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EXPERIMENTAL ARTICLES

# A Study of Nucleotide Sequences of *nifH* Genes of Some Methanotrophic Bacteria

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**Abstract**—Using a previously developed primer system, *nifH* gene fragments 450 nucleotides long were amplified, cloned, and sequenced for representatives of nitrogen-fixing methanotrophic bacteria of the genera *Methylococcus, Methylocystis,* and *Methylosinus.* Fragments of *nifH* genes were also detected and sequenced in representatives of the genera *Methylomonas* and *Methylobacter*, which were not considered diazotrophs until recently. Phylogenetic analysis revealed the remoteness of *nifH* gene sequences of methanotroph types I and II. At the same time, a close relationship was found between *nifH* of type I methanotrophs and representatives of γ-proteobacteria and between *nifH* genes of type II methanotrophs and representatives of α-proteobacteria. The results obtained in this study are in good accordance with the data of phylogenetic analysis based on 16S rRNA sequence comparison with the only exception being *Methylococcus capsulatus* strains, whose *nifH* genes proved to be closely related to *nifH* genes of *Methylocystis* and *Methylosinus* representatives. Our findings extend the database of primary sequences of *nifH* genes and allow the contribution of methanotrophs to the process of nitrogen fixation to be estimated.

*Key words*: nitrogen fixation, nitrogenase genes, *nifH*, methanotrophic bacteria, phylogeny.

Microorganisms that can fix molecular nitrogen belong to various genera of eubacteria and archaea, and the list of nitrogen-fixing prokaryotes increases permanently. In spite of the huge ecological and economical importance of nitrogen fixation, the knowledge of the biodiversity of nitrogen-fixing bacteria in natural habitats is far from being complete. This is in part caused by the fact that the application of selective nitrogen-free nutrient media does not detect a significant part of diazotrophs. Thus, the study of the biodiversity of diazotrophs at the present time is mainly based on the methods of molecular ecology, which do not imply isolation of pure cultures. The most common approach in such studies is based on sequence analysis of 16S rRNA genes (or their fragments). However, analysis of structural genes may be more informative, particularly in the ecological studies of diazotrophs, the most popular method in these studies is based on the analysis of nucleotide or amino acid sequences of the Fe-protein from the nitrogenase complex [1, 2]. The *nifH* gene is one of the most ancient and conservative structural genes. Its analysis makes it possible to study relationships of nitrogen-fixing microorganisms at different taxonomic levels. This approach promotes more complete estimation of the biodiversity of diazotrophs in various natural habitats (soil, aquatic, etc.) [3, 4].

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However, the use of this approach is complicated by the disproportion between a number of described diazotrophs and the amount of *nifH* gene sequences available in databases. Methanotrophic bacteria, which use methane as the sole source of carbon and energy, are also poorly studied with respect to nitrogen fixation. These microorganisms are widespread in soil and aquatic ecosystems. Based on their physiological and biochemical features, methanotrophs are divided into two groups: type I methanotrophs, including the genera *Methylomonas*, *Methylobacter, Methylomicrobium, Methylocaldum, Methylosarcina, Methylosphaera,* and *Methylococcus* and type II methanotrophs, including the genera *Methylocystis, Methylosinus, Methylocella,* and *Methylocapsa.* Subsequent genotyping and phylogenetic analysis demonstrated that methanotrophs belong to the following phylogenetic subdivisions: type I methanotrophs belong to γ-proteobacteria, and type II—to α-proteobacteria [5].

As early as in 1939, Harper [6] suggested that methanotrophs are involved in the process of nitrogen fixation; this idea was based on increased nitrogen content in soil exposed to natural gas whose main component was methane. Later, several methanotrophic bacteria capable of fixing atmospheric nitrogen were isolated from the same soil [7]. In spite of intense studies of the physiology and biochemistry of methanotrophs, the number of publications concerning nitrogen fixation by

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**Table 1.** List of strains of methanotrophic bacteria used in this study

| Group | Name of microorganism | Strain |
|---|---|---|
| Type I | *Methylomonas methanica* S1$^{T}$* | INMI 68 = UNIQEM 8 = ATCC35067 = IMET 10543 |
| | "*Methylomonas rubra*" 15m | INMI 23 |
| | "*Methylobacter vinelandii*" | INMI 87 = UNIQEM 17 |
| | "*Methylobacter chroococcum*"$^{T}$ | INMI 90 = UNIQEM 21 |
| | "*Methylobacter bovis*"$^{T}$ | INMI 89 = UNIQEM 14 |
| | "*Methylobacter bovis*" | INMI 98 = UNIQEM 15 |
| | *Methylococcus capsulatus* | INMI 14 |
| | *Methylococcus capsulatus* | INMI 115 |
| Type II | *Methylocystis parvus* OBBP$^{T}$ | INMI 93 = UNIQEM 38 = ATCC35066 = IMET 10483 |
| | "*Methylocystis methanolicus*"$^{T}$ | INMI 10 = UNIQEM 33 |
| | *Methylocystis echinoides*$^{T}$ | INMI 2 = UNIQEM 25 = IMET 10491 |
| | "*Methylocystis minimus*" | INMI 41 = UNIQEM 51 |
| | *Methylosinus sporium* 5$^{T}$ | INMI 22 = UNIQEM 61 = ATCC35069 = IMET 10545 |
| | *Methylosinus trichosporium* OB5b | INMI 40 = UNIQEM 77 |
| | *Methylosinus trichosporium* OB3b$^{T}$ | INMI 74 = UNIQEM 75 = ATCC35070 = IMET 10543 |

