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EXPERIMENTAL ARTICLES

# Comparative Characterization of Methanotrophic Enrichments by Serological and Molecular Methods

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**Abstract**—Three stable methane-oxidizing enrichment cultures, SB26, SB31, and SB31A, were analyzed by transmission electron microscopy and by serological and molecular techniques. Electron microscopy revealed the presence of both type I and type II methanotrophs in SB31 and SB31A enrichments; only type II methanotrophs were found in SB26 enrichment. *Methylosinus trichosporium* was detected in all three enrichments by the application of species-specific antibodies. Additionally, *Methylocystis echinoides* was found in SB26 culture; *Methylococcus capsulatus*, in SB31 and SB31A; and *Methylomonas methanica*, in SB31. The analysis with *pmoA* and *nifH* gene sequences as phylogenetic markers revealed the presence of *Methylosinus/Methylocystis* group in all communities. Moreover, the analysis of *pmoA* sequences revealed the presence of *Methylomonas* in SB31. *Methylocella* was detected in SB31 and SB31A enrichments only by *nifH* analysis. It was concluded that the simultaneous application of different approaches reveals more reliable information on the diversity of methanotrophs.

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*Key words:* methanotrophs, immunofluorescence, *pmoA* and *nifH* genes.

Methane-oxidizing bacteria (MOB) are unique in their ability to utilize $CH_4$ as a source of cellular carbon and energy [1]. The different methods applied for the analysis of the structure of methanotrophic communities include determination of the culturable methanotrophs by the most probable number method [2]; microscopic techniques such as immunofluorescence analysis (IFA) [3] and fluorescence in situ hybridization (FISH) [4]; phospholipids fatty acid analysis (PLFA) [5]; and molecular ecology methods mainly based on the application of polymerase chain reaction (PCR) [6]. Each of the above-mentioned techniques has both advantages and disadvantages. Cultivation recovers only a small fraction of the cells present in the environmental sample and does not allow the so-called uncultured forms to be analyzed. The limited number of fluorescent species-specific antibodies is the main weakness of IFA in the analysis of the structure of methanotrophic communities [3]. The investigation of specific phospholipids acids provides reliable estimates of the biomass and cell numbers of MOB, but does not reveal the taxonomic structure of the community [5]. The FISH method, which combines identification and enumeration of MOB, is based on the detection of rRNA fragments and depends on the physiological state of microorganisms [7]. The methods involving the analysis of *pmoA* and *mmoX*, the functional marker genes of methanotrophy, make it possible to detect both known and novel methanotrophs, but are not universal because *Methylocella* lacks the *pmoA* gene and only a few methanotrophs have the *mmoX* gene. Sequence analysis of *nifH*, the marker gene of nitrogen fixation, was recently demonstrated to be applicable for the successful identification of methanotrophic bacteria [8].

The indirect immune diagnostic technique for MOB analysis using polyclonal species-specific antisera was developed at the beginning of the 1980s [9] and was applied for the analysis of natural microbial communities of aquatic [10] and terrestrial [3] ecosystems. Publications dealing with the use of different experimental approaches, including immune methods, for evaluation of the structure of microbial communities, are scarce. For example, the biodiversity of nitrogen-fixing bacteria in the rhizosphere was evaluated simultaneously by molecular and serological techniques [11]; however, until now there were no such investigations of methanotrophic communities.

The goal of the present work was to assess the diversity of MOB in methane oxidizing enrichments from a *Sphagnum* peat bog soil by a combination of microscopic, serological and molecular techniques.

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## MATERIALS AND METHODS

**Object of study.** The methane-oxidizing enrichment cultures were obtained from peat samples from the Sosvyatskoe ombrotrophic bog (Zapadnaya Dvina Field Station of Institute of Forestry, Russian Academy of Sciences, Tver oblast, Russia). By the time of the study, the cultures exhibited low complexity of the bacterial population and uniform colonial growth on solid media, and contained soluble methane monooxygenase (sMMO) as determined by the naphthalene test. One of the cultures, SB31, was characterized by strong pink-orange pigmentation and by the cell growth in liquid mineral media as aggregated clumps. Incubation under intensive aeration (about one year after enrichment) resulted in the culture losing its pigmentation. The initial SB31 and the transformed SB31A enrichment cultures were supported and analyzed separately afterwards.

