*). In addition to the earlier performed determination of the nucleotide sequences of the 16S rRNA genes (*rrs*) of the type strain *O. trichoides* DG-6 and strains *Oscillochloris* sp. R, BM, and A19 [3], in the present work we sequenced the 16S rRNA gene of a newly isolated strain *Oscillochloris* sp. C6; the determined sequence was 1350 nucleotides long and corresponded to *Escherichia coli* positions 38–1447.

Comparative analysis of the nucleotide sequences of the 16S rRNA genes (*rrs*) of all the *Oscillochloris* strains investigated, including the type strain of the sole species of this genus, *O. trichoides* DG-6, revealed that they were highly homologous (97.8–100%). The *Oscillochloris* strains formed in the phylogenetic tree (Fig. 1), a coherent cluster that was distinct from the

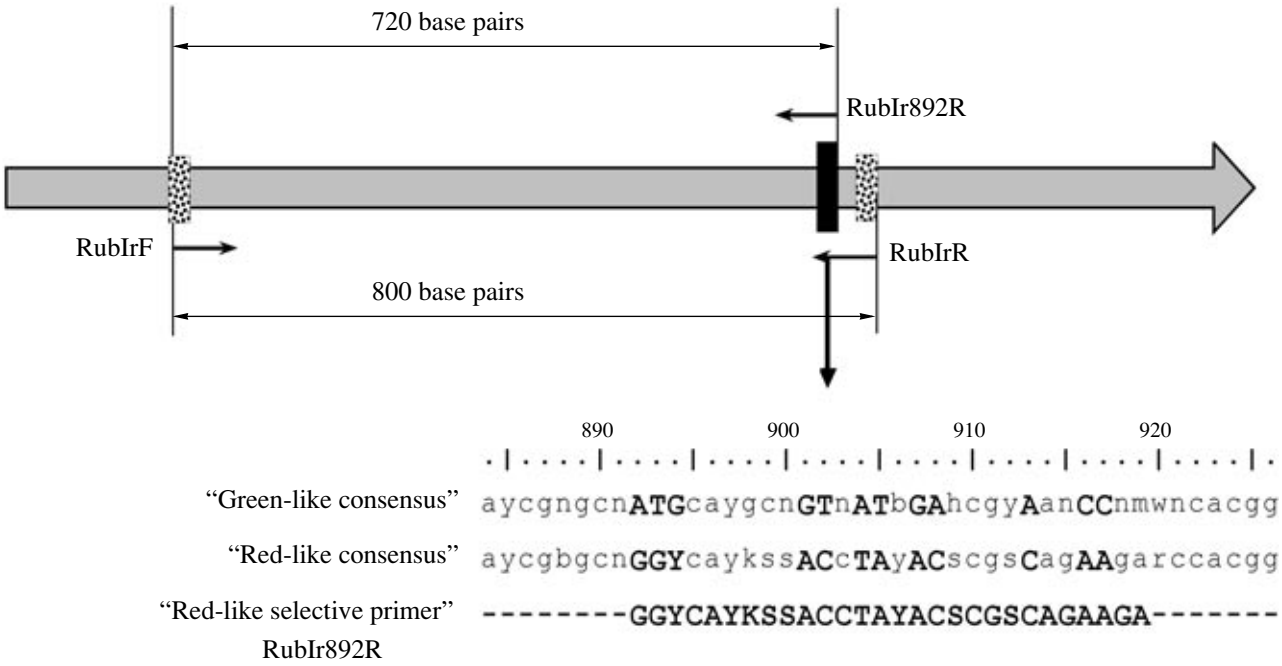

Fig. 2. Scheme of the location of conserved sites in the nucleotide sequence of the "red-like" form I *cbbL* gene. The conserved sites targeted by an earlier-designed primer system are shown by particolored rectangles, and that targeted by the newly designed additional reverse primer is shown by a black rectangle. The bold arrow points to the consensus sequences of the latter site (nucleotides different in the consensuses are shown in bold uppercase) and the nucleotide sequence of the newly designed primer (the numbering is according to the *cbbL* gene of *Rh. sphaeroides* HR).

representatives of other AFPB genera, from which the closest to the *Oscillochloris* strains was the AFPB enrichment culture termed *"Chloronema giganteum"* (89.9–91.0% homology).