Note: INMI is the personal collection of methanotrophic bacteria of V.F. Galchenko, Institute of microbiology, Russian Academy of Sciences, Moscow; UNIQEM is Unique and Extremophilic Microorganisms Database (http://inmi.da.ru), Institute of microbiology Russian Academy of Sciences, Moscow; ATCC is American Type Culture Collection, Rockville, Md.; IMET is Institute of Medical and Experimental Therapy, Jena, Germany.
* The type strain of the species.

methanotrophs is still limited. The data on the contribution of methanotrophs to the nitrogen balance in soil are scarce and contradictory. For example, using indirect methods, it was concluded that the contribution of methanotrophs to the nitrogen-fixing activity of rice fields is insignificant [8]. However, the analysis of $^{15}$N uptake led to a conclusion that methanotrophs played an important part in the process of nitrogen fixation in a wide range of rice soils of the Soviet Union [9]. The capacity for nitrogen fixation was demonstrated in *Methylococcus capsulatus* [10] and *Methylosinus trichosporium* [11] by the acetylene and $^{15}$N methods. The nitrogen-fixing activity of some representatives of the genera *Methylococcus, Methylocystis* and *Methylomonas* was also demonstrated [12]. Until recently, it was claimed in the studies of methanotrophic bacteria that only members of the genera *Methylococcus, Methylocystis* and *Methylosinus* can fix molecular nitrogen, as distinct from methanotrophs belonging to the genera *Methylomonas* and *Methylobacter* [5, 13].

Recently, Auman *et al.* [14] demonstrated the presence of nitrogenase activity in several strains of methanotrophs belonging to the genera *Methylomonas* and *Methylobacter.* Sequences of fragments of the *nifH* genes were also revealed. Unfortunately, only three strains used in that study were type strains of validly described species; other strains used were determined only at a generic level.

The main task of the present work was to investigate the *nifH* gene diversity in different genera of methanotrophic bacteria using a previously described system of universal *nifH* primers [15] with the aim of using the data obtained in further molecular ecological investigations on the role of methanotrophs in the process of nitrogen fixation.

## MATERIALS AND METHODS

**Microorganisms and cultivation conditions.** Microorganisms used in this work are listed in Table 1.

Strains of methanotrophic bacteria were grown as described earlier [16]. *Escherichia coli* and *Bacillus thuringiensis* strains were cultivated on LB agar media [17] at 37°C.

**DNA extraction.** DNA was extracted from bacteria using a combination of a modified method of Birnboim–Doly [18] and the Wizard-technology of the Promega company (United States). A small amount of bacterial biomass—one colony from agar plate or 25 μl of pellet from liquid culture—was suspended in 100 μl of buffer I (50 mM Tris–HCl, pH 8.0; 10 mM EDTA; 50 μg/ml of pancreatic RNase) to obtain a homogeneous suspension. Then, 125 μl of lysis buffer II (0.2 M NaOH; 1% sodium dodecyl sulfate) was added. The mixture was sonicated using a UZDN-2T (Russia) ultrasound disintegrator at a maximum power and 22 kHz for 2 min at 4°C. Then, the suspension was incubated at 65°C for 45 min and was cooled to room temperature. After that, 125 μl of counteractive buffer III (2.5 mM potassium acetate, pH 4.5) was added and

**Table 2.** Homology level of nucleotide sequences (lower triangle) and identities of translated amino acid sequences (upper triangle) in the *nifH* genes studied