**Transmission electron microscopy (TEM).** Microbial cells in the exponential growth phase were harvested by centrifugation and resuspended in 2.5% glutaraldehyde solution in a 0.2 M cacodylate buffer (pH 7.2). The preparation and analysis of ultrathin sections were carried out as described previously in [10].

**Immunofluorescence analysis of enrichment cultures** was done using the procedure described by Gal'chenko et al. [12]. We applied twelve polyclonal species-specific antisera which bind with the cell wall epitopes of the 12 methanotrophic species, namely, *Methylocystis echinoides, "Mcs. methanolicus," "Mcs. minimus," "Mcs. pyriformis," Mcs. parvus, Methylosinus sporium, Ms. trichosporium, Methylomonas methanica, "Methylobacter vinelandii," "Mb. bovis," "Mb. chroococcum,"* and *Methylococcus capsulatus.*

**DNA extraction of and PCR amplification of *pmoA* and *nifH* genes fragments.** Genomic bacterial DNA was extracted according to the method [8] from methanotrophic enrichment cultures grown in liquid medium. The *nifH* gene fragments were amplified in a Genius thermal cycler (Techne Ltd, Cambridge, United Kingdom) using the primers and the PCR steps described previously by Boulygina et al. [13]. The *pmoA* gene fragments were amplified with the primers A189f and A682r under the PCR conditions described by Holmes et al. [14]. The products obtained were checked for size and purity on 1.5% agarose gel stained with ethidium bromide and documented using BioDoc Analyze System (Biometra, Germany). The PCR fragments were purified with a Wizard PCR Preps kit (Promega, United States) according to the protocols of the manufacturers.

**Cloning, restriction fragments length polymorphism analysis, and sequencing.** Purified PCR products were cloned into a pGEM-3Zf (+) vector or pGEM-T easy vector system I (Promega, United States) and were transformed into competent *Escherichia coli* DH5α cells. The recombinant clones with *pmoA* inserts were grouped based on restriction analysis by using *Eco*RI and *Hinc*II restriction endonucleases. The clones with *nifH* inserts were grouped on the basis of "single-base" sequencing. The nucleotide similarity within each group was 100%. From two to five randomly chosen clones from each group were sequenced by the Sanger method using the Silver Sequencing kit (Promega, United States) as specified by the manufacturer with minor modifications [8].

**Phylogenetic analysis.** Sequence data of the *pmoA* and *nifH* gene fragments were analyzed using the BLAST software package (http:// www.ncbi.nlm.nih.gov/BLAST). ORF Finder software ackag (http:// www.ncbi.nlm.nih.gov/gorf/gorf.html) was used for translation. Alignment of the nucleotide sequences and of inferred amino acid sequences was done with the Basic Genebee Clustal W1.75 software package (http://www.genebee.msu.su/clustal). Phylogenetic trees were constructed on inferred amino acid sequences of the fragments of *nifH* and *pmoA* gene with TREECON for Windows software [15]. The trees were bootstrapped (500 replicates) using TREECON.

**Nucleotide sequence accession numbers.** The nucleotide sequences of new *pmoA* fragments have been deposited in the GenBank database under the accession numbers AY597817–AY597822. The nucleotide sequences of *nifH* PCR-fragments obtained in this study for environmental clones as well as for *Methylocapsa acidiphila* $B2^T$ and *Methylocella palustris* $K^T$ have been deposited in the GenBank database under the accession numbers AY597526–AY597544.

## RESULTS

**Transmission electron microscopy of enrichment cultures** revealed the MOB by the presence of a well-developed system of intra-cellular membranes. In the SB26 culture, only the type II membrane arrangement was identified in approximately 70% of cells (Fig. 1a, 1b). Both types of methanotrophic bacteria were revealed in the SB31 and SB31A communities. About 60% of the cells demonstrated peripherally arranged membranes characteristic of type II methanotrophs, and 10–15% of the cells demonstrated the stacks of membrane vesicles characteristic of type I methanotrophs (Figs. 1c, 1d). The nonmethanotrophic satellites were gram-negative rod-shaped bacteria.

**Immunofluorescence analysis** revealed only the type II methanotrophs (*Ms. trichosporium* and *Mcs. echinoides*) in SB26. At the same time, both type I and II methanotrophs were found in SB31 and SB31A cultures: *Ms. trichosporium, Mc. capsulatus*, and *Mm. methanica* in SB31; and *Ms. trichosporium* and *Mc. capsulatus* in SB31A.

