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SHORT COMMUNICATIONS

Inadequacy of Enrichment Culture Technique for Assessing the Structure of Methanotrophic Communities in Peat Soil

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The enrichment culture technique is one of the conventional approaches used to assess the composition of communities of methanotrophic bacteria in natural and anthropogenic ecosystems [1]. This technique has to be used because the number of cells of heterotrophic bacteria in samples usually exceeds the number of methanotrophs, significantly complicating their detection and identification. The use of enrichment cultures with methane as a selective growth substrate results in predominant development of the methanotrophic component of the microbial community and allows qualitative analysis of it.

The question as to how the composition of the enrichment cultures formed under laboratory conditions corresponds to the composition of methanotrophic communities in the original sample is of fundamental importance. It is known that varying pH, the total salt content in the medium, or the incubation temperature results in obtaining enrichment cultures of different composition from the same samples [2–4]. To date, it is commonly accepted that for the most adequate information about the composition of the methanotrophic community in situ to be obtained, the pH of the medium and the cultivation temperature should be approximated to those in the ecosystem studied. At the same time, not much attention is given to other conditions of enrichment culture setup, such as the presence and nature of a source of bound nitrogen in the medium and the culture aeration conditions. This investigation was undertaken to test the effect of these factors on the composition of laboratory methanotrophic enrichments and on how the latter corresponds to the methanotroph composition in the original sample. The direct molecular method for microorganism identification, the in situ hybridization with 16S rRNA-specific fluorescently labeled oligonucleotide probes (FISH), was used as the most objective referent method.

Sphagnum peat was sampled from a 10–20-cm layer of Bakcharskoe oligomesotrophic *Sphagnum* bog (56°51′ N, 82°51′ E) in Tomsk oblast in July 2006. The peat was homogenized by cutting with sterile scissors and mixed thoroughly. A 2-g portion of peat was fixed with 0.4% formaldehyde according to the method described earlier [5] for further analysis by FISH, and the remaining material was used for medium inoculation. Enrichment cultures were initiated in M2 medium (pH 5.0) [6] used in three modifications: (a) without a source of bound nitrogen; (b) with KNO_3 in an amount of 200 mg l^{-1}; and (c) with $(NH_4)_2SO_4$ in an amount of 200 mg l^{-1}. The media were dispensed in 20-ml portions into 150-ml glass flasks; 2 g of peat was added, the flasks were hermetically sealed, and methane was injected to a concentration of 20 vol %. Every two weeks of incubation, the gas phase in the flasks was renewed by blowing air through them and injecting a certain amount of methane. Incubation was carried out at 24°C under the following conditions: (a) vigorous shaking on a shaker with orbital movement (120 rpm); (b) slow shaking on a shaker with a three-dinemsional movement (50 rpm); or (c) without shaking. The culture suspension was sampled from the flasks and fixed with 0.4% formaldehyde after seven weeks of incubation in batch cultures with shaking and after nine weeks of incubation in batch cultures without shaking (in the latter case, the formation of enrichment cultures occurred later). Identification of methanotrophic bacteria in the original peat sample and in the enrichment cultures obtained was performed by FISH. Probes specific to type I methanotrophs (M84 + M705), type II *Methylosinus*/*Methylocystis* methanotrophs (M450), and the acidophilic methanotrophs *Methylocella palustris* (Mcell-1026) and *Methylocapsa acidiphila* (Mcaps-1032) [7] were used. The synthesis of Cy3-labeled probes was carried out by Syntol (Moscow, Russia). The preparation of specimens, their hybridization with the probes, and additional staining with a 1 μM solution of the universal DNA-specific fluorescent DAPI (4,6-diamidino-2-phenylindole) stain was carried out according to the technique described earlier [8]. The specimens were examined under a Zeiss Axioplan 2 epifluorescent microscope (Jena, Germany). The number of methanotrophic bacteria in the samples was determined by counting the number of probe-hybridized cells in 100 microscope fields with the subsequent

