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SOIL BIOLOGY

Multistep Introduction of Bacteria to Natural Substrates at Different Initial Inoculation Doses

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Abstract—The population dynamics of the saprotrophic *Pseudomonas fluorescens* 32 *gfp* bacteria and two conventionally pathogenic enterobacteria (*Escherichia coli* 0157:H7 and *Salmonella enterica* var. Typhimurium) were investigated in their inoculation at different doses into cattle excreta and their subsequent entering soil and plants and migration through the gastroenteric tract of invertebrates. All the introduced bacteria investigated are shown to be able to overcome ecological barriers as they migrate through the natural substrates and habitats. The introduce microorganisms maintain their high population density even at the lowest initial inoculation dose—10^5 CFU/g of dry matter. Plants were found to be a favorable substrate for the survival of the bacteria investigated (for enteropathogens, in particular). Enteropathogens are able to pass through the gastroenteric tract of invertebrates. Therefore, these organisms can function as incubators and carriers of enteroinfections in nature.

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INTRODUCTION

Studying the fate of microorganisms in natural substrates and, moreover, their activity has always been important for soil microbiologists [4, 5, 8, 20, 22]. Thousands of publications are devoted to studying the population dynamics of microorganisms capable of fixing nitrogen, decomposing xenobiotic substances, annihilating other pollutants, or stimulating the growth of plants and inhibiting their pathogens [1, 13, 16, 19, 23].

Presently, in developed countries, the movement for the production of ecologically pure products and for environment control over the unregulated chemization and spreading of genetically modified organisms has been expanded. The application of noncomposted or insufficiently composted manure to soils is often used in current practice and represents a serious hazard for humans to be infected by such strains of bacteria as *E. coli* O157: H7, *Salmonella* spp., *Campilobacter jejuni, Listeria monocytogenes*, and some others. Animals have been traditionally considered as reservoirs and spreaders of the enteropathogens mentioned. However, recent investigations have shown that the enteropathogens also survive successfully out of the animals' gastroenteric tracts [24]. Precisely this fact has induced extensive studies at foreign scientific centers [15, 17, 21].

Some common regularities in the behavior of the reducers introduced to the soil were established from the example of saprotrophic microorganisms. Usually, after the inoculation of bacteria in the presence of nutrients in the substrate, their number can increase to some degree [8]. With time, the number of the introducents, as a rule, decreases due to the loss of nutrients, competition, and predators. During some time, the rates of the introduced bacteria reproduction and dying off become equal, so that a quasi-stationary state may be reached [6]. The further fate of the organisms introduced is related to trophic, physicochemical, and biotic factors [3, 12].

Most researchers observe the behavior of introducents only in one substrate inoculated with microorganisms. In many cases, such an approach was justified. Moreover, a special task was often set—the introducent should be eliminated after the performance of its function. Unfortunately, this fact does not always take place, as is evidenced by the natural circulation of microorganisms useful and hazardous and very dangerous for humans, animals, and plants. Thus, when only observing the introducent's behavior at the first step of the introduction, the results obtained cannot meet the modern requirements from the standpoint of the ecology of the microorganisms or, moreover, from the standpoint of their distribution in different ecological niches. The problems of the transfer of microorganisms through some interrelated natural substrates, which can be called "multistep" introduction, have become as topical as ever. The probability and even the commonness of these events is evident. However, there are only a few works experimentally studying multistep introduction, and to reveal some common regularities and use them in practice is difficult [9, 18].

The aim of the studies was to reveal the dynamics of the survival of bacteria introduced in different amounts to cattle feces and to determine their further movement to soil, plants, and through the gastroenteric tracts of invertebrates.

MATERIALS AND METHODS

The microorganisms and the media for their growth. The microorganisms were genetically labeled bacteria capable of synthesizing green fluorescing protein (GFP): *Salmonella enterica* var. Typhimurium MAE 110 *gfp* (further named *S.*Typhimurium) was received from Dr. Yuta Remling (Microbiological Center, Royal Institute, Stockholm, Sweden), *Escherichia coli* O157:H7 *gfp* was received from the Laboratory of A. van Bruggen (University of Wageningen, the Netherlands), and *Pseudomonas fluorescens* 32 *gfp* was supplied by Dr. R.J. Saler (Faculty of Biology, Arkansas University, USA). The investigations were performed with antibiotic-resistant and avirulent strains (the genes of virulence were removed).