**Analysis of *pmoA* and *nifH* genes.** PCR products of the predicted sizes (525 bp for *pmoA* and 470 bp for *nifH*) were obtained from all three enriched cultures (data not shown). The corresponding clone libraries

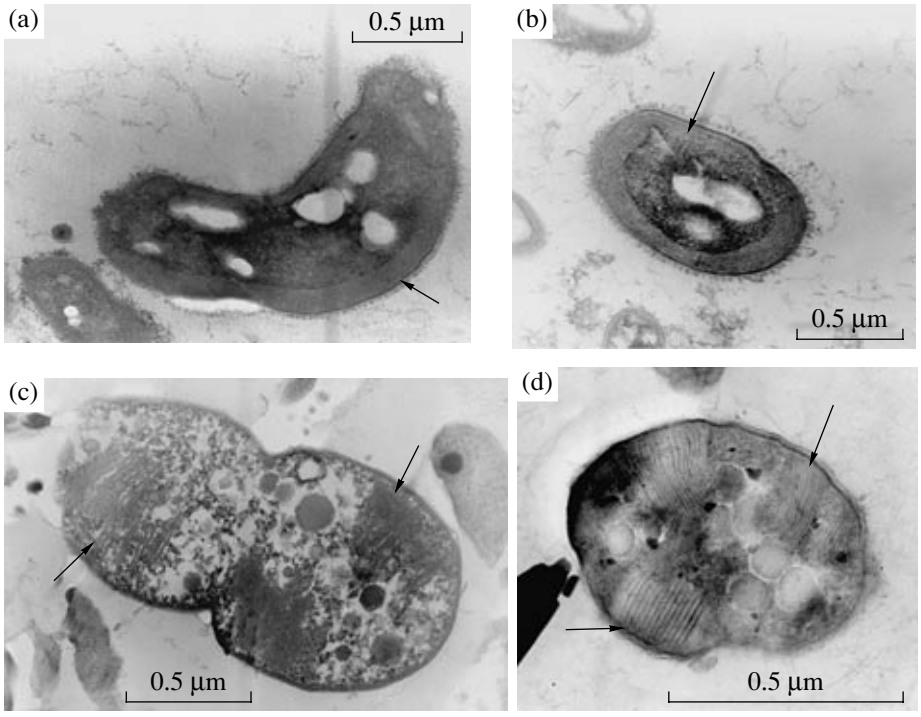


**Fig. 1.** Transmission electron micrographs depicting the ultrastructure of the representative cells of type II ((a) and (b)) and type I ((c) and (d)) methanotrophs in SB26, SB31 and SB31A cultures. The arrows show the intracellular membranes position.

were constructed and a total of 162 *pmoA* clones and 176 *nifH* clones were grouped based on restriction analysis and "single-base" sequencing.

Nucleotide sequences from every group of *pmoA* and *nifH* clones were translated to amino acid sequences. Obtained de novo sequences and referent sequences from GenBank were used for the construction of phylogenetic trees. The tree generated on the basis of inferred PmoA sequences (Fig. 2) showed that clone 31-26m from the SB31 community was similar to *Methylomonas methanica* (92.6% of identity). The other five clones from all three communities (26-15m, 31-4m, 31A-4m, 31A-25m, 31A-28m) had almost identical protein sequences (98.2 to 100% identity) and were closest to *Methylocystis echinoides* (96.5–100% identity).

Since some of the *nifH* sequences received by GenBank were shorter then those received in this study, the deduced sequences of only 110 amino acids were used in the phylogenetic analysis. The resulting tree represented on Fig. 3. shows that the majority of clones from all enrichments formed a tight cluster with *Methylocystis minimus* and *Mcs. echinoides* (94–96 % identity). Clone 26-4n was most similar to *Methylocystis echinoides* (99% identity); clones 26-3n and 26-5n, to *Mcs. minimus* (98 and 99%, respectively). Clones 31-5n and 31A-1n formed a cluster with *Methylocella palustris*, and the identity values were 100 and 97%, respectively. Clone 31-3n grouped together with *Azospirillum brazilensis* (97% identity) and *Rhizobium phaseoli* (95% identity).

## DISCUSSION

Methanotrophs have been recognized as a group of microorganisms which occupy a specific niche in the ecosystems. Due to their unique ability to metabolize methane aerobically, methanotrophs play an important role in the regulation of global methane emission and in maintaining the ecological balance.

