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EXPERIMENTAL ARTICLES

Taxonomic Composition of Denitrifying Bacteria in Soddy Podzolic Soil

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Abstract—The taxonomic composition of denitrifying bacteria in soddy podzolic soil was studied by the succession analysis method. This method revealed a significant variation in the taxonomic composition of denitrifying microorganisms in the course of succession. In contrast to succession analysis, the single microbiological analysis of soil samples reflected only the late stage of succession and thus led to an underestimation of the major members of succession. Myxobacteria were found to be the most active denitrifiers at the early stages of succession, whereas bacilli dominated at its late stages. The bacilli were represented by three facultatively anaerobic species: *Bacillus cereus, Bac. circulans*, and *Bac. polymyxa.*

Key words: denitrification, bacteria, taxonomic composition, succession, soil

The ability to denitrify is widely distributed among soil bacteria and has been experimentally demonstrated in representatives of the genera *Bacillus, Pseudomonas, Micrococcus*, and *Achromobacter* [1–4]. However, the spectra of bacterial taxa capable of denitrification in various types of soil have not yet been studied in detail.

The aim of this study was to elucidate the taxonomic composition of the bacterial complex responsible for dissimilatory nitrate reduction in soddy podzolic soil during the succession initiated by soil wetting and the introduction of nitrate and glucose.

MATERIALS AND METHODS

Soddy podzolic soil samples collected from experimental fields of a lysimetric station in Moscow oblast, Odintsovo region, were studied. Five grams of soil were placed in penicillin bottles and wetted with 1 ml of a solution of glucose and potassium nitrate to give 2.5 mg of glucose and 0.1 mg of nitrogen per 1 g of soil. In this case, the soil moisture content was 50%. The bottles were hermetically sealed and incubated at 27°C. Samples were taken on days 1, 7, 15, and 30 of the experiment. Then 1 g of soil was placed in 100 ml of sterile tap water and exposed to 22-kHz ultrasound (44A) for 2 min on an UZDN-1 disintegrator (Russia). The prepared soil suspensions were used to inoculate test tubes with nutrient medium containing (g/l) K_2HPO_4, 1.6; KH_2PO_4, 1; $MgSO_4 \cdot 7H_2O$, 0.3; NaCl, 0.5; KNO_3, 3; yeast extract, 0.1; agar, 20; and one of the following carbon sources (g/l): sucrose, 10; starch, 6; and peptone, 6. After one-week incubation at room temperature, the enrichment cultures thus obtained were plated, in five replicates, onto solid nutrient media of the same composition, but containing 2% agar. To inhibit fungal growth, all the media used were supplemented with 100 μg/ml nystatin. Bacteria were counted after two weeks of cultivation at room temperature. To discriminate bacteria belonging to different taxonomic groups, colonies of different morphological types on each examined plate were enumerated separately. Three to five representatives of each colony morphotype were isolated in pure cultures and identified on the basis of their physiological, biochemical, and micromorphological characteristics (the shape, size, and motility of cells, specific features of their life cycle, mycelium formation, the type of cell division and separation, the presence of cocci in aged cultures, and the character of spore formation) [5, 6].

Denitrifying activity was assessed in pure bacterial cultures obtained at different stages of succession. For this purpose, two-day-old cultures were placed, at a concentration of 10^6 cells/ml, in 15-ml sealable flasks with liquid nutrient medium of the above-described composition. Immediately after inoculation, the gas phase of the flasks was replaced with argon to create anaerobic conditions. Denitrifying activity was determined by the accumulation of nitrous oxide (N_2O) in the gas phase. For this purpose, 1 ml of acetylene was injected to the gas phase of the flasks, and they were incubated at 27°C for one week. The concentration of N_2O was determined on a 3700/4 gas chromatograph equipped with a thermal-conductivity detector and a 3-m column packed with Polysorb-1. The carrier gas was helium at a flow rate of 25 ml/min. The amount of the nitrous oxide formed was calculated per million of bacterial cells. Microorganisms were enumerated in a Goryaev counting chamber.