Moreover, the comparison of the 16S rRNA gene sequences of the *Oscillochloris* strains with the 16S rRNA gene sequences, available from the GenBank database, revealed only one remotely related (92.8–93.1% homology) environmental 16S rDNA clone, which represented soil AFPB (accession no. AY921893). In the phylogenetic tree that we constructed, this clone occupied an intermediate position between the cluster of *Oscillochloris* strains and the *"Chloronema giganteum"* branch.

Detection and sequencing of *cbb* genes. To detect *cbb* genes in the AFPB strains *O. trichoides* DG-6 and *Oscillochloris* sp. R and C6, we initially used an earlier designed system of oligonucleotide primers [16]. However, in these experiments we failed to obtain PCR products corresponding to fragments of *cbb* genes (either of "green-like" or "red-like" form I RuBisCO or of form II RuBisCO), although in positive controls the primer system was effective. An analogous (negative) result was obtained when we used our primer system to amplify *cbb* genes in the gram-positive bacterium *Sulfobacillus acidophilus* NAL, although, for this organism, a fragment of the *cbbL* gene of "red-like" form I RuBisCO has been detected and sequenced (GenBank accession number U75301).

In order to improve our primer system, we explored the possibility of designing an additional primer for "red-like" form I RuBisCO genes that would be more sensitive and selective with respect to these genes (and discriminative with respect to the "green-like" genes). As a result of comparative analysis of the gene fragments available at that moment in the database and the newly sequenced gene fragments, a conserved site of the consensus sequence of "red-like" genes was chosen that exhibited the highest number of nucleotide substitutions, as compared to the consensus sequence of the "green-like" genes. Proceeding from this site, a new degenerated reverse primer was designed that was intended for the selective amplification of fragments of "red-like" *cbbL* genes of a wide range of bacteria (Fig. 2).

Testing of the newly designed primer employed the DNA of *S. acidophilus* NAL as a template. As a result, a 720-bp PCR product was obtained and sequenced. Its subfragment proved to be 99.7% homologous to the 387-bp *cbbL* gene fragment available in GenBank; this attested to the successful operation of the new primer system.

Using the improved primer system, we obtained PCR products of the expected size (about 720 bp) for all the *Oscillochloris* strains studied. Complete

