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EXPERIMENTAL ARTICLES

Quantity and Taxonomic Composition of Ultramicrobacteria in Soils

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Abstract—The number, physiological state, and taxonomic composition of ultramicrobacteria and archaea in various soils (alluvial meadow, sod-podzolic, leached chernozem, and peat) were studied. In all of the tested soil samples, a large number of ultramicrobacteria (tens and hundreds of millions of cells per 1 g of soil) was revealed by fluorescence microscopy. The portion of cells with intact membranes was larger among ultramicrobacteria than among the ordinary-size cells (95–98 and 50%, respectively). Ultramicrobacteria were characterized by high taxonomic diversity and included representatives of the main phylogenetic groups widespread in soils, such as *Archaea, Actinobacteria, Cytophaga*, and *Proteobacteria*. The results indicate that ultramicrobacteria are widespread in soils in a viable state and are involved in soil processes.

Key words: ultramicrobacteria, soil nanoforms of microorganisms, physiological state, viability, phylogenetic groups.

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Direct microscopic observations in the previous century revealed that bacteria in soils were smaller than those cultivated in laboratory as pure cultures. Indigenous soil microflora contained mainly cell forms of 0.4–0.6 μm and sometimes smaller cells of 0.2–0.3 μm [1–5]. In the last decade, due to the application of electron microscopic and molecular biological methods, bacteria of small size termed "ultramicrobacteria," "nanobacteria," and "nanoforms" were shown to be widespread in natural environments [6–12].

The use of terms "nanoforms of bacteria," "ultramicrobacteria," and "nanobacteria" to denote ultrasmall (less than 0.2 μm) prokaryotes seems to be rather debatable; in our opinion, the term ultramicrobacteria is the most suitable for a primary designation of ultrasmall prokaryotes [12, 13]. In the present work, the cells obtained by filtration through membrane filters with a pore diameter of 0.2 μm were designated as ultramicrobacteria; obviously, the cells of less than 0.2 μm in diameter also belonged to this group.

In recent years, the isolation and investigation of ultramicrobacteria have attracted the attention of researchers because of increasing interest in the development of nanotechnologies.

The study of soil ultrasmall bacteria and archaea is of much importance since they represent a large group of microorganisms with unknown functions in the biosphere, which can be used in modern biotechnological processes.

The aim of this work was the isolation and enumeration of soil ultramicrobacteria and the study of their taxonomic composition to gain insight into their role in natural ecosystems.

MATERIALS AND METHODS

The study was carried out with air-dry soil samples stored in the laboratory for no more than a month. The samples were obtained from the upper horizons of the following soils: alluvial meadow (Moscow oblast), sod-podzolic (Bryansk oblast), peat soil (West Dvina Station, Institute of Forestry, Russian Academy of Sciences, Tver oblast), and leached chernozem (Irkutsk oblast, Prebaikal valley). The samples of peat soil were taken from different horizons of raised peat bog: P0 (6–10 cm), P1 (10–14 cm), and P2 (14–30 cm).

To isolate ultramicrobacteria, we modified the method [14]: soil suspension (10 g of soil per 100 ml of sterile tap water) was sonicated for desorption of the cells from soil particles on an UZDN-1 ultrasonic disintegrator (22 kHz, 0.44 A, 2 min) in an ice bath and centrifuged at 3000 *g* for 10 min. The supernatant was filtered through a membrane filter with the pore diameter of 0.2 μm and then concentrated by centrifugation (8000 *g*, 10 min) if required.

The number and physiological state of bacteria and archaea were determined with the use of a two-component L7012 fluorescent dye (LIVE/DEAD *Bac*Light bacterial viability kit) according to the manufacturer's instructions [15]. Bacterial cells with intact membranes ("live") showed green fluorescence, whereas the cells with damaged membranes ("dead") were red colored. Stained specimens were examined in an Axioskop 2 plus microscope (Carl Zeiss, Germany)

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Table 1. The quantity of bacteria and archaea in different soil types ($\times 10^6$ cells/g; staining with L7012)

Soil, horizon	Alluvial meadow, A1 (1–10 cm	Sod-podzolic, A1 (1–10 cm)	Leached chernozem, A1 (1–15 cm)	Peat soil, P0 (6–10 cm)
Cells of the ordinary size	1400 ± 110	4300 ± 390	3100 ± 200	4300 ± 300
Ultramicrobacteria	80 ± 10	40 ± 5	100 ± 8	500 ± 35

Table 2. The quantity of bacteria and archaea ($\times 10^9$ cells/g) and the level of cells with intact membranes (%) in the main horizons of raised peat bog (staining was performed with an L7012 dye)