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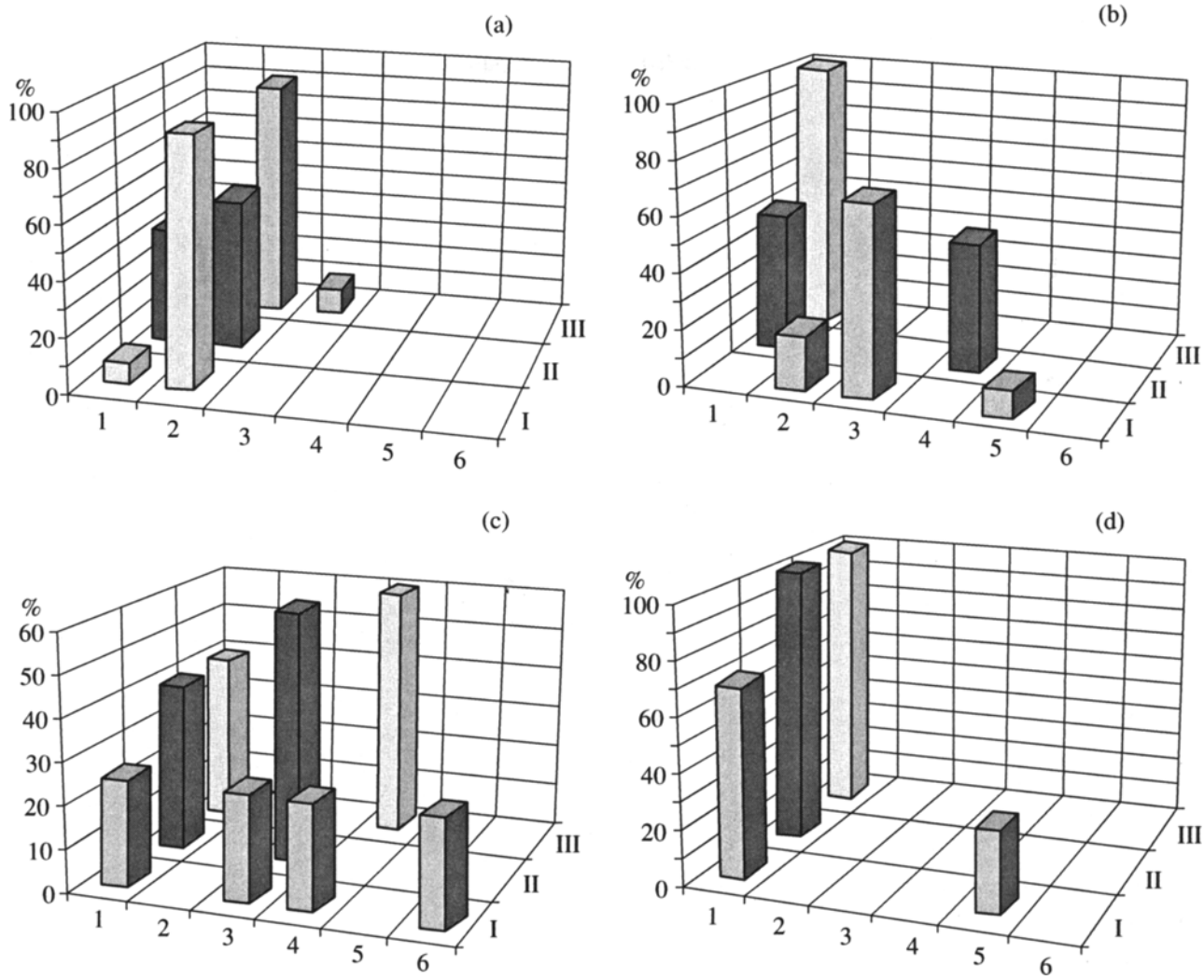

Fig. 1. Composition of the bacterial complex of soddy podzolic soil as shown by the results of succession analysis: (a) day 1, (b) day 7, (c) day 15, and (d) day 30 after the initiation of succession; (1) *Bacillus*, (2) *Myxobacterales*, (3) *Arthrobacter*, (4) *Aquaspirillum*, (5) *Coryneform*, and (6) *Rhodococcus;* (I) sucrose, (II) starch, and (III) peptone.

RESULTS

The taxonomic composition of the soil-inhabiting bacterial complex varied in the course of succession, as revealed by the bacterial count on media with nitrates and different carbon sources. At the early stage of succession (on day 1 after the initiation of succession), myxobacteria dominated the bacterial community, comprising 50 to 90% of the total number of bacteria detected on different media. Bacilli and arthrobacters were the minor succession components (Fig. 1a).

By day 7 after the initiation of succession, the proportion of myxobacteria was significantly reduced, whereas representatives of the genus *Bacillus* (detected on medium with peptone and starch) or *Arthrobacter* (detected on medium with sucrose) became dominant (Fig. 1b).

On day 15, the genus *Aquaspirillum*, which is detected on medium with sucrose and peptone, also became dominant (additionally to the two aforementioned genera). Analysis on medium with sucrose showed that bacteria of the genera *Bacillus, Arthrobacter, Aquaspirillum*, and *Rhodococcus* were present in the community in approximately equal amounts (Fig. 1c).

On day 30 after the initiation of succession, the hierarchical structure of the bacterial complex dramatically changed: bacilli became the only dominants on all of the media used (Fig. 1d).

Thus, 30-day microbiological analysis on media with nitrates and different carbon sources revealed the succession of dominants in the soil-inhabiting bacterial complex. The dominants were represented by both gram-negative (myxobacteria and spirilla) and gram-positive (bacilli and coryneforms) bacteria.