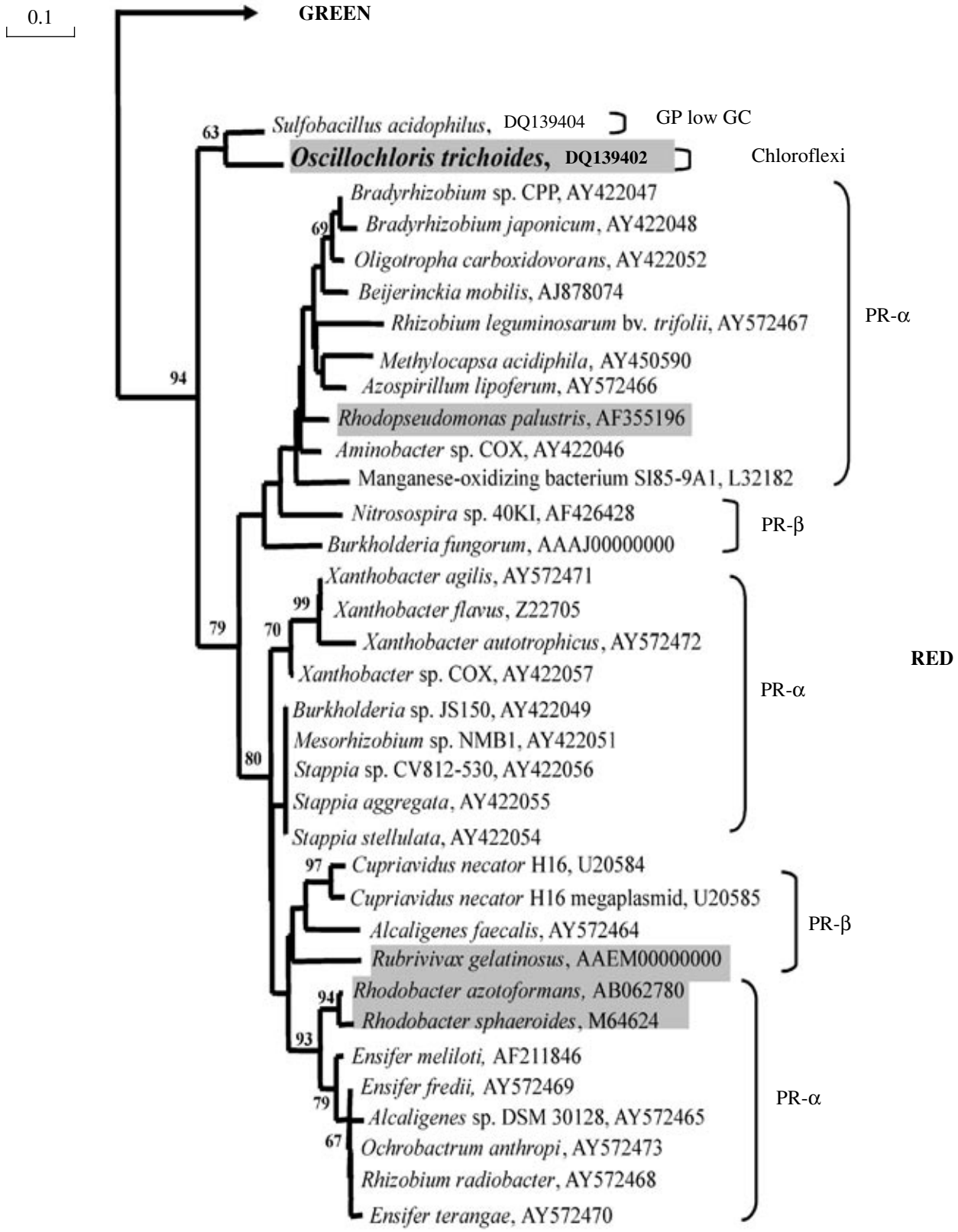

Fig. 3. Unrooted phylogenetic tree of autotrophic bacteria possessing "red-like" form I RuBisCO. The tree was constructed based on comparative analysis of conceptual translations of *cbbL* genes, with the use of the neighbor-joining algorithm. The sequences determined in the present study are set in bold. A gray background shows the sequences of phototrophic bacteria. Numerals show the significance of the branching order as determined by bootstrap analysis (values above 60 were considered significant). The scale bar shows evolutionary distance corresponding to 10 substitutions per 100 amino acid residues.

sequencing of these products yielded nucleotide sequences that were virtually identical in all of the *Oscillochloris* strains (99.4–100% homology). Preliminary screening in the GenBank database showed that the newly sequenced genes belonged to the "red-like" *cbbL* gene family.

Phylogenetic analysis of *cbbL* genes. After conceptual translation of the *cbbL* gene fragments of the *Oscillochloris* strains studied and their alignment with the amino acid sequences of "red-like" form I RuBisCO of other bacteria available from GenBank, comparison of 232 positions was carried out. The topologies of phylogenetic trees constructed on the basis of this alignment were the same irrespective of the algorithm used, namely the neighbor-joining (Fig. 3), maximum parsimony, and Fitch–Margoliash algorithms (data not shown).

