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EXPERIMENTAL ARTICLES

Tolerance of Soil Bacterial Complexes to Salt Shock

E. V. Lapygina, L. V. Lysak, and D. G. Zvyagintsev

Faculty of Soil Science, Moscow State University, Vorob'evy gory, Moscow, 119899 Russia

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Abstract—Investigations showed that bacteria present in soil are resistant to one-day exposure to a saturated solution of ammonium nitrate and can well develop when transferred to laboratory nutrient media. The evaluated number of bacteria in NH_4NO_3-treated soil samples was nearly the same as in native soil samples, while it was 1.5–2.5 times smaller in the former than in the latter case when microbial succession in the soil samples was initiated by wetting them. Bacteria (particularly gram-negative ones) occurring at the early stages of succession were the most sensitive to salt stress. Bacteria in soil were found to be much more resistant to salt stress than the same bacteria isolated in pure cultures.

Key words: salt shock, soil bacterial complex, lysis.

The use of granulated fertilizers leads to the formation of zones with high salt concentrations around the fertilizer granules. This can alter the structure and normal functioning of soil microbial complexes [1, 2]. The data available in the literature do not provide a coherent answer to the question of what salt concentrations can affect soil bacterial complexes over short and long periods of time after the fertilizer application.

This prompted us to study the effect of short-term (one day long) exposure to a saturated solution of ammonium nitrate on the bacterial complex of soddy meadow soil.

MATERIALS AND METHODS

Experiments were carried out with model microcosms which represented 100-g samples of a soddy meadow soil in the Klyazma River floodplain in Moscow oblast. Native soil samples were stored at –4°C. Immediately before the experiment, a soil sample was wetted with sterile tap water to soil moisture content comprising 60% of the field soil moisture capacity. A weighed portion (1 g) of the wetted soil sample was placed in a 100-ml saturated (64%) solution of NH_4NO_3 (alternatively, solid ammonium nitrate was added in an amount sufficient to create a saturated solution of this salt in a 1-g soil sample) and kept at 10°C for 1 day. The control samples were wetted with sterile tap water and also kept at 10°C for 1 day. After that, both experimental and control soil samples were incubated at 20°C at a constant soil moisture content.

On the 1st, 3rd, 7th, 14th, 30th, and 45th days of the experiment, the soil samples were analyzed microscopically with the use of luminescent dyes, viz., acridine orange (AO) and fluorescein diacetate (FDA), to determine, respectively, the total number of bacterial cells and the number of metabolically active cells [3]. The taxonomic structure of the soil bacterial complex was determined as follows: The soil sample was suspended using a UZDN-1 ultrasonic disintegrator (22 kHz; 0.44 A; 2 min), and the appropriate dilutions of the soil suspension were plated onto a glucose–peptone–yeast extract (GPY) agar supplemented with nystatin [3]. Soil bacteria were identified to a generic level based on the colonial, morphological, cultural, physiological, and biochemical characteristics using the identification criteria of Bergey's Manual [4]. The structure of soil bacterial complexes was described in terms of modern ecological indices [5].

To study the effect of salt shock on bacteria grown on GPY agar, bacterial colonies were washed off from the agar surface with sterile tap water, and a 1-ml aliquot of the washings was mixed with 100 ml of the saturated NH_4NO_3 solution (or sterile tap water for the control). After 1-day incubation at 10°C, the control and experimental mixtures were analyzed for the number of soil bacteria.

RESULTS AND DISCUSSION

Changes in the bacterial population of soil samples induced by wetting are shown in Fig. 1. The maximum number of bacterial cells (8.3–9.4 billion cells/g soil) stained with acridine orange was observed in the control soil samples within the first week of the experiment. By the 14th day, the number of bacterial cells decreased to about 4 billion cells/g soil and then remained at this level until the end of the experiment (45 days). These data somewhat disagree with the earlier observations indicating that the bacterial population of chernozem and serozem soils reaches a maximum on the 14th to 16th day of succession induced by wetting the soils [6, 7]. This disagreement can be explained by different types of soils and different con-

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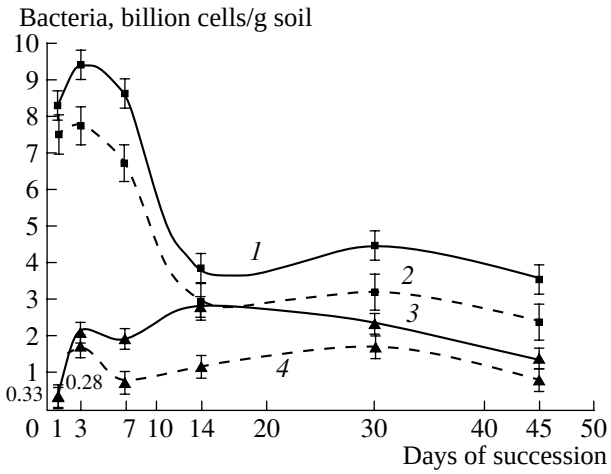

Fig. 1. The effect of ammonium nitrate on the number of the total (AO staining) and metabolically active (FDA staining) bacterial cells detected microscopically in soil in the course of succession induced by wetting: (*1*) the total bacteria in the control soil; (*2*) the total bacteria in the soil treated with ammonium nitrate; (*3*) metabolically active bacteria in the control soil; and (*4*) metabolically active bacteria in the soil treated with ammonium nitrate.

