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EXPERIMENTAL ARTICLES

Estimation of Abundance Dynamics of Gram-Negative Bacteria in Soil

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Abstract—Bacterial succession in soil was studied for two variants of initiation (moistening and moistening with addition of glucose). To determine the numbers of viable gram-negative bacteria, the modified nalidixic acid method was applied. The numbers of gram-negative bacteria revealed by this method were 2 to 3.5 times higher than those determined by the traditional method. In a developing community, the highest total bacterial numbers were observed on day 7; afterwards their numbers decreased and stabilized at a level exceeding four- to fivefold the initial one. In both experimental variants, the highest numbers of viable gram-negative bacteria were revealed on day 15 (75–85% of the total bacterial numbers). Morphology of these bacteria suggests their classification as cytophagas (chitinophagas) utilizing chitin from the dead fungal mycelium.

Key words: bacterial succession, bacteria, gram-negative bacteria, dynamics of bacterial numbers, viability of gram-negative bacteria.

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In soil–microorganism systems, regular fluctuations of microbial qualitative and quantitative composition occur, as well as of rates and direction of microbial processes. These events are described as succession of a microbial soil community [1]. Microbial succession is studied in detail in model experiments under stable conditions; for this purpose, soil samples are studied under controlled conditions of temperature, humidity, etc. A succession is initiated either simply by moistening or by moistening along with introduction of an organic compound. Such experiments permit determination of the patterns of the composition and functioning of soil microbial complexes and the degree of its organization, as well as finding approaches to the problem of regulation of microbial activity in soil [1]. Studies of microbial components of soil in model experiments resulted in a wider range of microbial species and genera associated with specific types of soil [2–6].

Succession changes were found to occur in a certain sequence, the highest densities of the populations of different components of the microbial complex not coinciding in time. Fungi develop mostly at the earlier stages; the subsequent maximums of bacteria and actinomycetes are due to utilization of the dead fungal mycelium [1, 7]. Initially, organisms with high rates of substrate colonization, not adapted to competition (r strategy) predominate; highly competitive organisms with low energy consumption for maintenance (K strategy) become dominant at later stages [8, 9]. Dynamics of the ratio and numbers of the members of different taxonomic and physiological groups (for example, of gram-positive and gram-negative bacteria) is important for analysis of the bacterial component of microbial communities. This information is necessary for assessment of the actual and potential microbial functional activity in soils.

The goal of the present work was to estimate the numbers of gram-negative bacteria in the course of microbial succession in a laboratory soil model system after initiation by moistening or moistening with addition of glucose.

MATERIALS AND METHODS

Object of research. Microbial communities were studied in chernozem soil samples collected in the Alekhin Central Chernozem State Biosphere Nature Preserve [10]. The humus content of the upper soil layers was 9–12%. The upper part of the humus horizon (10–30 cm) was used for analyses. Microbial succession was initiated either by moistening (control) or by moistening with addition of 0.1% glucose. The soil was moistened to 60% of soil water capacity.

Total bacterial numbers. Prior to microbiological analysis, the samples were sonicated in a low-frequency UZDN-1 device (22 kHz, 0.44 A, 2 min) [11].

Total bacterial numbers were determined by fluorescence microscopy [12]. Suspensions of soil samples (0.02 ml) were applied to degreased microscope slides

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and spread over a 4 cm^2 area. After complete drying, the preparations were fixed by flaming. Twelve preparations were prepared for each soil sample. The preparations were stained for 2–3 min with acridine orange (1 : 10000).

The number of cells per 1 g of soil was calculated from the equation $N = S_1an/vS_2c$, where N is the number of bacteria per 1 g soil; S_1 is the preparation area (μm^2); a is the number of cells per microscope field (averaged for all the preparations); n is a dilution factor (ml); v is the volume of a drop applied to the slide, ml; S_2 is the area of a microscope field (μm^2); and c is the weight of the soil sample (g).

Enumeration of gram-negative bacteria. The Kogure method [13] was used as a basis, which has been initially proposed for enumeration of viable marine bacteria, especially those not growing on standard media. The method involves nalidixic acid, an inhibitor of DNA replication active only against gram-negative bacteria. In the standard method, the soil suspension was supplemented with1 ml of nalidixic acid (0.002 vol %) and yeast extract (0.025%), incubated for 6 h in the dark at 18°C, and used to prepare the slides for microscopy (see above). In the preparation, 50 microscope fields were examined and the percentage of elongated or changed cells was determined. According to recommendations of [13, 14], the cells exceeding 6–8 μm were counted. A modification of the method was also used. Prior to fixation, the preparations prepared from the soil suspension supplemented with nalidixic acid and yeast extract were incubated in a moist chamber for 3 or 6 h at 28°C. The slides were then fixed and stained with acridine orange.