Moreover, the topology of the trees constructed did not differ from that of earlier published trees, based on the comparative analysis the "red-like" form I RuBisCO [18]. As it was with the earlier published trees, the topology of our tree for "red-like" form I RuBisCO, despite the restricted phylogenetic diversity of the bacteria possessing it, did not coincide with the topology of trees based on the commonly accepted analysis of the 16S rRNA genes (*rrs*): α- and β-proteobacteria did not form separate clusters in the RuBisCO tree.

At the same time, the analysis of *cbbL* genes testified to a separate phylogenetic position of the *Oscillochloris* representatives, which, with a high level of bootstrap support (94), formed in the phylogenetic tree a separate branch that was distinct from the cluster of proteobacteria possessing "red-like" genes (the level of identity of amino acid sequences was 73.1–85.3%). According to data from *cbbL* gene analysis, the only organism relatively close to the AFPB under study was the gram-positive facultatively chemoautotrophic bacterium *S. acidophilus* (the level of identity of amino acid sequences of 89.2%), which fell into the monophyletic cluster formed by the *Oscillochloris* strains. The level of bootstrap support however was not high (63). Not a single sequence of an uncultivated organism was found in GenBank that would fall into the cluster under consideration.

Detection, sequencing, and phylogenetic analysis of *nifH* genes. The use of an earlier designed primer system [17] yielded PCR products of the expected length (about 450 bp), with DNA of all the *Oscillochloris* strains studied. Sequencing of the PCR products showed that they were 99.1–99.6% homologous in the strains studied. Preliminary screening in the GenBank database showed the affiliation of the newly determined nucleotide sequences with the *nifH* gene family.

After conceptual translation of the *nifH* gene fragments of the *Oscillochloris* strains studied and their alignment with the amino acid sequences of *nifH* genes of various phylogenetic lineages of prokaryotes available from GenBank, comparison of 150 positions was carried out. The topologies of phylogenetic trees constructed on the basis of this alignment, using neighbor-joining (Fig. 4), maximum parsimony, and Fitch–Margoliash algorithms (data not shown) exhibited inconsiderable differences in the order of certain branches (with a low level of bootstrap support).

As compared to the diversity of the autotrophs that possess the "red-like" form I RuBisCO, the phylogenetic diversity of diazotrophs is rather large, including representatives of most subdivisions of phototrophs: purple sulfur and nonsulfur proteobacteria, green sulfur bacteria, heliobacteria, and cyanobacteria. The topology of the nitrogenase tree that we constructed showed a significant correlation with the 16S rRNA tree; this correlation was in agreement with the results obtained by other researchers [19]. The distinctions observed can be explained by certain methodical difficulties encountered in the phylogenetic analysis of *nifH* genes, as well as by peculiarities of the evolution of these genes, including their lateral interspecies transfer [14].

In the nitrogenase tree, the *Oscillochloris* strains studied did not show relatedness with any of the previously studied diazotrophs, including diazotrophs representing the phylogenetic subdivisions of phototrophic bacteria (the degree of amino acid sequence identity was not higher than 72.5%). The *Oscillochloris* strains formed a separate branch, with a branching point close to the root of the eubacterial phylogenetic domain, although the level of bootstrap support was relatively low (68). As with the *cbbL* genes of *Oscillochloris*, not a single sequence of an uncultivated organism was found in GenBank that would belong to the *Oscillochloris nifH* gene lineage.

DISCUSSION

Although the AFPB strains of the genus *Oscillochloris* were isolated from geographically remote freshwater habitats, they proved to be identical phylogenetically, according to both the traditional analysis of 16S rRNA (*rrs*) genes and the analysis of the genes specific for their metabolism, namely RuBisCO genes and nitrogenase genes. These results do not contradict the data of phenotypic analysis, obtained earlier, including data from a detailed study of their carbon metabolism, which also did not reveal considerable distinctions between these strains [3, 13]. Therefore, based on the entire complex of the data obtained, it may be concluded that all of the strains investigated represent one species, *O. trichoides*; thus, the genus *Oscillochloris* remains monotypic, i.e., it contains a single species.