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 | 22 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | **100** | 99 | 91 | 91 | 85 | 85 | 85 | 86 | 85 | 91 | 89 | 89 | 74 | 74 | 74 | 74 | 74 | 71 | 74 | 76 | 74 | 80 |
| 2 | 99 | **100** | 92 | 89 | 86 | 86 | 86 | 85 | 86 | 92 | 88 | 88 | 72 | 72 | 72 | 72 | 72 | 69 | 72 | 74 | 72 | 79 |
| 3 | 83 | 84 | **100** | 98 | 93 | 93 | 93 | 92 | 93 | 100 | 90 | 90 | 74 | 75 | 75 | 74 | 74 | 70 | 74 | 75 | 74 | 80 |
| 4 | 81 | 81 | 93 | **100** | 93 | 93 | 93 | 95 | 93 | 97 | 93 | 93 | 74 | 75 | 75 | 77 | 77 | 74 | 77 | 79 | 74 | 80 |
| 5 | 77 | 78 | 91 | 88 | **100** | 100 | 99 | 95 | 100 | 93 | 92 | 92 | 72 | 74 | 72 | 75 | 77 | 74 | 77 | 79 | 74 | 79 |
| 6 | 77 | 78 | 91 | 88 | 100 | **100** | 99 | 95 | 100 | 93 | 92 | 92 | 72 | 74 | 72 | 75 | 77 | 74 | 77 | 79 | 74 | 79 |
| 7 | 78 | 79 | 92 | 90 | 96 | 96 | **100** | 96 | 99 | 93 | 92 | 92 | 72 | 74 | 72 | 75 | 77 | 74 | 77 | 79 | 74 | 79 |
| 8 | 79 | 79 | 84 | 85 | 88 | 88 | 90 | **100** | 95 | 92 | 93 | 93 | 74 | 75 | 74 | 77 | 77 | 74 | 77 | 79 | 72 | 80 |
| 9 | 77 | 78 | 91 | 88 | 10 | 100 | 96 | 88 | **100** | 93 | 92 | 92 | 72 | 74 | 72 | 75 | 77 | 74 | 75 | 77 | 70 | 79 |
| 10 | 83 | 84 | 100 | 94 | 91 | 91 | 92 | 85 | 91 | **100** | 90 | 90 | 74 | 75 | 75 | 74 | 74 | 70 | 74 | 75 | 74 | 80 |
| 11 | 81 | 79 | 84 | 85 | 85 | 85 | 86 | 84 | 85 | 85 | **100** | 100 | 79 | 82 | 80 | 79 | 79 | 75 | 79 | 80 | 79 | 82 |
| 12 | 81 | 79 | 84 | 85 | 85 | 85 | 86 | 84 | 85 | 85 | 100 | **100** | 79 | 82 | 80 | 79 | 79 | 75 | 79 | 80 | 79 | 82 |
| 13 | 68 | 67 | 63 | 61 | 63 | 63 | 63 | 63 | 63 | 63 | 71 | 71 | **100** | 95 | 95 | 86 | 86 | 83 | 86 | 88 | 97 | 89 |
| 14 | 66 | 66 | 61 | 62 | 59 | 59 | 58 | 61 | 59 | 61 | 69 | 69 | 83 | **100** | 97 | 82 | 82 | 78 | 82 | 83 | 95 | 88 |
| 15 | 69 | 68 | 63 | 64 | 58 | 58 | 63 | 63 | 58 | 63 | 72 | 72 | 85 | 88 | **100** | 80 | 80 | 77 | 80 | 82 | 97 | 86 |
| 16 | 66 | 64 | 62 | 66 | 61 | 61 | 63 | 60 | 61 | 63 | 70 | 70 | 78 | 75 | 76 | **100** | 97 | 96 | 97 | 99 | 83 | 95 |
| 17 | 67 | 67 | 62 | 65 | 62 | 62 | 64 | 62 | 62 | 62 | 72 | 72 | 84 | 78 | 79 | 96 | **100** | 96 | 95 | 96 | 83 | 95 |
| 18 | 67 | 66 | 61 | 64 | 59 | 59 | 63 | 61 | 59 | 61 | 70 | 70 | 82 | 76 | 79 | 98 | 97 | **100** | 93 | 95 | 80 | 92 |
| 19 | 69 | 67 | 63 | 67 | 61 | 61 | 65 | 65 | 61 | 63 | 71 | 71 | 78 | 75 | 77 | 95 | 94 | 95 | **100** | 99 | 83 | 95 |
| 20 | 67 | 66 | 61 | 67 | 60 | 60 | 63 | 66 | 60 | 62 | 70 | 70 | 76 | 75 | 76 | 94 | 93 | 94 | 99 | **100** | 85 | 96 |
| 21 | 70 | 69 | 63 | 63 | 63 | 63 | 63 | 66 | 63 | 64 | 73 | 73 | 88 | 86 | 88 | 73 | 76 | 76 | 75 | 74 | **100** | 81 |
| 22 | 63 | 63 | 61 | 63 | 57 | 57 | 60 | 61 | 57 | 61 | 67 | 67 | 75 | 77 | 78 | 81 | 82 | 81 | 80 | 81 | 76 | **100** |

Note: Strain designation are as follows: 1 – *Mcs. minimus* 41, 2 – *Mcs. echinoides* 2, 3 – *Mcs. methanolicus* 10, 4 – *Mcs. parvus* 93, 5 – *Ms. trihosporium* 40, 6 – *Ms. trihosporium* 74, 7 – *Ms. sporium* 22, 8 – *Methylosinus* LW3, 9 – *Ms. trichosporium* OB3b, 10 – *Methylocystis* LW5, 11 – *Mc. capsulatus* 114, 12 – *Mc. capsulatus* 115, 13 – *Mm. methanica* 68, 14 – *Mm. rubra* 23, 15 – *Mm. methanica* S1, 16 – *Mb. marinus* A45, 17 – *Mb. chroococcum* 90, 18 – *Mb. vinelandii* 87, 19 – *Mb. bovis* 89, 20 – *Mb. bovis* 98, 21 – NR1624, 22 – NR1620.

the mixture was vortexed and centrifuged for 10 minutes at 10000*g* in an Eppendorff 5415C minicentrifuge. 500 μl of Wizard Maxi Preps resin (Promega, United States) was added to the supernatant, and further extraction was continued according to the recommendations of Promega Company for the Wizard Preps set. The concentration of recovered DNA was 15–100 μg/ml. The obtained DNA was of good quality ($\lambda_{260} : \lambda_{280} > 1.8$) and suitable for amplification. The RNA was present in trace amounts: less than 1% according to data from electrophoretic analysis.

**Amplification and fragment purification.** Amplification of *nifH* gene fragments was performed in a Cetus 480 thermocycler (Perkin Elmers, Sweden) using an earlier described pair of primers: F1 (5'-TAYGGIAARGGIGGIATYGGIAARTC-3') and R6 (5'-TCIGGIGARATGATGGC-3'), where I is inosine, R is A or G, and Y is T or C [15]. The PCR mixtures (20 μl) had the following composition: 1 × PCR buffer (17 mM $(NH_4)SO_4$, 6 mM Tris–HCl, pH 8.8, 2 mM $MgCl_2$), 1 nM of each dNTP, 10–50 ng of DNA, 100 pM of each primer and 2 units of BioTaq DNA Polymerase (Dialat Ltd, Russia). The temperature regime was as follows: first cycle: 94°C, 3 min; 50°C × 3 min; and 72°C , 3 min; next 5 cycles: 94°C, 30 s; 50°C, 2 min; and 72°C, 30 s; next 30 cycles: 94°C, 30 s; 40°C, 30 s; and 72°C, 30 s; final extension step: 72°C, 7 min. PCR products were analyzed by electrophoresis in 1% agarose gel stained with EtBr.