For statistical treatment of the results (average, variance, and confidence intervals), the STATGRAPHICS and STATISTICA software packages were used. For bacterial numbers, the standard deviation (δ_{n-1}) did not exceed 10%.

RESULTS AND DISCUSSION

The initial amount of bacteria determined in chernozem samples by fluorescence microscopy was 0.5×10^9 cells per gram soil (Fig. 1). On the third day of succession, bacterial numbers in the variants with moistening and with moistening and addition of glucose increased to 1.7 and 3.5×10^9 cells/g, respectively. On the seventh day, the highest bacterial numbers were revealed in both variants: 2.6×10^9 cells/g in the control (moistening) and 4.3×10^9 cells/g in the variant with glucose; these values exceeded the initial ones fivefold and almost ninefold, respectively. Bacterial numbers decreased on the twelfth day and subsequently stabilized at a level three to four times higher than the initial one. The dynamics of bacterial numbers was similar in the control variant and in the variant with succession initiated by addition of glucose, indicating high levels of easily available organic compounds in the soil samples.

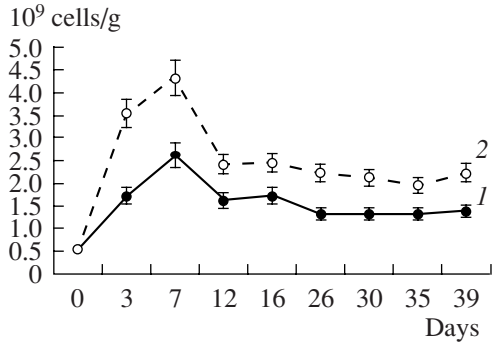

Fig. 1. Dynamics of total bacterial numbers in chernozem in the course of succession initiated by moistening (*1*) and moistening with introduction of glucose (*2*).

The nalidixic acid method [13] was used to enumerate gram-negative bacteria in the developing microbial community. Nalidixic acid inhibits division of gram-negative bacteria; viable cells therefore become elongated and are easily identified by fluorescence microscopy. According to the standard procedure, nalidixic acid was added to soil suspensions and the preparations were made after 6 h incubation at 18°C with shaking.

This method revealed low numbers of viable gram-negative bacterial cells in the soil under study; their difference in size from the cells in the preparations without nalidixic acid was less pronounced (Fig. 2a, b). A modification of this method resulted in more reliable enumeration of gram-negative bacteria in soil samples. Immediately after addition of nalidixic acid, the soil suspension was applied to microscope slides which were then incubated in a moist chamber for 3 or 6 h at 18°C. The slides were then fixed, stained, and analyzed.

The comparative results of enumeration of gram-negative bacteria by the standard and modified method are presented on Fig. 3. On the 7th and 15th day of succession, the numbers of gram-negative bacteria determined by the modified method were 2–3.5 times higher than those determined by the traditional Kogure method. The photographs of gram-negative cells in the preparations made by the Kogure method and the modified method illustrate another positive result (Fig. 2). In the first case, the size of elongated gram-negative cells did not exceed 10 μm (Fig 2a, b), while the modified method yielded gram-negative cells 15–20 μm long (Figs. 2c–2f); at later stages of succession, the cells could reach 40–80 μm (Fig. 2g, 2h). Compared to 3-h incubation of the preparations, incubation for 6 h resulted in detection of 1.5–2 times more gram-negative bacteria (Fig. 3).

The modified Kogure method was used to study the dynamics of the numbers of gram-negative bacteria in a developing soil community. The number of gram-negative bacteria in the initial soil sample was low,