Analysis of the created collection of 120 strains, including representatives of the genera *Arthrobacter,*

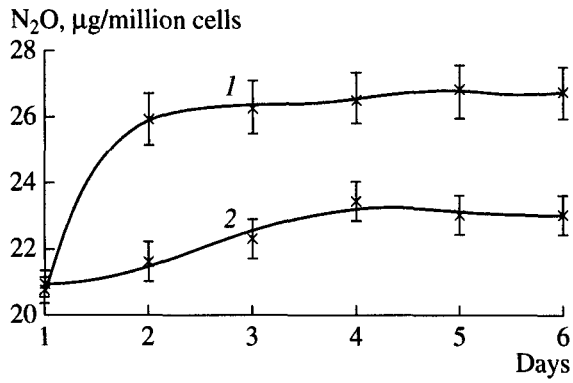

Fig. 2. Dynamics of nitrous oxide production by myxobacteria isolated from soddy podzolic soil at the early stages of succession: (*1*) strain no. 1; and (2) strain no. 2.

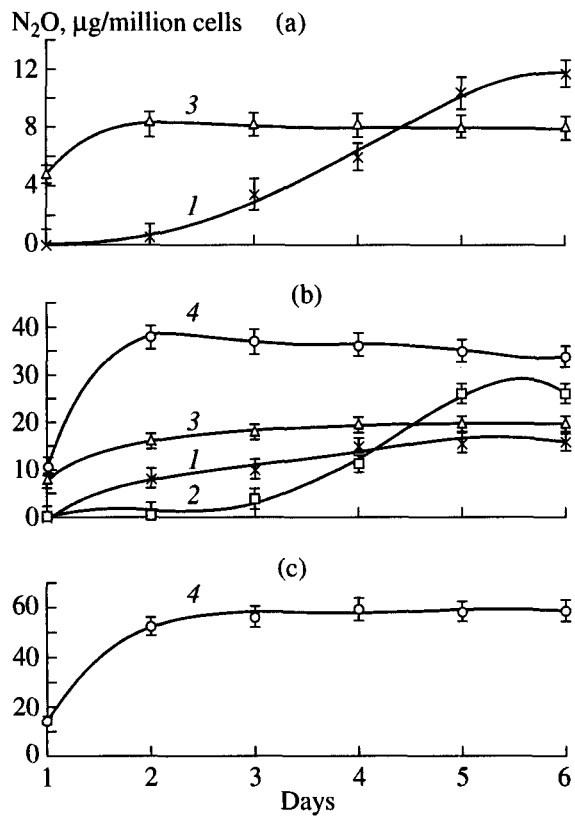

Fig. 3. Dynamics of nitrous oxide production by *Bacillus* species: (a) *Bac. cereus*, (b) *Bac. circulans*, and (c) *Bac. polymyxa*; (*1*) day 1, (*2*) day 7, (*3*) day 15, and (*4*) day 30 after the initiation of succession.

Aquaspirillum, *Bacillus*, and *Rhodococcus* and the order *Myxobacterales* showed that 60% of these strains were capable of dissimilatory nitrate reduction. All the nitrate-reducing strains were found to belong to the order *Myxobacterales* and the genus *Bacillus*. The maximal denitrifying activity was demonstrated by myxobacteria (27 μg N_2O/million cells) (Fig. 2) and by representatives of the species *Bac. cereus* (12 μg N_2O/million cells), *Bac. circulans* (35 μg N_2O/million cells), and *Bac. polymyxa* (60 μg N_2O/million cells) (Figs. 3a, 3b, and 3c). All these three bacillar species were facultative anaerobes.

DISCUSSION

The taxonomic composition of denitrifying bacteria in soddy podzolic soil was studied by the method of succession analysis. This method allowed significant transient variations in the taxonomic composition of soil microorganisms during succession to be revealed, whereas the single microbiological analysis of soil samples reflected only the late stage of succession and led to an underestimation of the main members of succession.

Earlier, only *Pseudomonas denitrificans* and *Bac. polymyxa* were detected among the denitrifiers of soddy podzolic soil [1, 2]. In the present work, using the succession method, we managed to isolate the myxobacterial strains that were able to reduce nitrates to nitrous oxide. To the best of our knowledge, no data are available in the literature on the denitrifying activity of myxobacteria, except for in our previous publication [7], in which we reported on the accumulation of nitrous oxide in amounts of < 1 μg/million cells by the myxobacteria isolated from chernozem soil. However, the myxobacterial strains isolated in this work from soddy podzolic soil produced 25 times more nitrous oxide than those previously reported, which allowed the important role of myxobacteria in the process of denitrification at the early stages of succession to be established. The collection of soil isolates created during this work may provide for further investigations of the role of myxobacteria in the soil nitrogen cycle.

The majority (about 70%) of bacillar isolates were found to be active denitrifiers at the late stages of succession. The data obtained indicate a significant role of facultatively anaerobic bacilli in the dissimilatory nitrate reduction.

Recently, the species *Bac. polymyxa* has been reclassified into the species *Paenibacillus polymyxa* [8]. It is believed that representatives of this genus are active nitrogen fixers. The data presented here and those obtained by other authors [3] indicate that *Paenibacillus polymyxa* is also an active denitrifier.

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