PCR fragments were purified on low-melting-point agarose using Wizard PCR Preps kit (Promega, United States).

**PCR fragment cloning and sequencing.** Purified PCR fragments were cloned into the *Hin*cII site of the pGEM-3Zf(+) vector polylinker (Promega, United States) using competent cells of *E. coli* DH5α for transformation. Plasmid DNA was extracted and purified using a Wizard MiniPrep kit (Promega, United States) according to the recommendations of the manufacturer.

Sequencing was performed by the Sanger method [19] using a Silver Sequencing kit (Promega, United

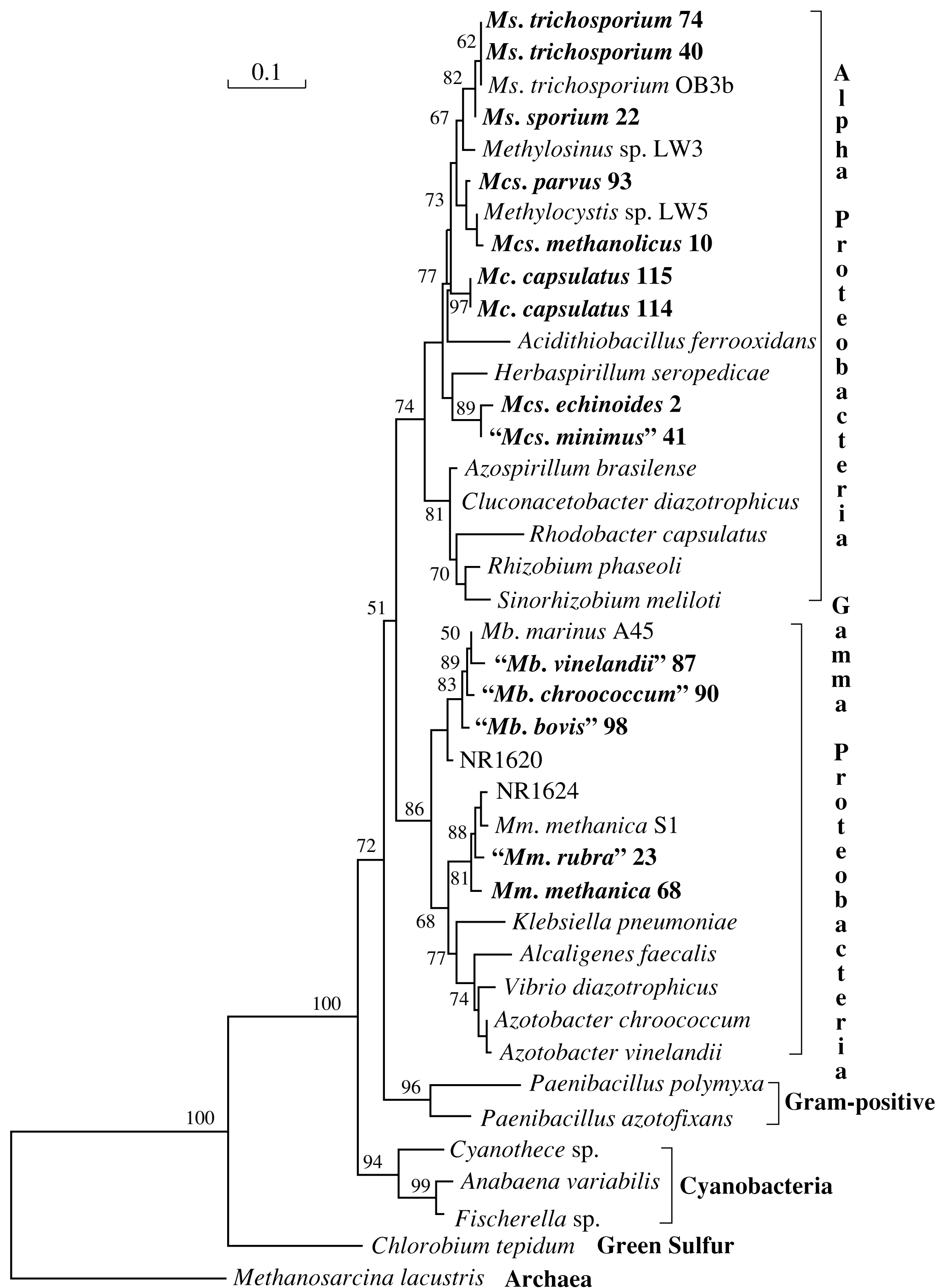


**Fig. 1.** Phylogenetic tree constructed based on translated amino acid sequences of *nifH* gene fragment sequences. The Genbank accession numbers of *nifH* gene sequences of reference microorganisms are indicated in parentheses: *Mb. marinus* A45 (AF378714); *Mm. methanica* (AF378717); *Methylosinus* sp. LW3 (AF378720); *Ms. trichosporium* (AF378724); *Methylocystis* sp. LW5 (AF378719); NR1624 (AF035508); NR1620 (AF035504); *Rhodobacter capsulatus* SB100 (X07866); *Azospirillum brasilense* str. ATCC29145 (M64344); *Gluconacetobacter diazotrophicus* str. PAL5 (AF030414); *Sinorhizobium meliloti* taxon:382 (V01215); *Rhizobium phaseoli* taxon:396 (M15942); *Herbaspirillum seropedicae* Z78 (Z54207); *Acidithiobacillus ferrooxidans* str. ATCC33020 (M15238); *Alcaligenes faecalis* taxon:511 (X96609); *Azotobacter chroococcum* taxon:353(M24596)); *Azotobacter vinelandii* taxon:354 (M20568); *Klebsiella pneumoniae* taxon:573 (AF303353); *Vibrio diazotrophicus* ATCC33466 (AF111110); *Paenibacillus azotofixans* ATCC35681 (U23649); *Paenibacillus polymyxa* CF43 (AJ223996); *Cyanothece* sp. ATCC51142 (AF003336); *Fischerella* sp. UTEX1903 (U73140); *Anabaena variabilis* ATCC29413 (U89346); *Chlorobium tepidum* ATCC49652 (AF065617); *Methanosarcina lacustris* taxon:170861 (AF296355). The scale bar corresponds to 10 substitutions per 100 amino acid sites (evolutionary distances). Numbers indicate the statistical confidence of the branching order determined by bootstrap analysis of 500 alternative trees (values of less than 50 are not shown).