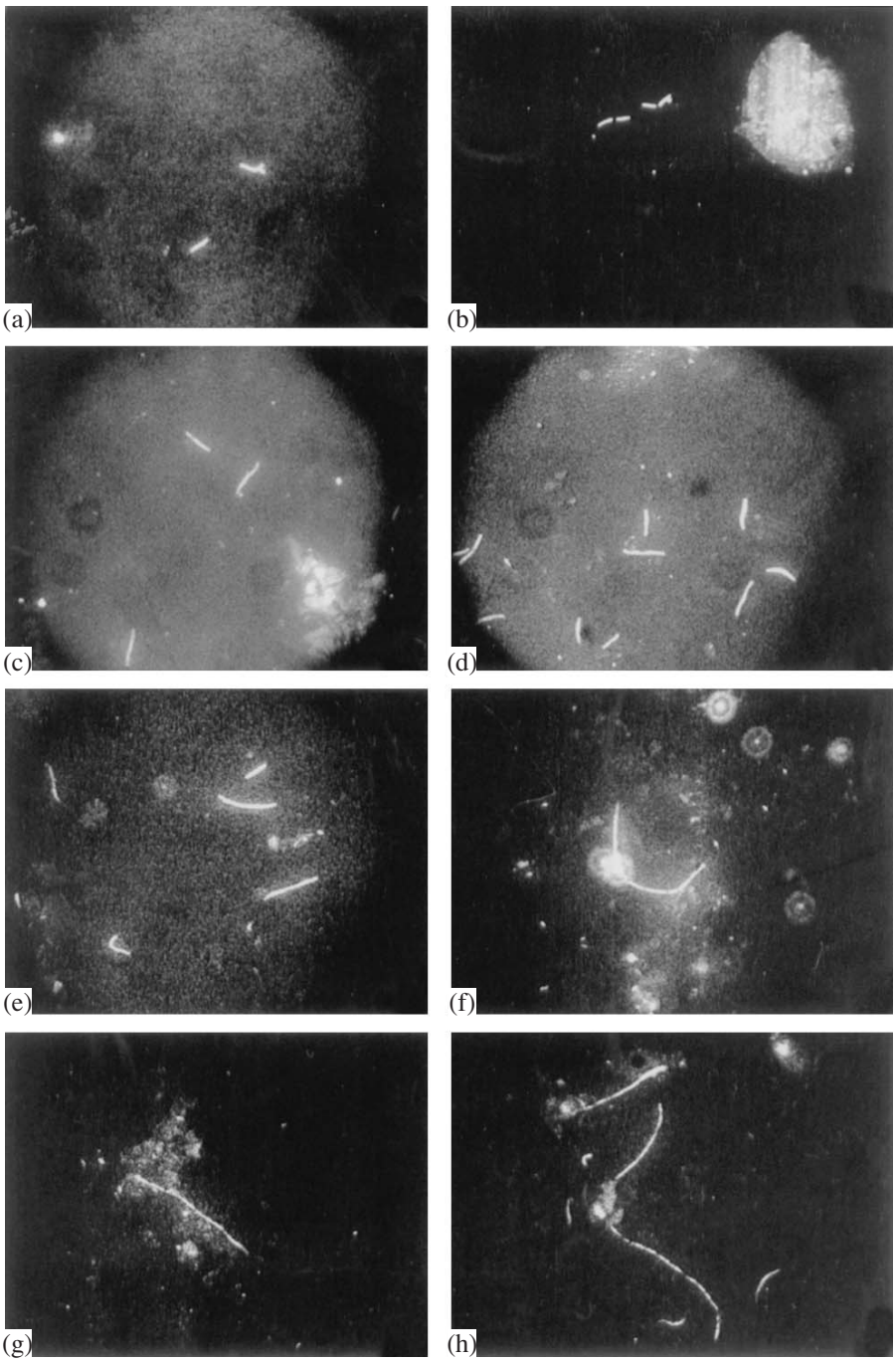

Fig. 2. Cells of gram-negative bacteria in chernozem soil: standard method Kogure (a, b); modified method, 3 h incubation with nalidixic acid (c) and 6 h incubation (d); gram-negative bacteria revealed by the modified method at the last stages of succession (e–h). Magnification ×1000.

0.1×10^9 cells/g soil (Fig. 4); they constituted ~20% of the total bacterial number. In the course of succession, their numbers on the 15th day increased by an order of magnitude in the control and by 1.5 orders of magnitude in the variant with glucose; they subsequently decreased and stabilized close to the initial level by the end of the experiment. The relative ratio of gram-negative bacteria in the bacterial pool also changed. In the original soil, it was 17.5% (Fig. 5); on the 7th day the number of gram-negative bacteria decreased to 7.2 and 3.8% in the control and in the variant with glucose, respectively. This was possibly due either to increased numbers of gram-positive bacteria or to an increased number of cells incapable of growth or requiring additional stimuli for growth. The first explanation seems more probable. The highest numbers of viable gram-

negative bacteria (84 and 77% for the control variant and the variant with glucose, respectively) occurred on the 15th day. By the end of the experiment, the ratio of gram-negative bacteria decreased to 7.6 and 5.5% of the total bacterial number, respectively; these values are lower than their ratio on initiation of succession. In the experimental variant with glucose addition, the percentage of viable gram-negative bacteria was always somewhat lower than in the control, probably due to the higher total number of bacteria resulting from the preferential growth of gram-positive bacteria (Fig. 1).