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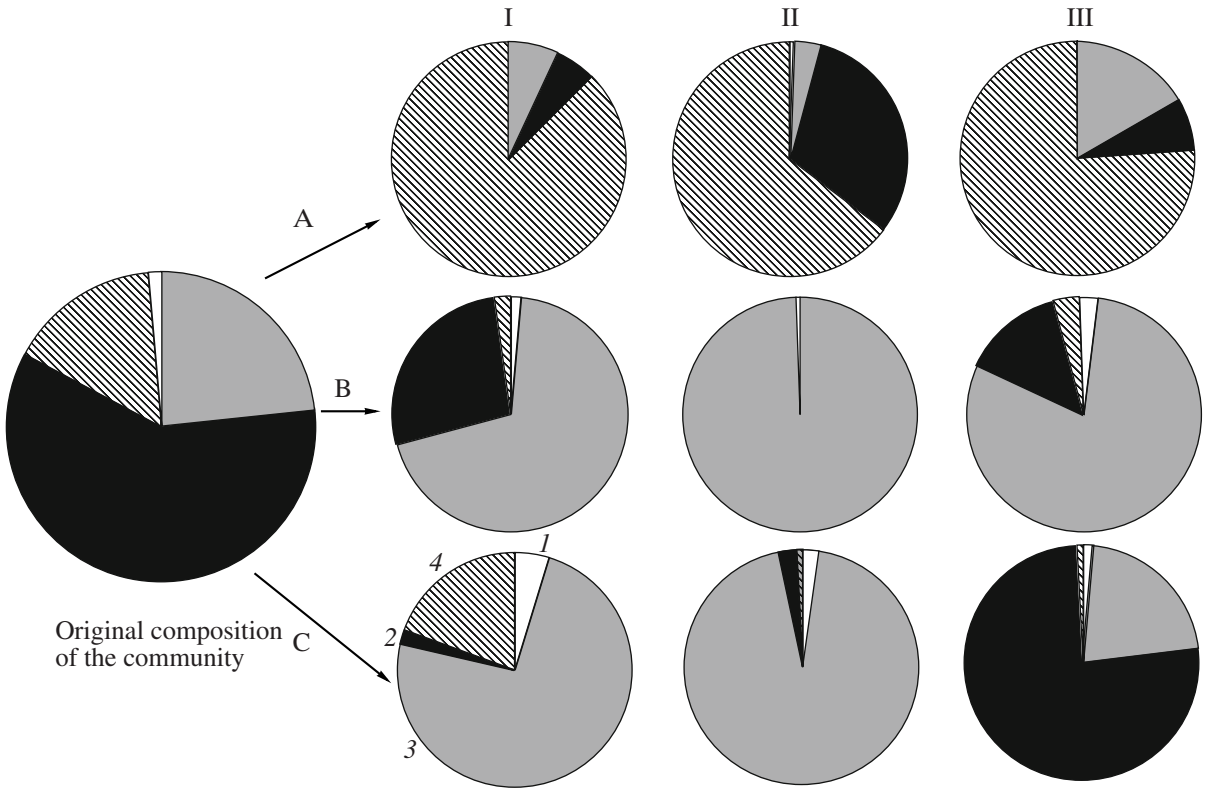

Fig. 1. Structure of the communities of methanotrophic bacteria in the native peat sample and in the enrichment cultures obtained from it as dependent on the aeration conditions and the presence of a source of bound nitrogen in the medium: A, vigorous shaking; B, slow shaking; C stationary conditions; I, medium without bound nitrogen; II, medium with KNO_3; III, medium with $(NH_4)_2SO_4$; *1*, type I methanotroph cells revealed by hybridization with M84 + M705 probes; *2*, *Methylocella palustris* cells (Mcell-1026 probe); *3*, cells of the *Methylosinus/Methylocystis* representatives (M450 probe); *4, Methylocapsa acidiphila* cells (Mcaps-1032 probe).

calculation of the size of the corresponding populations per 1 g of wet peat or per 1 ml of enrichment culture.