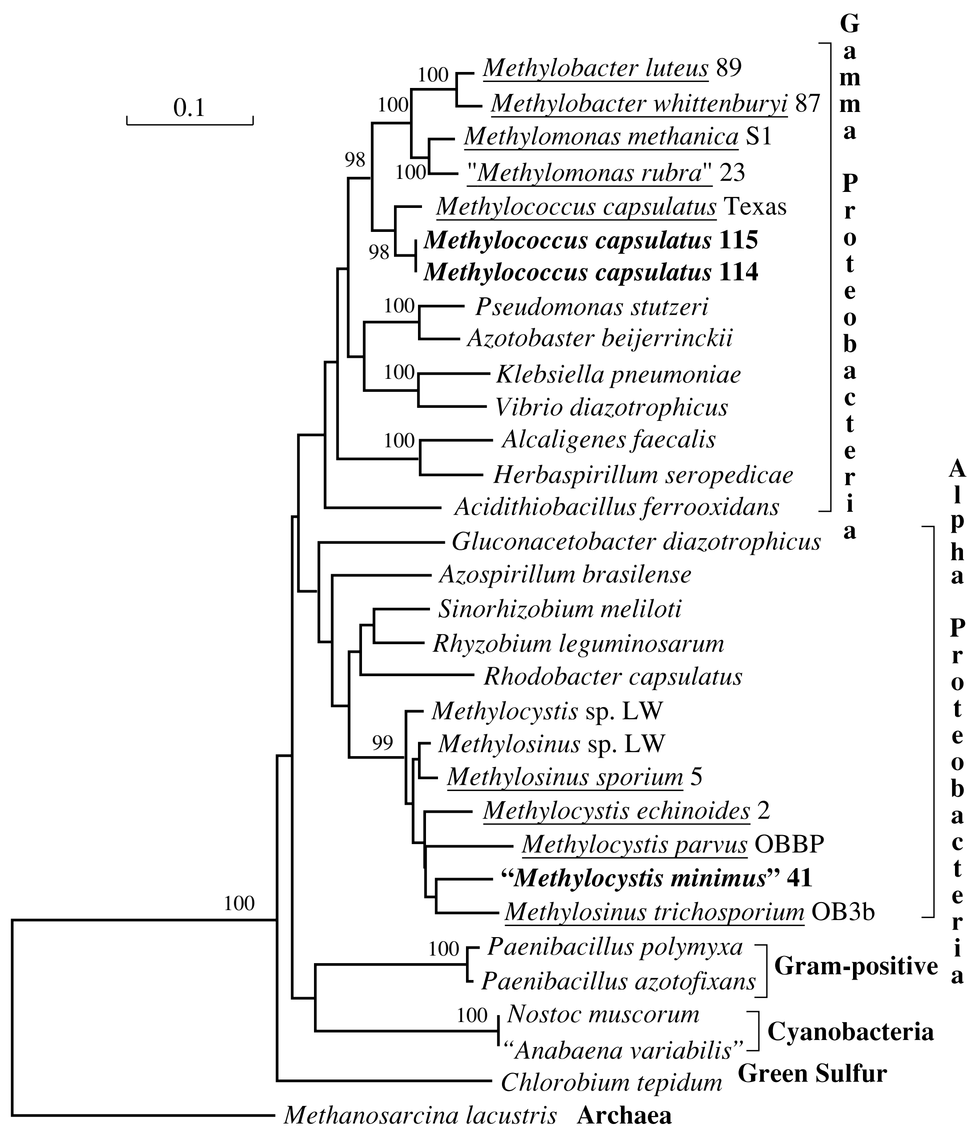


**Fig. 2.** Phylogenetic tree constructed based on 16S rRNA nucleotide sequences. The Genbank accession numbers of 16S rRNA sequences of reference microorganisms are indicated in parentheses: *Herbaspirillum seropedicae* DSM *6445*$^{T}$ (Y10146); *Azospirillum brasilense* NCIMB 11860$^{T}$ (Z29617); *Gluconacetobacter diazotrophicus* ATCC 49037$^{T}$ (X75618); *Rhodobacter capsulatus* ATCC 11166$^{T}$ (D16428); *Sinorhizobium meliloti* ATCC 9930$^{T}$ (X77121); *Vibrio diazotrophicus* ATCC 33466$^{T}$ (X56577); *Klebsiella pneumoniae* ATCC *1*3883$^{T}$, (Y17656); *Azotobacter vinelandii* AMOP (L40329); *Pseudomonas stutzeri* ATCC 17598 (AJ006104); *Alcaligenes faecalis* ATCC 8750$^{T}$ (M22508); *Paenibacillus azotofixans* NRRL B-14372 (D78318); *Paenibacillus polymyxa* IAM 13419$^{T}$ (D16276); *“Anabaena variabilis”* IAM M-3 (AB016520); *Nostoc muscorum* PCC 7120 (X59559); *Chlorobium tepidum* NZC (M58468); *Acidithiobacillus ferrooxidans* ATCC 23270$^{T}$ (FA465604); *Methanosarcina lacustris* SZ$^{T}$ (AF432127); *Methylosinus trichosporium* OB3b$^{T}$ (M29024); *Methylocystis parvus* OBBP$^{T}$ (M29026); *Methylosinus sporium* 5$^{T}$, (M95665); *Methylocystis echinoides* 2$^{T}$ (L20848); *“Methylocystis minimus”* 42 (L20844); *Methylococcus capsulatus* Texas$^{T}$ (X72770); *“Methylomonas rubra”* VKM-15m (M95662); *Methylomonas methanica* S1$^{T}$ (AF304196); *Methylobacter luteus* 89 (L20839); *Methylobacter whittenburyi* 87 (L20841). Strains identical or close to methanotrophs studied in this work are underlined. The scale bar corresponds to 5 substitutions per 100 nucleotides (evolutionary distances). Numbers indicate the statistical confidence of the branching order determined by bootstrap analysis of 100 alternative trees (values of less than 90 are not shown).

States) in accordance with the recommendations of the manufacturer (with minor modifications). Electrophoresis was run on a Macrophore (Pharmacia, Sweden) and SQ3 Sequencer (Hoefer, United States) in 0.19-mm-thick polyacrylamide gels. Universal plasmid primers SP6 and T7, as well as the earlier developed primers F1 and R6 were used for sequencing in both directions.

**Amplification and sequencing of 16S rRNA gene fragments** were performed as described earlier [20].

**Phylogenetic analysis.** Preliminary analysis of new sequences was performed with the BLAST package (www.ncbi.nlm.nih.gov/BLAST). The ORF Finder program (http://www.ncbi.nlm.nih.gov/gorf/gorf.html) was used for translation. Multiple alignments of nucleotide and protein sequences were performed using the

Basic Genebee Clustal W1.75 package (http://www.genebee.msu.su/clustal). Phylogenetic analysis of the data was performed using the TREECON package [21]. Statistic reliability of the branching points was computed using bootstrap analysis by analyzing 500 bootstrap resamplings.