Horizons	Cells of the ordinary size		Ultramicrobacteria	
	Quantity	Level of cells with intact membranes	Quantity	Level of cells with intact membranes
P0 (6–10 cm)	4.3 ± 0.3	47	0.5 ± 0.04	97
P1 (10–14 cm)	7.8 ± 0.4	50	0.5 ± 0.03	98
P2 (14–30 cm)	2.7 ± 0.2	48	0.1 ± 0.02	95

Note: The level of cells (%) was calculated from the average values of their quantity.

with a Filter set 09 (450–490 nm). For cell counting, at least 90 fields of vision were examined. The total number of bacteria in soil suspensions, the quantity of nanoforms in the filtrate, and the portion of cells with damaged and intact membranes were enumerated. The number of bacteria (ultramicrobacteria) per 1 g of soil was estimated by the standard procedure used for acridine orange staining [16].

The taxonomic position of bacteria was characterized by the molecular biological method of fluorescent in situ hybridization (FISH) with 16S rRNA-targeted, fluorescently labeled oligonucleotide probes (Sintol, Russia) specific for the following phylogenetic groups of prokaryotes: *Archaea* (ARCH915), *Actinobacteria* (HGC69a), *Cytophaga* (CF319a), *Alphaproteobacteria* (ALF1b), *Betaproteobacteria* (BET42a), and *Gammaproteobacteria* (GAM42a). The specimen preparation, hybridization, and staining were performed as described earlier [17–19]. The specimens were examined under an Axioskop 2 microscope (Carl Zeiss, Germany) with the use of a Filter set 15 (546 nm) for Cy3-labeled probes and a Filter set 09 (450–490 nm) in the case of staining with acridine orange. The number of individual phylogenetic groups was determined by counting the cells hybridizing with specific probes in 100 fields of vision with subsequent calculation of the cell number per gram of air-dry soil sample. The level of individual phylogenetic groups was calculated on the basis of their quantity.

RESULTS AND DISCUSSION

The total number of the ordinary-size and ultrasmall cells of bacteria and archaea are given in Table 1. The number of ultrasmall bacteria in the upper horizons of various soils varied from 40 to 500×10^6 cells/g, which was an order of magnitude less than the quantity of the ordinary-size cells (Table 1). Ultramicrobacteria were revealed in all of the soil types studied (10^7–10^8 cells/g) with the highest content in the chernozem and peat soil ($100–500 \times 10^6$ cells/g), while their content in the sod-podzolic and alluvial meadow soils was lower. These results imply active involvement of these organisms in soil processes.

In our experiments, the share of ultramicrobacteria varied from 1 to 11% of the total number of bacteria that is lower than the level of ultrasmall cells revealed in permafrost soil (up to 43%) [8, 11] and is comparable with the data on insignificant amount of bacterial cells of less than 0.25 μm in diameter and 0.5 μm in length in soil samples examined by light and scanning electron microscopy [4]. It should be noted that the number and the share of bacterial nanoforms in these works were estimated by other methods.

As seen from Table 2, ultramicrobacteria were revealed in all of the horizons of the raised peat bog; the patterns of the changes of their number with depth were different from those of the ordinary-size bacteria. In P0 and P1 horizons, the numbers of ultramicrobacteria were high and almost equal, whereas the quantity of the ordinary-size bacteria differed by almost twofold.

The study of the physiological state of ultramicrobacteria by using a two-component L7012 fluorescent dye revealed that 95–98% cells were green colored, which is indicative of their intact membranes.

In all of the studied soil samples, the share of the ordinary-size cells with intact membranes was 47–50%. Almost the same level of "live" cells with intact membranes was observed in the pure culture of *Pseudomonas aurantiaca* incubated for a long time (about 6 months) under nitrogen limitation [20].

High level of ultramicrobacterial cells with intact membranes is indicative of their higher tolerance to

Table 3. Taxonomic composition of ultramicrobacteria from the raised peat bog profile (%)

Horizons	Phylogenetic groups				Unidentified clones
	Archaea	*Actinobacteria*	*Cytophaga*	*Proteobacteria*	
P0 (6–10 cm)	11	10	8	14	57
P1 (10–14 cm)	16	5	4	17	58
P2 (14–30 cm)	14	14	6	15	51

Note: The level of cells belonging to individual phylogenetic groups (%) was calculated from the average values of their quantity.

Table 4. Taxonomic composition of the ordinary-size cells from the raised peat bog profile (%)

Horizons	Phylogenetic groups				Unidentified clones
	Archaea	*Actinobacteria*	*Cytophaga*	*Proteobacteria*	
P0 (6–10 cm)	7	14	14	20	45
P1 (10–14 cm)	13	16	10	17	44
P2 (14–30 cm)	14	14	10	15	47

Note: The level of cells belonging to individual phylogenetic groups (%) was calculated from the average values of their quantity.

the impact of unfavorable environmental factors in soil in comparison with the ordinary-size bacteria.