S. Typhimurium MAE 110 *gfp* was determined on the medium containing the following (g/l): yeast extract (5), bacterial peptone (10), and agar (17), and 1 l of distilled water; pH 7.2–7.4. The medium was sterilized with nalidixic acid (antibiotic, 50 mg/l) at a pressure of 0.5 atm for 30 min. After autoclaving, kanamycin (50 mg/l) sterilized by filtration was added. *E coli* O157:H7 *gpf* was isolated on the medium containing the following (g/l): yeast extract (5), bacterial peptone (10), NaCl (10), agar (17), and 1 l of distilled water; pH 7.2–7.4. The medium was sterilized at a pressure of 0.5 atm for 30 min. After the autoclaving, ampicillin sterilized by filtration (50 mg/l) was added. The *P. fluorescens* 32 *gfp* was grown on the medium containing the following (g/l): bacterial peptone (2), K_2HPO_4 (1.4), $MgSO_4 \cdot 6H_2O$ (1.5), glycerin (15 ml/l), agar (17), and 1 l of distilled water; pH 7.0–7.2. The medium was sterilized at a pressure of 0.5 atm for 30 min. After the autoclaving, kanamycin sterilized by filtration (50 mg/l) and rifampicin sterilized by filtration (50 mg/l) antibiotics were added [10].

The bacteria for the introduction to the cattle excreta (CE) were grown in the same way on liquid media. The temperature for the bacteria growth was 37°C for *S.* Typhimurium MAE 110 *gfp* and *E coli* O157:H7 *gpf* and 25°C for *P. fluorescens* 32 *gfp*.

Cattle excreta (CE) were collected on the farm "Moskovskii konnyi zavod no. 1" (Odintsovskii district, Moscow oblast); the CE pH was 7.1. The ration for the animals included (kg/ind./day): green mass (2.5), hay (1), dry combined fodder (3.0), and salt on green mass (40 g/ind./day). Fresh feces were used in the tests. The initial moisture of the excreta averaged 85%.

Soil. The soil (soddy-podzolic) samples were taken in the territory of the Botanical Garden of Moscow State University nearby growing plants of sea buckthorn (*Hippophae rhamnoides* L.). Organic fertilizers were not applied during several previous years. Among the mineral fertilizers, ammonium nitrate (NH_4NO_3) was used in low doses. The soil was taken from a depth of 0 to 15 cm; then, it was sieved using a 2 mm-meshed sift and stored in polyethylene bags at room temperature. At the moment of the sampling, soil moisture was 6%. The soil contained the following: total carbon (39.6 mg/g), total nitrogen (2.87 mg/g), ammonium nitrogen (3.75 μg/g), nitrate nitrogen (85.5 μg/g), and phosphorus (PO_4) (17.7 μg/g). The pH of the soil was 6.6. The soil was composed of clay (11.65%), sand (31.8%), and silt (56.55%).

Plants. The capability of the bacteria to colonize plants was studied using cress (*Lepidium sativum*) of the Vitaminnyi variety (producer—Agrofirma Aelita, germination—92.5%).

Invertebrates. In studying the probability of the bacteria's survival in passing through a gastroenteric tract, French snails (*Helix pomatia*) collected in the Botanical Garden of Moscow State University were used. In the laboratory, the snails were kept in glass vessels at 18–20°C and fed cabbage leaves and fine slices of carrots.

The determination of the water-soluble organic carbon in the cattle excreta, the soil, and their mixture. Water extracts of the CE (1 g), the soil (1 g), and their mixture were prepared and filtered through a nylon bacterial filter. The C_{org} content was determined after its oxidation with $K_2Cr_2O_7$ in H_2SO_4 and the following recalculation using a calibrated curve plotted after the same reactions with different glucose concentrations.

Inoculation of the cattle excreta with the bacteria. In the suspensions grown in a liquid medium with the cells being at the initial stationary stage of growth, the bacteria were counted using a luminescence microscope (MIKMED 2 LYUMAM RPO 11, St. Petersburg OMO). The cells were concentrated by centrifuging (Backman J 6 B) at 5000 rev for 20 min at 4–5°C. The biomass was washed one time with water and suspended again in distilled water.

The CE mass was placed into a polyethylene bag with the CE to air ratio of ~1 : 5. The bacterial suspension was gradually added several milliliters at a time to the bags and evenly distributed over the surface. After its addition, the substrate was mixed carefully with a spreader and then in the closed bag by hand. The suspension volume was selected so that the final moisture of the CE did not exceed 90%.

The cattle excreta (500 g) were put into plastic vessels (0.7 l), closed using polyethylene film, and incubated in the dark at 18°C.