The domination of gram-negative bacteria of the 15th day of succession, when the total amount of the bacterial component decreases, may be explained as a result of a simultaneous drastic decrease in the length of fungal mycelium [1]. Consequently, additional organic material arrives to the soil as chitin, a component of fungal cell walls. In the course of prokaryotic succession in soils, gram-negative bacteria usually initially predominate [15]; at later stages, the ratio of actinomycetes increases [1, 16], correlating with a decrease of fungal biomass [17]. Actinomycetes are believed to specialize in utilization of dead fungal mycelium due to the chitinase activity, which is widely represented within this prokaryotic group. Appearance of moribund fungal mycelium in soil results in a paradox when quick-growing streptomycetes dominate in a mature system developing at the late stage of succession initiated by moistening or introduction of organic compounds (monomeric or polymeric) and characterized by low growth rates; at the initial stage of succession, in a young system with high growth rates, slowly growing streptomycete populations are present [18, 19].

However, cytophagas, the most active cellulose destructors in aerobic soil, can also utilize other polysaccharides, including chitin. Chitinophagas, slender gram-negative flexible rods up to 40 μm long, decomposing chitin, but not cellulose, belong to the cytophaga group [20]. Morphology of the viable gram-negative bacteria dominating at the last stages of the succession (15th day and after) (Fig. 2e–2h) indicates that the bacterial community is represented by chitin-degrading cytophagas that participate in decomposition of chitin from the cell walls of dead fungi along with actinomycetes. Higher numbers of viable gram-negative bacteria in experimental variants without glucose addition do not contradict this conclusion.

Thus, our research resulted in a modification of the Kogure method initially developed to determine the viability of marine bacteria; the method was applied for determination of the viability of gram-negative bacteria directly in soil. Dynamics of the numbers of viable gram-negative bacteria in a developing microbial community was investigated for the first time. The numbers of gram-negative bacteria determined by the modified method were found to be higher than those determined by the traditional method. The bacterial component of the mature bacterial community (15th day and after)

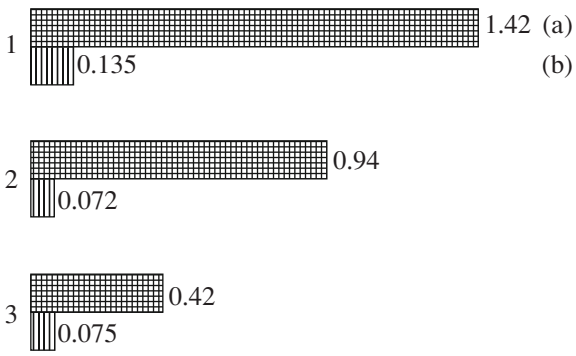

Fig. 3. Quantities of gram-negative bacteria determined by different methods (the numbers on the graph indicate bacterial quantities in 10^9 cells/g soil): modified method, 6 h incubation with nalidixic acid (1); modified method, 3 h incubation with nalidixic acid (2); standard method Kogure (3). Days of succession: 15 (a) and 7 (b).

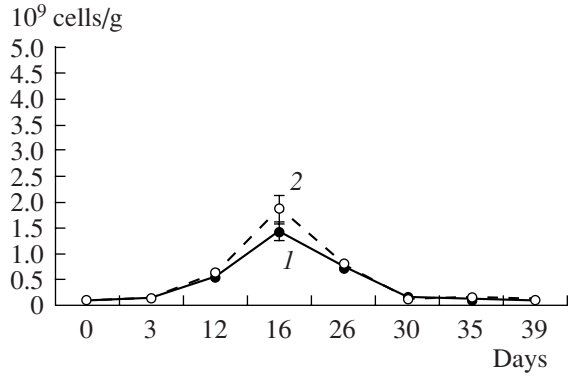

Fig. 4. Dynamics of the numbers of viable gram-negative bacteria in the course of microbial succession in chernozem soil: control (*1*) and additional introduction of glucose (*2*).

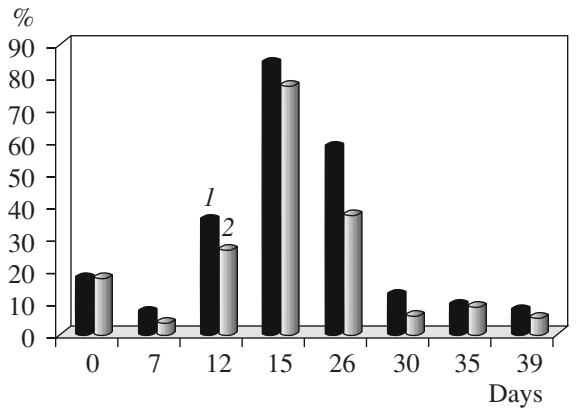

Fig. 5. The ratio (%) of gram-negative bacteria in the total bacterial population at different stages of succession in chernozem soil: control (*1*) and additional introduction of glucose (*2*).

was shown to contain mostly cytophagas (chitinophagas [20]).

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