In this study, we chose typical methane-oxidation enrichments as a model and examined the diversity of MOB with a combination of different methods in order to compare the potentials of these methods. Comparative studies of methanotroph diversity using different methods are not numerous. The culture-based MPN method was applied together with FISH to study the methanotrophic communities of rice paddy soils [16] and together with analysis of a *pmoA* clone library to study lake sediments [17]. The analysis of two different biomarkers, namely PLFA and Pmoa, was used to characterize the microorganisms that oxidize atmospheric methane [18]. In our study, we applied three different approaches in order to estimate the diversity of MOB; our results are summarized in the table. Electron microscopy analysis revealed the presence of type II methanotrophs in the SB26 community and type I and II methanotrophs in SB31 and SB31A. These findings were confirmed by further research, and every additional analysis

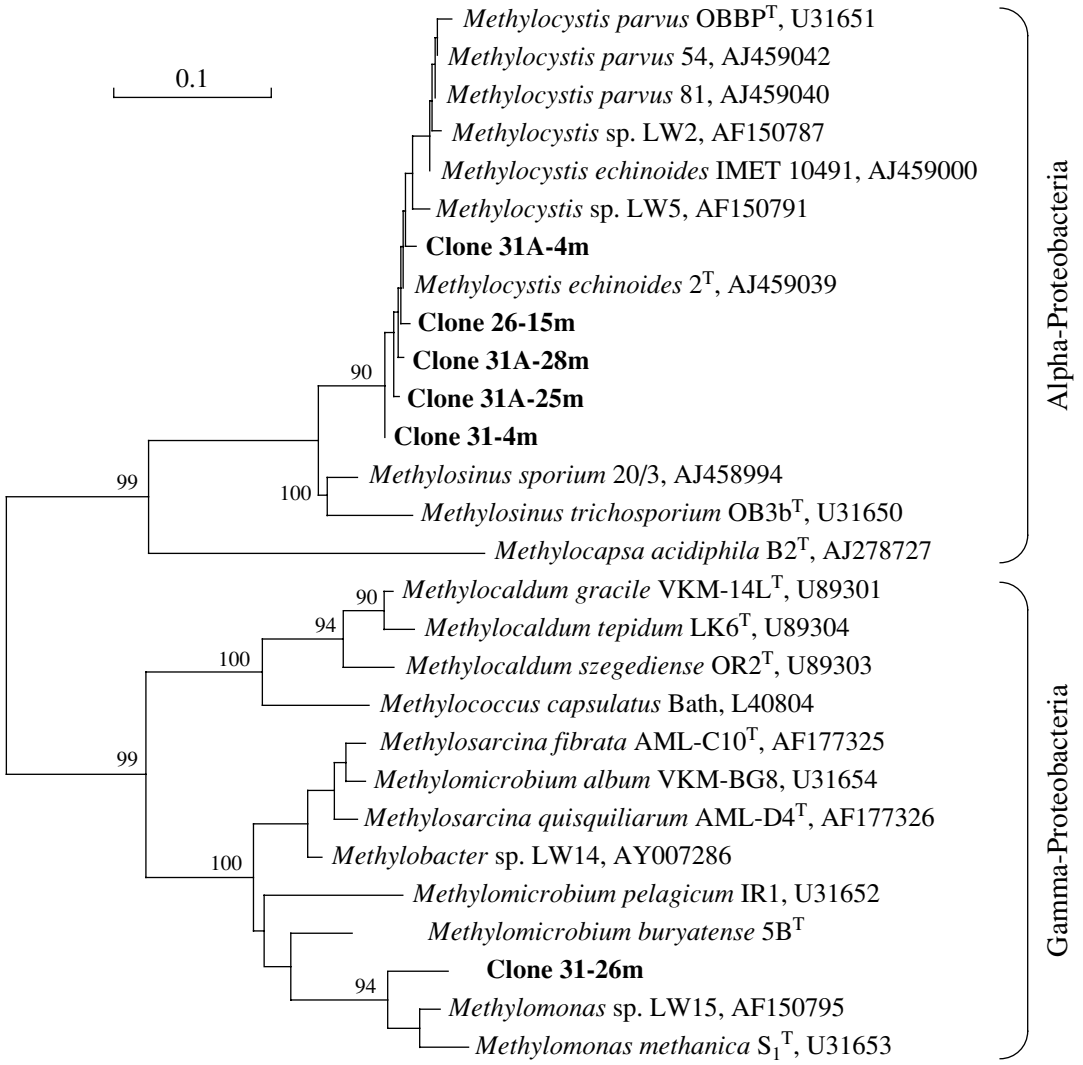


**Fig. 2.** Phylogenetic tree based on partial sequences (175 amino acids) of *PmoA*. Sequences obtained in this study are shown in bold. GenBank accession numbers are indicated next to bacterial names. The scale bar corresponds to 10 substitutions per 100 amino acid sites (evolutionary distances). Bootstrap values were expressed as a percentage of 500 replications and shown at branch points (values of less than 50 are not shown).