According to the data of the analysis of amino acid sequences of RuBisCO and nitrogenase, *Oscillochloridaceae* representatives are not related to any subdivision of phototrophs (purple sulfur or nonsulfur proteobacteria, green sulfur bacteria, heliobacteria, or cyanobacteria). In the phylogenetic trees constructed based on this analysis, *Oscillochloridaceae* representatives

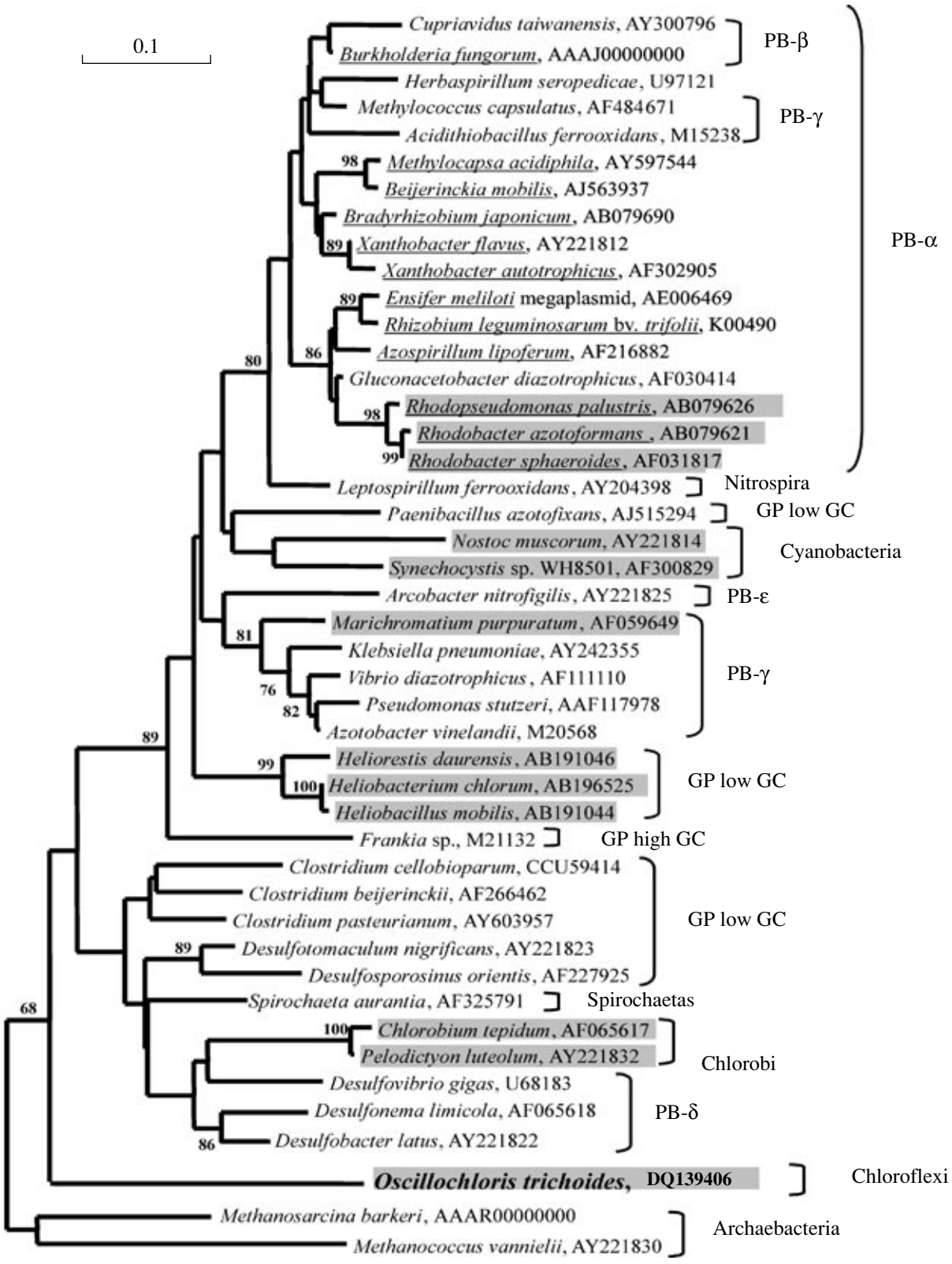

Fig. 4. Unrooted phylogenetic tree of nitrogen-fixing bacteria, constructed based on comparative analysis of conceptual translations of *nifH* genes, with the use of the neighbor-joining algorithm. The sequences determined in the present study are set in bold. Underlined are sequences of phototrophic bacteria possessing "red-like" form I RuBisCO and presented in Fig. 3. A gray background shows the sequences of phototrophic bacteria. Numerals show the significance of the branching order, as determined by bootstrap analysis (values above 65 were considered significant). The scale bar shows evolutionary distance, corresponding to 10 substitutions per 100 amino acid residues.

belong to a separate phylogenetic lineage; this agrees with the conclusion from the traditional analysis of 16S rRNA genes, which assigns all AFPB to one of the main evolutionary lineages of eubacteria. The proximity of the branching point of this lineage to the roots of the eubacterial nitrogenase tree, as well as the earlier revealed [20] proximity to the roots of the 16S rRNA gene tree, suggests an ancient origin of AFPB.

At the same time, analysis of *cbbL* genes revealed a considerable similarity of their sequences in the gram-negative phototrophic bacteria of the family *Oscillochloridaceae* and in the gram-positive facultatively chemoautotrophic bacteria of the species *S. acidophilus.* By now, the presence of an active RuBisCO has been demonstrated for all the species of the genus *Sulfobacillus* [21, 22]; however, its type and nucleotide sequence of the *cbb* genes remain unknown (an exception is *S. acidophilus*). In the future, obtaining these data for other members of the species *Sulfobacillus* should reveal whether the similarity of the *cbbL* genes of *O. trichoides* and *S. acidophilus* is a result of vertical (divergent) evolution or, which is more probable, it is due to horizontal interspecies gene transfer.