**Nucleotide sequence accession numbers.** The nucleotide sequences of cloned *nifH* gene fragments of methanotrophic bacteria listed in Table 1 were submitted to GenBank under accession numbers AF484658–AF484678. The nucleotide sequence of 16S rRNA gene fragments of "*Methylocystis minimus*" 41 and *Methylococcus capsulatus* 114 and 115 were submitted to GenBank under accession numbers AF486572–AF486574.

## RESULTS

**Detection of the fragments of *nifH* genes in methanotrophic bacteria.** Primary screening for the *nifH* gene presence was performed in methanotrophs (including type strains) from the collection of the Institute of microbiology, Russian Academy of Sciences (Table 1). In these experiments, *E. coli* and *Bacillus thuringiensis* DNAs were used as negative controls. PCR fragments of expected size (450 bp) were observed for all strains tested, including representatives of the genera *Methylomonas* and *Methylobacter.* These fragments were absent in the control reactions. It should be pointed out that this analysis included the *"Methylomonas rubra"* strain that did not produce the PCR fragment in the study of Auman *et al.* [14]. The differences in the results can be explained by a higher efficiency of the primer system that we used.

**Analysis of *nifH* gene fragment sequences.** Preliminary screening in GenBank confirmed the relatedness of the cloned PCR fragments to the family of *nifH* genes. By their similarity values, the obtained nucleotide sequences fell into two clusters. The first cluster included representatives of the genera *Methylococcus, Methylocystis*, and *Methylosinus*, and the second cluster included representatives of *Methylomonas* and *Methylobacter.* The similarity level of nucleotide sequences was higher than 75% within each cluster and did not exceed 70% between the clusters. The identities of the deduced amino acid sequences were 85 and 80%, respectively. The levels of similarity of nucleotide sequences between strains of the same species were 98–100%, and the deduced amino acid sequences were identical. Nucleotide sequences of clones of the same strains differed from each other within the standard sequencing error (less than 1%).

A BLAST search was performed throughout GenBank to reveal *nifH* gene sequences most closely related to the sequences obtained in this study. A high identity level was found between uncultured bacterium NR1624 [AF035508] and *Methylomonas methanica* (97% at the amino acid level) and between uncultured bacterium NR1620 [AF035504] and *"Methylobacter chroococcum"* (95%). Table 2 shows the similarity levels for the sequences obtained in the present study, some sequences from the work of Auman *et al.* [14], and two sequences retrieved from BLAST searches.