has extended our knowledge of the diversity of MOB in the communities under investigation.

IFA revealed the presence of type II methanotrophs from the *Methylosinus/Methylocystis* group in all three communities; *Methylomonas* and *Methylococcus* in SB31; and *Methylococcus* in SB31A. Direct microscopic enumeration of MOB by the serological procedure provides important information concerning the structure of the community in situ; however, the avail-

Comparison of methanotrophic diversity in enriched cultures as determined by different methods

| Culture | TEM | IFA | Analysis of *pmoA* | Analysis of *nifH* |
|---|---|---|---|---|
| SB26 | Type II methanotrophs | *Methylosinus trichosporium*; *Methylocystis echinoides* | *Methylocystis echinoides* | *Methylocystis echinoides*; "*Methylocystis minimus*"; |
| SB31 | Type I and type II methanotrophs | *Methylosinus trichosporium*; *Methylococcus capsulatus*; *Methylomonas methanica* | *Methylocystis echinoides*; *Methylomonas methanica* | *Methylocystis echinoides*; "*Methylocystis minimus"*; *Methylocella palustris* |
| SB31A | Type I and type II methanotrophs | *Methylosinus trichosporium*; *Methylococcus capsulatus* | *Methylocystis echinoides* | *Methylocystis echinoides*; "*Methylocystis minimus*"; *Methylocella palustris* |