The estimation of the quantity and the share of individual phylogenetic groups of bacteria and archaea was performed by the FISH method, which made it possible to determine the taxonomic structure of the metabolically active part of microbial communities in different natural systems without isolation of pure cultures.

Among bacteria and archaea (including both ultrasmall forms and the ordinary-size cells) inhabiting three horizons of the *Sphagnum* raised peat bog, the representatives of the following phylogenetic groups were revealed: *Archaea, Actinobacteria, Cytophaga*, and *Proteobacteria* (Tables 3 and 4). The portion of proteobacteria recorded is the sum of the alpha-, beta-, and gammaproteobacteria.

Predominance of gram-negative bacteria (proteobacteria and cytophagas) and a decrease in the level of actinobacteria were revealed in all of the studied layers of the peat bog, which is typical of peat soils and agrees well with the results of other researchers obtained by conventional plating procedures [21] and the FISH method [19].

The taxonomic composition of the ordinary-size microorganisms was somewhat similar to that of ultramicrobacteria with predominance of gram-negative bacteria (24–27 and 21–22%, respectively) and a lower content of both actinobacteria (14–16 and 5–14%, respectively) and archaea (7–14 and 11–16%, respectively); along the whole profile, the share of proteobacteria (15–20 and 14–17%, respectively) was higher than that of cytophagas (10–14 and 4–8%, respectively). The level of archaea increased with depth from 7 and 11%, respectively, in the upper horizons to 14–16% in the lower horizons. Ultramicrobacteria differed from the ordinary-size bacteria by a higher level of archaea and unidentified clones and a lower portion of actinobacteria in P0 and P1 horizons.

By using the FISH method, we designated the phylogenetic position of less than half of ultramicrobacteria (Table 3) and of a larger number of clones among the ordinary-size bacteria (Table 4). The relatively high level of unidentified clones among ultramicrobacteria (from 51 to 58%) can be explained both by inability of the used probes to reveal the representatives of poorly known phylogenetically separated groups of bacteria and archaea and by a special dormant state of ultramicrobacteria with thickened cell walls, which prevented the probe from penetrating inside the cells.

It should be noted that the level of unidentified clones in unfiltered soil suspension (44–47%) was lower than in the fraction of ultramicrobacteria, although the distribution of individual taxa was similar in these samples. This finding suggests that representatives of several or even many taxa exist in soil as both large and small cells. The presence in the development cycle of the pure culture of the ultramicrobacterium *Kaistia adipata* of both small coccoid cells (0.4–0.8 μm) and ultramicrobacteria (0.2–0.3 μm) [9] confirms this assumption. The share of "true nanobacteria" (a phylogenetically separated taxonomic group) having ultrasmall size during the whole development cycle seems to be low in natural ecotopes. This supposition requires careful verification and further investi-

gations with the use of various electron microscopic and molecular biological techniques.

Based on our findings on a high level of cells with intact membranes and considerable amount of unidentified clones among ultramicrobacteria, it can be suggested that a part of the ultramicrobacterial population is represented by living cells exhibiting low activity. These cells are probably in some sort of a dormant state. This assumption is in agreement with the hypothesis that in soils, especially under nitrogen and carbon limitation, and in the ecotopes characterized by extreme low temperatures (permafrost soils), a significant number of cells were in a dormant state [22]. It is not inconceivable that ultrasmall cells of bacteria and archaea, as well as other resting forms of prokaryotes (spores, cysts, refractory cells, etc.), form a pool of prokaryotes promoting the preservation of microbial biodiversity in soil.

Our results indicate that the number of ultramicrobacteria in soils was large and reached millions of cells per gram of soil; they contained a higher level of cells with intact membranes (95–98%) than the ordinary-size cells (47–50%). Ultramicrobacteria were characterized by high phylogenetic diversity and included the representatives of the main groups of soil microorganisms. The share of unidentified clones among ultramicrobacteria was higher than among bacteria and archaea of the ordinary size, probably due to our insufficient knowledge of this taxon as well as to a special dormant state of ultramicrobacteria in soils.

The data on the relatively high number of ultramicrobacteria in soil samples, their taxonomic diversity and a peculiar physiological state allow us to assume that some representatives of the phylogenetic groups of common soil microorganisms are present in soil in the form of ultrasmall cells that can promote the preservation of cell viability under unfavorable conditions and the cell involvement in soil processes.

These results demonstrate that ultramicrobacteria are worthy of special attention and intense investigation with the use of modern microscopic and molecular biological techniques.

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