Amino acid sequences of reference microorganisms from the database representing the main phylogenetic groups and some methanotrophic bacteria from the study of Auman *et al.* [14] were included in the phylogenetic analysis together with translated new sequences. Since some of the sequences retrieved from GenBank were shorter than others, only the region 110 amino acid residues long was taken for analysis. The resulting phylogenetic tree is shown in Fig. 1. The addition of new amino acid sequences did not influence the general topology of the tree, and it was similar to analogous trees based of amino acid sequences coded by *nifH* genes [14].

The results of phylogenetic analysis of amino acid sequences coded by *nifH* genes were compared with the data of 16S rRNA analysis of the same microorganisms (Fig. 2). To perform the latter analysis, we additionally sequenced 16S rRNA fragments of *"Mcs. minimus"* strain 41 and *Mc. capsulatus* strains 114 and 115. Sequences of corresponding regions of 16S rRNA genes for reference strains of methanotrophic bacteria and various representatives of diazotrophs were used. Topologies of both trees were similar to a high degree. Thus, representatives of type I methanotrophs were phylogenetically close to representatives of γ-proteobacteria, and type II methanotrophs were close to representatives of α-proteobacteria. *Methylococcus capsulatus* strains were the only exception: the sequences of their *nifH* genes were closer to type II methanotrophs, forming a separate cluster within the alpha subdivision of proteobacteria. It should be noted that representatives of the genus *Methylocystis* fell into two relatively distant clusters by their *nifH* gene sequences. The fact that *Mcs. parvus* and *Mcs. methanolicus* formed a compact group with representatives of the genus *Methylosinus* is in good agreement with data of 16S rRNA analysis. The sequences of *"Mcs. minimus"* and *Mcs. echinoides* were even farther from type II methanotrophs than *Methylococcus capsulatus* strains.

## DISCUSSION

As pointed out above, the data concerning the ability of methanotrophic bacteria to fix molecular nitrogen are scarce and contradictory. The common opinion is that among all methanotrophic bacteria only representatives of the genera *Methylococcus, Methylocystis*, and *Methylosinus* are diazotrophs. At the beginning of this study, we were unable to find any *nifH* gene sequences of methanotrophic bacteria in GenBank. Therefore, the detection of nucleotide sequences of these genes (which play a key role in the nitrogenase operon) in all of the strains studied, including representatives of the genera *Methylomonas* and *Methylobacter* is more,

although indirect, evidence for the ability of all methanotrophs to fix molecular nitrogen.

A direct demonstration of nitrogenase activity in some representatives of the genera *Methylomonas* and *Methylobacter* provided by the study of Auman *et al.* [14] is evidence of the fact that type I methanotrophs can be diazotrophs. Furthermore, the data of the BLAST search confirmed this fact, because the uncultured freshwater diazotrophs NR1620 and NR1624 turned to be phylogenetically closely related to type I methanotrophs.

Topological correlation of the phylogenetic trees based on the 16S rRNA and nitrogenase genes of methanotrophs confirms that the nitrogenase genes can be used as phylogenetic markers in molecular ecological studies. Certain differences in the topologies of "nitrogenase" and "ribosomal" trees (Fig. 1 and Fig. 2) can be explained by methodological difficulties in the usage of *nifH* genes for phylogenetic analysis: due to the high variability of the 3'-end region, the design of universal primers for the amplification of the entire gene is impossible. This determines the relatively small size of compared fragments (350–450 bp, or 110–150 amino acid residues), which, together with the considerable conservatism of nitrogenase gene sequences, may reduce the accuracy of phylogenetic analysis.

Also the comparison of the topologies of both trees compels one to return to the repeatedly discussed question concerning the involvement of the horizontal transfer of the nitrogenase gene in diazotroph evolution. The possibility of such transfer between methanotrophs belonging to types I and II is to be ruled out because of the significant divergence of the *nifH* gene sequences of these two groups. This divergence suggests the independent origin of these genes.

The situation with *Methylococcus capsulatus* deserves special attention. It can be assumed that the *nifH* gene appeared in this microorganism as a result of horizontal gene transfer from type II methanotrophs. Nominally assigned to type I methanotrophs, this microorganism is often considered as a special group X based on some physiological and biochemical features [5]. The results of phylogenetic analysis of the *Methylococcus capsulatus nifH* gene may be an argument that this group is transitional between type I and II methanotrophs.

It should be pointed out that our results revealed a relatively high level of intergeneric (up to 15%) and interspecies (up to 12%) divergence of *nifH* gene sequences of methanotrophs; at the same time, they were almost completely identical in strains of the same species (less than 2% differences). This must allow identification of diazotrophs in natural communities to the species level without cultivation and demonstrates the necessity for extending the database of nitrogenase gene sequences for different $N_2$-fixing microorganisms, including representatives of additional genera of methanotrophic bacteria.

Within the framework of this study, we did not set the task of studying the $N_2$-fixing ability of methanotrophs and focused on the search for *nifH* gene fragments in the genomes of methanotrophs and on the study of their primary structure to estimate the possibility of using these data for future ecological studies. However, the obtained data can be used not only in studies of the contribution of methanotrophs to the soil nitrogen balance but also in the studies of the biochemistry of nitrogen fixation.