Among AFPB, the autotrophic assimilation of carbon dioxide via the Calvin cycle and nitrogen fixation have been demonstrated only for members of the family *Oscillochloridaceae* [9, 11]; therefore, phylogenetic analysis of the genes encoding key enzymes of this pathway does not allow the phylogenetic relationships within the AFPB lineage to be traced. It should be mentioned that complete sequencing of the chromosomal DNA of strain *C. aurantiacus* J-10-f1 (GenBank accession number AAAH00000000) revealed a region homologous to the *nifH* gene, whereas regions homologous to other genes of the nitrogenase complex were not found. Comparison of the region homologous to the *nifH* gene with *nifH* genes of various nitrogen-fixing bacteria, including genes of the *O. trichoides* strains studied in the present work, showed its considerable divergence (no more than 48.6% identity of amino acid sequences obtained by conceptual translation). This may indicate a changed function of the gene or the loss of function (silent gene). However, its presence in the genome of *C. aurantiacus* allows one to assume that nitrogen fixation was intrinsic to ancestor forms of AFPB but was gradually lost by representatives of this lineage, with the exception of the members of the family *Oscillochloridaceae*.

In conclusion, it might be mentioned that the lack in the databases of sequences of *rrs, cbbL*, and *nifH* genes closely related to the genes of the strains studied in the present work, suggests that representatives of the family *Oscillochloridaceae* are not widespread in natural habitats. The data obtained in our work on the primary structure of the *rrs, cbbL*, and *nifH* genes of *Oscillochloridaceae* members will allow molecular markers to be revealed useful for the detection of representatives of this family in various natural environments.

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