The total number of methanotrophic bacteria revealed in the *Sphagnum* peat sample with the probes used in this work was 2.3×10^7 cells/g^{-1} of wet peat. This accounted for 3% of the total number of microbial cells revealed in the peat using DAPI staining. Representatives of *Methylocella palustris*, constituting 54% of the total number of the cells of methanotrophic bacteria revealed, were the most numerous group of methanotrophs in the sample studied (Fig. 1). The other numerically important components of the original methanotrophic community were representatives of the *Methylosinus/Methylocystis* group (30% of the total number of the methanotrophs revealed) and the species *Methylocapsa acidiphila* (15%). Type I methanotrophic bacteria were not numerous, accounting for 1% of the total number of the methanotrophic cells revealed in peat. These results agree well with the earlier reported data on the numerical predominance of type II methanotrophs in acid *Sphagnum* bogs of the boreal zone [7].

During incubation in the flasks with methane, the total number of methanotrophic bacteria in the culture medium increased by 1.5 to 2 orders of magnitude. As a result, in the enrichment cultures sampled for analysis, methanotrophs constituted up to 40% of the total number of microorganism cells (Fig. 2). The analysis of the structure of methanotrophic communities in the enrichment cultures obtained under different conditions revealed that it differed significantly from that in the original peat sample (Fig. 1). The enrichment culture aeration conditions exerted a decisive influence on the composition of the methanotrophic community. Thus, when the incubation flasks were shaken vigorously, a selective advantage was gained by *Methylocapsa acidiphila* representatives, which became the main component of the methanotrophic communities formed under these conditions (Fig. 2A). The proportion of these bacteria constituted 64 to 88% of the total number of methanotrophs, being maximum in the medium without the source of bound nitrogen. The proportion of *Methylosinus/Methylocystis* cells in these enrichment cultures was insignificant, varying between 4 and 17%. On the contrary, the main component in the methanotrophic communities formed under conditions of slow shaking or stationary conditions was the *Methylosinus/Methylocystis* group (Fig. 2B), whose representatives accounted for 70 to 99% of the total number of metha-