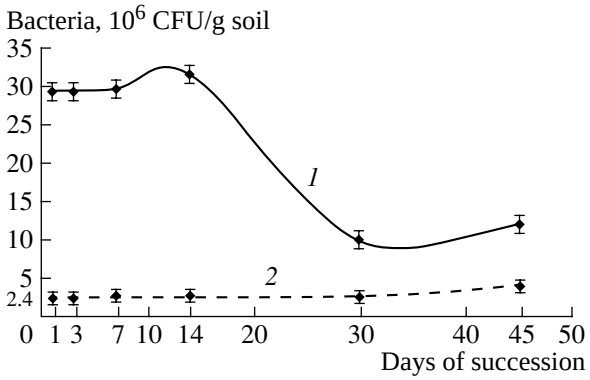

Fig. 2. The effect of ammonium nitrate on the number of saprotrophic bacterial cells detected in soil in the course of succession induced by wetting: (*1*) the control soil and (*2*) the soil treated with ammonium nitrate. Saprotrophic bacteria were enumerated using GPY agar.

ditions of storage of soil samples in the present and previous experiments. According to Zvyagintsev [6], the considerable decrease in the bacterial population of soil samples observed by the 14th day of succession is caused by the depletion of easily metabolizable substrates.

The exposure of soil samples to NH_4NO_3 resulted in the number of soil bacteria in them being 1.2 to 1.5 times smaller than in the control samples throughout the experiment. In this case, the general trends of bacterial populations in the salt-treated and control soil samples were the same. These results suggest that bacteria occurring in soil are highly resistant to short-term exposure to ammonium nitrate and can well develop when transferred to laboratory nutrient media.

Within the first day of incubation in the presence of ammonium nitrate, soil bacteria were more resistant to this salt than in later terms, so that only 10% of salt-exposed cells underwent lysis (Table 1). It could be suggested that soil bacteria occur in a dormant state characterized by an increased resistance to unfavorable environmental factors, which is similar to a state of some microorganisms during starvation [8, 9]. The initiation of microbial succession in the soil samples by wetting resulted in the reduced resistance of soil bacteria to lysis: the relative number of lysed bacterial cells increased to 18–22% within a period of 3 to 7 days of succession and to 25–30% within a period of 14 to 45 days.

The enumeration of metabolically active bacteria with the use of FDA (this luminescent dye undergoes hydrolysis by esterase inside bacterial cells with the formation of strongly fluorescing fluorescein) gave results that were essentially different from those obtained with acridine orange (Fig. 1). It can be seen that the number of bacterial cells detected with FDA (i.e., metabolically active cells possessing esterase activity) was always smaller than the number of cells detected with AO. The minimal number of metabolically active cells (0.28 and 0.33 billion cells/g soil) was observed on the 1st day of succession, when most bacterial cells obviously had no time to transit from the dormant state, in which they occurred in native soil. In the control soil, the number of metabolically active cells increased tenfold (to 2.8 billion cells/g soil) by the

Table 1. The percentage of ammonium nitrate–sensitive bacterial cells in soil samples

Day of experiment	AO staining	FDA staining
1	10	15
3	18	17
7	22	63
14	26	59
30	29	27
45	32	42

Table 2. The percentage of metabolically active bacterial cells in soil samples

Day of experiment	Control	Soil treated with NH_4NO_3
1	4	4
3	22	22
7	22	10
14	73	40
30	52	53
45	39	33