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## REFERENCES

1. Ben-Porath, J. and Zehr, J.P., Detection and Characterization of Cyanobacterial *nifH* Genes, *Appl. Environ. Microbiol.*, 1994, vol. 60, pp. 880–887.
2. Ueda, T., Suga, Y., Yashiro, N., and Matsuguchi, T., Remarkable $N_2$-Fixing Bacterial Diversity Detected in Rice Roots by Molecular Evolutionary Analysis of *nifH* Gene Sequences, *J. Bacteriol.*, 1995, vol. 177, pp. 1414–1417.
3. Zehr, J.P., Mellon, M., Braun, S., Litaker, W., Steppe, T., and Paerl, H.W., Diversity of Heterotrophic Nitrogen Fixation Genes in a Marine Cyanobacterial Mat, *Appl. Environ. Microbiol.*, 1995, vol. 61, pp. 2527–2532.
4. Zani, S., Mellon, M.T., Collier, J.L., and Zehr, J.P., Expression of *nifH* Genes in Natural Microbial Assemblages in Lake George, New York, Detected by Reverse Transcriptase PCR, *Appl. Environ. Microbiol.*, 2000, vol. 66, pp. 3119–3124.
5. Hanson, R.S. and Hanson, T.E., Methanotrophic Bacteria, *Microbiol. Rev.*, 1996, vol. 60, pp. 439–471.
6. Harper, H.J., The Effect of Natural Gas on the Growth of Microorganisms and Accumulation of Nitrogen and Organic Matter in Soil, *Soil Sci.*, 1939, vol. 48, pp. 461–453.
7. Davis, J.B., Coty, V.F., and Stanley, J.P., Atmospheric Nitrogen Fixation by Methane-Oxidizing Bacteria, *J. Bacteriol.*, 1964, vol. 88, pp. 468–472.
8. De Bont, J.A., Lee, K.K., and Boudin, D.F., Bacterial Oxidation of Methane in the Rice Paddy, *Ecol. Bull.*, 1978, vol. 26, pp. 91–97.
9. Chistyakova, I.K., Kalininskaya, T.A., and Miller, Yu.M., Methane as a Source of Carbon and Energy for Nitrogen-Fixing Bacteria in Soils under Rice, *Izv. Akad. Nauk SSSR, Ser. Biol.*, 1982, no. 3, pp. 428–432.
10. Dalton, H. and Whittenbury, R., The Acetylene Reduction Technique as Assay for Nitrogenase Activity in the Methane Oxidizing Bacterium *Methylococcus capsulatus* Strain Bath, *Arch. Microbiol.*, 1976, vol. 109, pp. 147–151.
11. De Bont, J.A. and Mulder, E.G., Nitrogen Fixation and Co-Oxidation of Ethylene by Methane-Utilizing Bacteria, *J. Gen. Microbiol.*, 1976, vol. 83, pp. 113–119.

12. Romanovskaya, V.A., Lyudvichenko, E.S., Sokolov, I.G., and Malashenko, Yu.R., Fixation of Molecular Nitrogen by Methane-Oxidizing Bacteria, *Mikrobiol. Zh.,* 1980, vol. 42, pp. 683–692.
13. Green, P.N., Overview of the Current State of Methylotroph Taxonomy, *Microbial Growth on C1 Compounds*, Murrel, J.C. and Kelly, D.P., Eds., Andover: Intercept Ltd., 1993, pp. 253–265.
14. Auman, A.J., Speake, C.C., and Lidstrom, M.E., *nifH* Sequences and Nitrogen Fixation in Type I and Type II Methanotrophs, *Appl. Environ. Microbiol.*, 2001, vol. 67, pp. 4009–4016.
15. Marusina, A.I., Boulygina, E.S., Kuznetsov, B.B., Tourova, T.P., Kravchenko, I.K., and Gal'chenko, V.F., A System of Oligonucleotide Primers for the Amplification of *nifH* Genes of Different Taxonomic Groups of Prokaryotes, *Mikrobiologiya*, 2001, vol. 70, pp. 86–91.
16. Gal'chenko, V.F., Andreev, L.V., and Trotsenko, Yu.A., *Taksonomiya i identifikatsiya obligatnykh metanotrofnykh bakterii* (Taxonomy and Identification of Obligate Methanotrophic Bacteria), Kalakoutskii, L.V., Ed., Pushchino: NTsBI, 1986, pp. 1–96.
17. Sambrook, J., Fritsch, E.F., and Maniatis, T., *Molecular Cloning: A Laboratory Manual*, 2nd ed., Cold Spring Harbor: Cold Spring Harbor Lab., 1989.
18. Birnboim, H.C. and Doly, J., A Rapid Alkaline Extraction Procedure for Screening Recombinant Plasmid DNA, *Nucleic Acids Res.*, 1979, vol. 7, no. 6, pp. 1513–1523.
19. Sanger, F., Nicklen, S., and Coulson, A.R., DNA Sequencing with Chain-Terminating Inhibitors, *Proc. Natl. Acad. Sci. USA*, 1977, vol. 84, pp. 5463–5467.
20. Zhilina, T.N., Garnova, E.S., Tourova, T.P., Kostrikina, N.A., and Zavarzin, G.A., *Amphibacillus fermentum* sp. nov. and *Amphibacillus tropicus* sp. nov., New Alkaliphilic, Facultatively Anaerobic, Saccharolytic Bacilli from Lake Magadi, *Mikrobiologiya*, 2001, vol. 70, pp. 825–837.
21. Van de Peer, Y. and De Wachter, R., TREECON for Windows: A Software Package for the Construction and Drawing of Evolutionary Trees for the Microsoft Windows Environment, *Comput. Appl. Biosci.*, 1994, vol. 10, pp. 569–570.