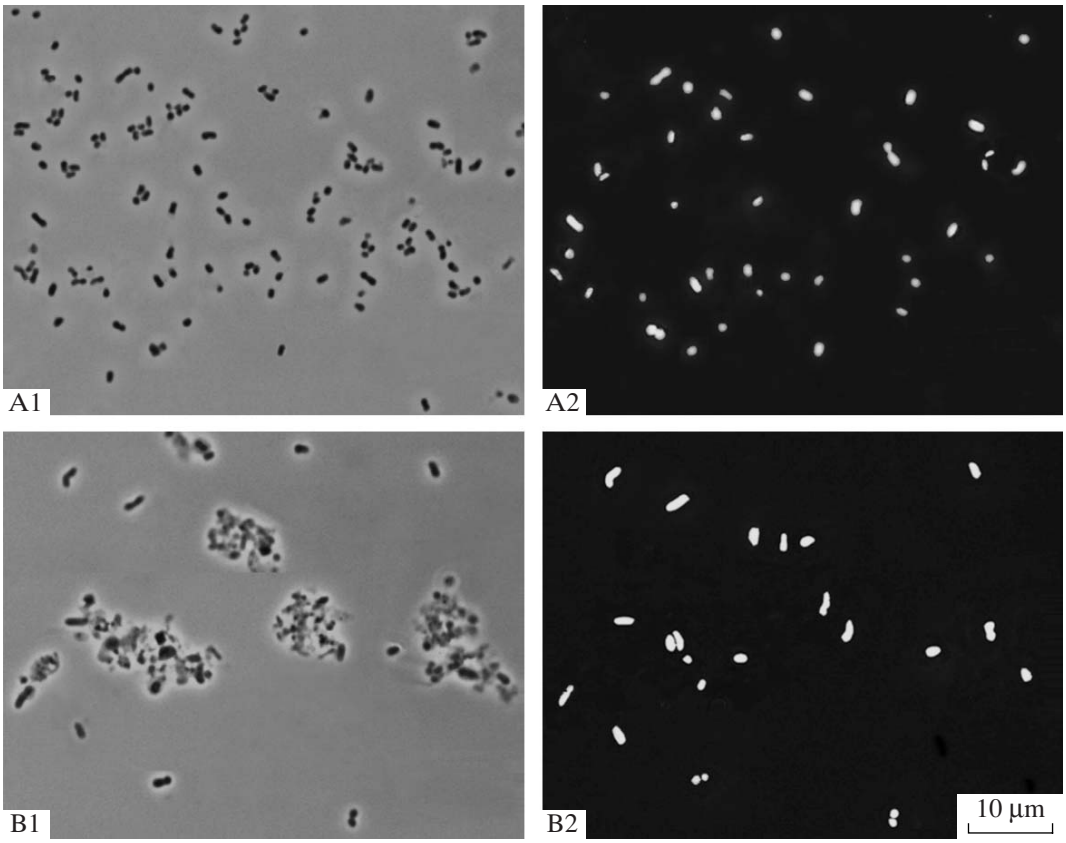

Fig. 2. Composition of the methanotrophic enrichment cultures formed under conditions of vigorous shaking (A) or slow shaking (B). A1 and B1, phase contrast; A2 and B2, fluorescent micrographs of hybridization with Cy3-labeled probes Mcaps-1032 and M450, respectively.

notroph cells. The reason for these contrasting patterns was the different sensitivity of the nitrogenases of *Methylocapsa acidiphila* and *Methylosinus/Methylocystis* methanotrophs to oxygen [9]. *Methylocapsa acidiphila* is the only methanotroph known to be capable of actively fixing N_2 at high partial pressure of oxygen (pO_2 0.18–0.19 bar), which is achieved by vigorous shaking of the experimental flasks. Representatives of the genera *Methylosinus, Methylocystis*, and *Methylocella* are also capable of nitrogen fixation; however, they carry out this process only at decreased oxygen concentrations (pO_2 0.05–0.15 bar) and, thus, gain a selective advantage under conditions of slow shaking.

In most of the batch cultures studied, the availability of bound nitrogen in the medium influenced the structure of the methanotrophic community of enrichment cultures less significantly (Fig. 1). This may be accounted for by the fact that all type II methanotrophs inhabiting *Sphagnum* peat possess nitrogen fixation capacity and take advantage of it successfully if the source of bound nitrogen in the medium has been exhausted. Nevertheless, when the enrichment cultures were obtained under stationary conditions, the structure of the methanotrophic communities in the nitrogen-free batch culture differed radically from that in the batch culture with $(NH_4)_2SO_4$. The predominant component of the community in the former case was representatives of the *Methylosinus/Methylocystis* group, and in the latter case, *Methylocella palustris*.

In none of the batch cultures analyzed were type I methanotrophs a numerically significant component; their proportion never exceeded 4%. This result agrees well with the fact that no acidophilic representatives have been described so far among the methanotrophs of this group.

Thus, the use of the FISH method has made possible the first objective comparison of the compositions of methanotrophic bacteria in the original peat sample and in the enrichment cultures obtained from it. It turned out that none of the enrichment cultures obtained under various conditions reflected adequately enough the actual structure of the methanotrophic community present in native peat. Thus, by the example of peat soil, we showed that the enrichment culture technique is inadequate for the assessment of the in situ structure of methanotrophic communities. The accuracy of this conclusion for other natural habitats requires separate experimental tests; however, it may be asserted that both the incubation conditions and the presence of a source of bound nitrogen in the medium significantly

influence the composition of the methanotrophic enrichment cultures formed under laboratory conditions.

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