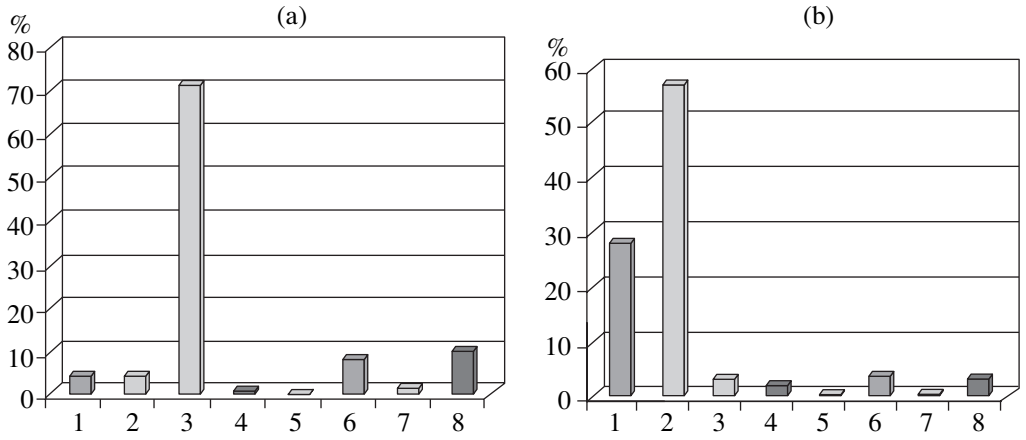

Fig. 3. Taxonomic structure of bacterial complexes in (a) the control soil and (b) the soil treated with NH_4NO_3: (1) *Streptomyces*, (2) *Bacillus*, (3) *Arthrobacter*, (4) *Rhodococcus*, (5) *Micrococcus*, (6) *Myxococcus*, (7) *Spirillum*, and (8) other gram-negative bacteria.

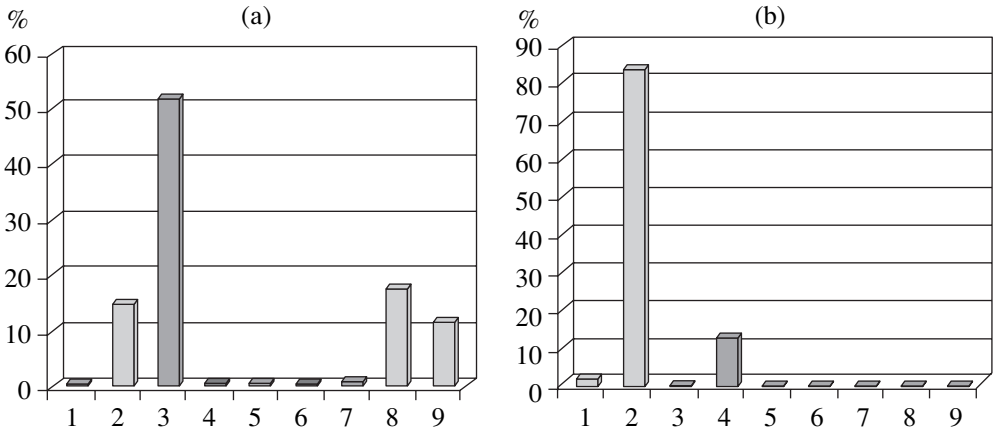

Fig. 4. Taxonomic structure of (a) the control bacterial population grown on GPY agar and (b) the saprotrophic bacterial population treated with NH_4NO_3: (1) *Streptomyces*, (2) *Bacillus*, (3) *Arthrobacter*, (4) *Rhodococcus*, (5) *Micrococcus*, (6) *Myxococcus*, (7) *Spirillum*, (8) other gram-negative bacteria, and (9) *Cytophaga*.

14th day of succession and then decreased about twofold (to 1.5 billion cells/g soil) by the end of the experiment. In the soil treated with ammonium nitrate, the number of metabolically active cells somewhat increased by the 3rd day of the experiment, decreased to almost the initial level by the 7th day, and then tended to increase to a level which was almost the same as in the control.

The treatment of the soil with ammonium nitrate within the first day of the experiment did not substantially decrease the number of metabolically active cells, indicating their high salt tolerance. Within a period of 7 to 14 days, the number of metabolically active cells sensitive to NH_4NO_3 decreased by 2.5 times. The relative number of metabolically active cells susceptible to lysis amounted to 15–17% within 1 to 3 days of succession (Table 1) and to 60% within 7 to 14 days. This quantity somewhat decreased thereafter, comprising 30–40% within a period of 30–45 days of succession. Such a population dynamics indicates that fast-growing bacteria (*r*-strategists), which prevail at the early stages of succession, are less salt tolerant than K-strategists, which prevail at the late stages of succession [6].

The ratio of FDA-reacting metabolically active cells to all AO-reacting cells (N_{FDA}/N_{AO}) (Table 2) for both control and NH_4NO_3-treated soils was minimal (4%) after 1 day of succession. In the control and experimental soils, this ratio reached maxima (73 and 53%, respectively) by the 14th and 30th days of succession. Taking into account the fact that FDA stains gram-negative bacteria better than gram-positive ones [11], it may be suggested that both control and experimental soils occurring at the late stages of succession are dominated by metabolically active gram-positive bacteria.