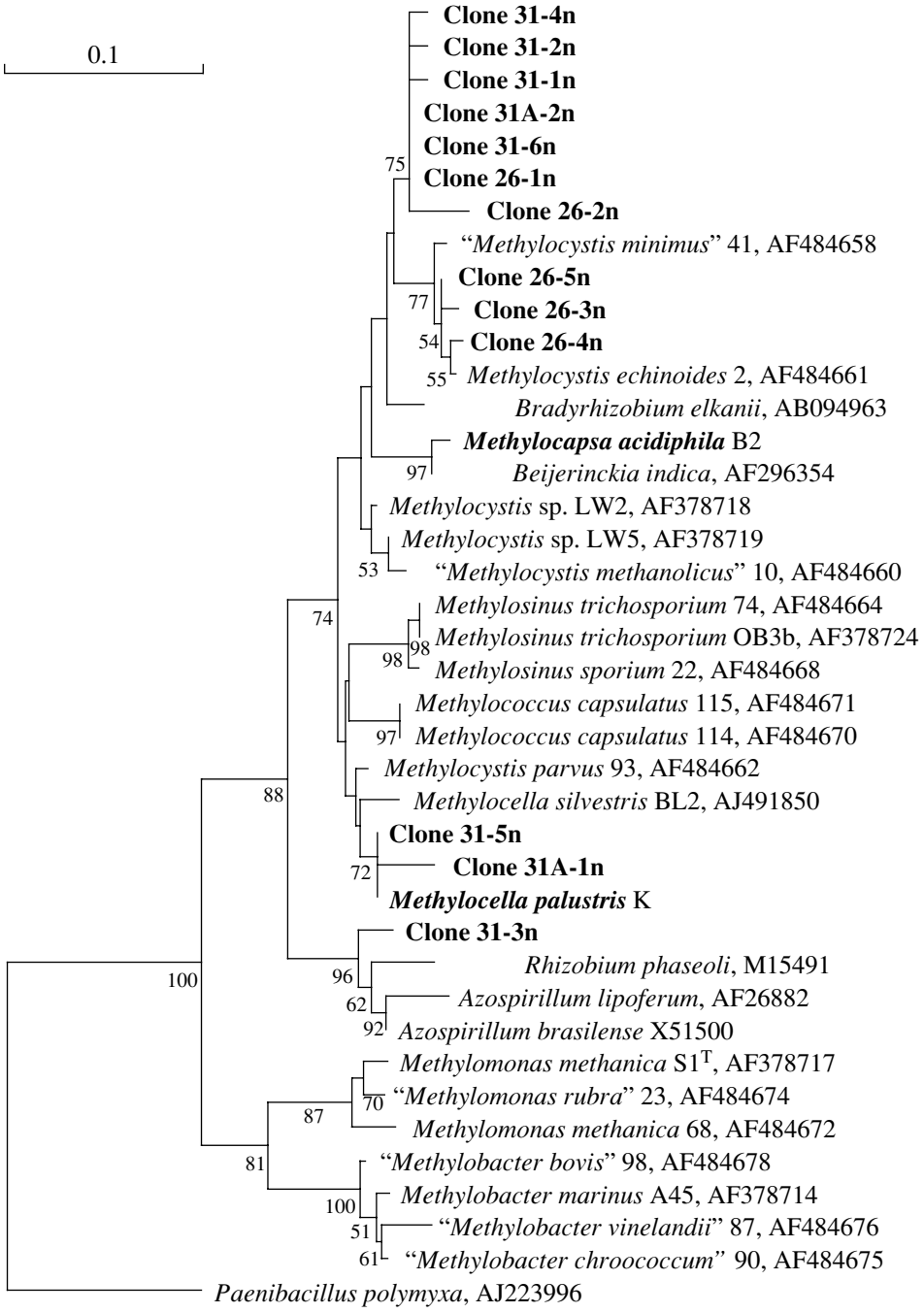


**Fig. 3.** Phylogenetic tree based on partial sequences (110 amino acids) of *nifH*. Sequences obtained in this study are shown in bold. GenBank accession numbers are indicated next to bacterial names. The scale bar corresponds to 10 substitutions per 100 amino acid residues (evolutionary distances). Bootstrap values were expressed as a percentage of 500 replications and shown at branching points (values of less than 50 are not shown).

ability of the antibodies is limited, as pure cultures of methanotrophs are required. As a result, it is not possible to analyze uncultured MOB.

The application of functional genes, unlike 16S rRNA, allows us to restrict the biodiversity analysis to the microorganisms with definite functions. Methane monooxygenase (MMO) is the key enzyme for methane oxidation in methanotrophs, and particulate MMO (pMMO) is present in all known methanotrophs with the exception of *Methylocella* [19]. The analysis of the *pmoA* gene encoding the 27 kDa subunit of pMMO is widely applied in ecological study, and we have also chosen this gene for our research. The results of *pmoA* gene analysis are generally in good agreement with IFA data. *Methylocystis* species were revealed in all com-

munities, and *Methylomonas*, in SB31. As was mentioned above, immune analysis demonstrated the presence of pink-colored *Mm. methanica* in SB31, but not in SB31A. The same results were obtained by *pmoA* gene analysis.

The diversity of methanotrophs was also evaluated by analysis of the *nifH* gene encoding dinitrogenase reductase subunit of the nitrogenase. Previously we demonstrated that the *nifH* gene-based phylogenies for methanotrophic bacteria are in good agreement with those based on 16S rRNA, with the exception *Mc. capsulatus* [8]. Unlike the serological and *pmoA* analyses, this technique did not reveal the presence of *Methylomonas*. Neither *pmoA* nor *nifH* studies detected members of the *Methylococcus* genus.

The main difference between IFA and molecular analyses is that the latter did not reveal the presence of *Methylococcus*; we believe this to be the result of several factors. First, according to the TEM data, the portion of type I methanotrophic cells in mixed cultures was comparatively low (10–15%). These findings are in agreement with the analysis of *pmoA* clone library, in which sequences close to *Methylomonas* accounted for less than 4%. This circumstance was probably the reason why we did not manage to detect *Methylomonas* by *nifH* and *Methylococcus* by *pmoA* and *nifH* investigations. Moreover, the high degree of degeneracy of the primers used, the DNA losses at the extraction steps, and the differences in PCR efficiency also can distort the final results [20]. Finally, the cross-reactivity and species specificity of the antibodies was examined and confirmed for pure collection cultures, whereas the immune characteristics of methanotrophs in the environment may be different. As a result, *Methylosinus trichosporium* and *Methylococcus capsulatus* were detected by IFA, but not by molecular methods.

Thus our research has demonstrated that the simultaneous application of different experimental approaches makes it possible to obtain better information about microbial diversity.

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