To determine the taxonomic affiliation of NH_4NO_3-resistant soil bacteria, we analyzed the saprotrophic bacterial complex of soils using GPY agar, which allows a sufficiently wide range of soil bacteria to be identified to a generic level [12]. As can be seen from the dynamics of bacterial population in the control soil

in the course of succession induced by wetting (Fig. 2), the population was maximum (28–30 million CFU/g soil) within a period of 1 to 14 days of succession and decreased about threefold (to 8–12 million CFU/g soil) over a period of 30 to 45 days. The population of saprotrophic bacteria in the NH_4NO_3-treated soil was about an order-of-magnitude less abundant (2–4 million CFU/g soil).

The decrease in the bacterial population of the NH_4NO_3-treated soil within 1 to 14 days of the experiment suggests that ammonium nitrate–sensitive bacteria prevail at the early stages of succession. In the control soil, the maximum numbers of the total and metabolically active bacterial cells were observed on the 14th day of succession.

The exposure of the soil to NH_4NO_3 resulted in the fraction of coryneform and gram-negative bacteria being decreased and that of bacilli and streptomycetes being increased. These changes in the structure of the saprotrophic bacterial complex were especially profound in the early terms of succession induced by soil wetting. Figure 3 presents the taxonomic structure of the soil bacterial complexes as determined on the 7th day of succession. The bacterial complex of the control soil was dominated by arthrobacters (70%), whereas gram-negative bacteria of the genera *Myxococcus, Polyangium, Cytophaga, Flavobacterium, Azotobacter, Spirillum, Aquaspirillum, Azospirillum, Pseudomonas,* and *Vibrio* added up to 20%, and the minor bacteria (streptomycetes, bacilli, rhodococci, and micrococci) added up to the remaining 10%. After soil treatment with NH_4NO_3, bacilli became predominant (60%), streptomycetes became subdominant (25%), and arthrobacters became a minor component of the soil bacterial complex. The fraction of rhodococci somewhat increased (in agreement with the reported salt tolerance of these bacteria [13]), and that of gram-negative bacteria decreased from 20 to 7%. Gram-negative bacteria, which cannot produce resting forms, were not detected at all. The high resistance of bacilli, streptomycetes, and myxobacteria to high salt concentrations may be explained by their ability to form resting forms (spores and cysts), which are more resistant to unfavorable environmental conditions than the respective vegetative cells.

It is known that bacteria present in soil, first, may be protected from unfavorable conditions by some soil components [6] and, second, may occur in specific states (as cystlike and dwarf cells) characterized by enhanced resistance to unfavorable environmental factors [8, 9]. Bearing this in mind, we compared the taxonomic structure and salt resistance of bacteria occurring in the soil and those grown on the surface of GPY agar inoculated with soil suspension dilutions. As can be seen from the results presented in Fig. 4, the control bacterial cells washed off from GPY agar were dominated by, in order of decreasing relative number, arthrobacters (52%), bacilli (15%), *Cytophaga* (12%), and gram-negative bacteria of the genera *Myxococcus, Polyangium, Flavobacterium, Spirillum, Aquaspirillum, Azospirillum,* and *Vibrio* (about 20% of the total), the rest being minor bacteria (micrococci, rhodococci, and streptomycetes). The exposure of the washed bacteria to NH_4NO_3 resulted in the bacterial complex being dominated by bacilli (84%). The fraction of rhodococci increased to 13%, whereas arthrobacters, micrococci, and gram-negative bacteria were in the minority. Gliding bacteria, which were salt-tolerant in the soil, appeared to be salt-sensitive after washing them from GPY agar. These findings can be explained by the prevalence of metabolically active cells in bacterial colonies grown on nutrient agar, whereas bacteria present in soil occur primarily in a resting state [9, 15]. The fact that bacilli and rhodococci are the most salt-resistant bacteria in the washings obtained from agar agrees well with the literature data on their survival in saline environments [16].

To conclude, both total and metabolically active bacterial cells present in soil are highly resistant to short-term exposure to ammonium nitrate, which is widely used as an artificial mineral fertilizer. Fast-growing bacteria (*r*-strategists), which prevail at the early stages of succession induced by soil wetting, are more sensitive to NH_4NO_3 than K-strategists, which prevail at the late stages of succession. The salt exposure of bacteria grown on the surface of GPY agar inoculated with the soil suspension dilutions led, on the background of reduced bacterial population, to an increase in the fraction of bacilli and streptomycetes and to a considerable decrease in the fraction of coryneform bacteria, gram-negative bacteria, and, what is more important, azotobacters, which are essential components of the soil microbial complexes. The bacteria present in soil were found to be more salt-resistant than the bacteria grown on the surface of nutrient agar after plating the soil suspension dilutions. This finding can be explained by the protective effect of some soil components on soil bacteria and by the fact that these bacteria predominantly occur in soil in a resting state, which is distinguished by an increased salt tolerance.

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