The mixing of the CE with the soil. In every particular case, the moment of mixing the CE containing the introduced bacteria with the soil was determined experimentally after the attainment of the quasi-stationary population density of the introducers (the plateau on the curve) in the substrate analyzed. Before mixing with

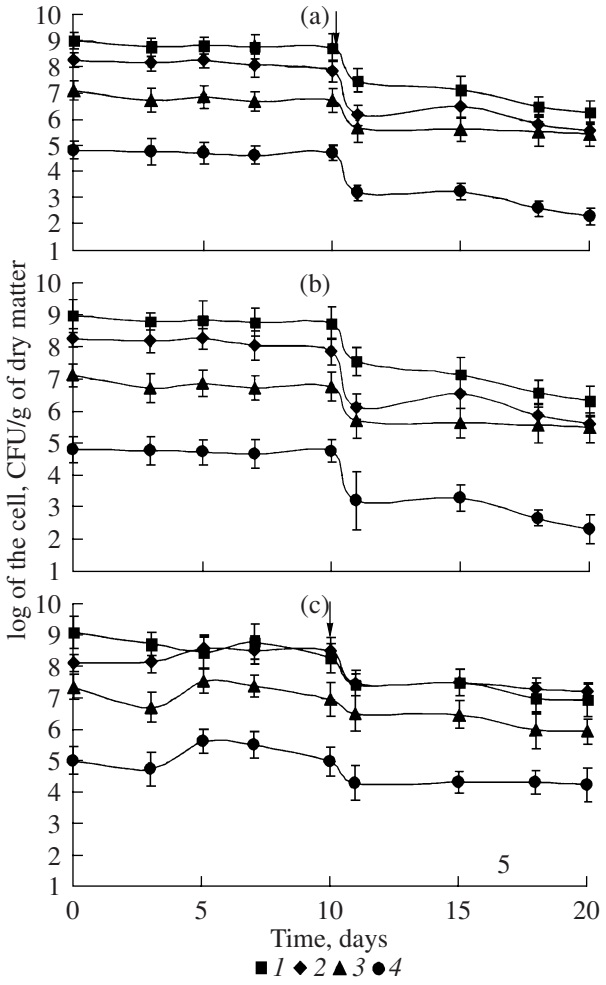

Fig. 1. The dynamics of the survival of *S. Typhimurium* MAE 110 *gfp* (a), *E. coli* O157:H7 *gpf* (b), and *P. fluorescens* 32 *gfp* C—25°C (c) in their introduction to CE with the following input to the soil depending on their initial concentration; the arrows show the time of the mixing with the soil. The initial concentration, cell/g of dry CE: *1*—10^9, *2*—10^8, *3*—10^7, and *4*—10^5 CFU/g.

the soil, the moisture of the CE was 65%, and the moisture of the soil was brought up to 14%. At this moisture, the CE to the soil ratio was 1 : 6 (425 g of soil and 75 g of CE); in the recalculation for the dry weight, the ratio was 1 : 16. Immediately after these procedures, the moisture of the soil–CE mixture was about 15%.

The growing of the plants. Cress seeds (0.5 g) were sown in pots containing a mixture of the CE and the soil with an introducent. The pots were closed with polyethylene film to preserve the soil moisture and placed under a fluorescent lamp. After 5 days, when the germs reached a height of ~3 cm, the plants were counted in each pot, and 30 individuals were cut (the aboveground part). The cut plants were placed into centrifuge tubes (40 ml), and 10 ml of sterile distilled water was added. The tubes were closed with rubber corks enveloped in foil and shaken on a vibration mixer for 30 s. The plants were taken out of the tubes with forceps, dried on filter paper (the water drops were soaked up), and weighed in the moist state at first and then after drying them to constant weight. Swabs from each of the 30 plants were centrifuged at 8000 rpm for 20 min at room temperature, the supernatant liquid was poured off, and the sediment was suspended again in 1 ml of sterile distilled water. In the suspension, the number of *gfp*-containing bacteria was determined using a microscope and plating on a selective nutrient medium.

For counting the bacterial population on the cress roots, 30 roots were taken out of the soil, the soil clumps were carefully removed from them by shaking, and they were three times washed with sterile distilled water. The plant roots were placed into centrifuge tubes with sterile distilled water. They were closed with corks, shook on vibration mixer for 30 s, the supernatant liquid was poured off, and the procedure was repeated. The clean roots after the removal of the water drops were weighed wet at first and then after their drying. In the supernatant liquid, the population of *gfp*-bacteria was determined in the way described above, and the mass of the rhizosphere soil was determined after the drying of the soil.

The feeding of the French snails on the cress. In each plastic pot, one actively feeding snail was placed. Before the performance of the tests, the snails were not fed for 24 h. In each pot, 50 cut plants were put and then the bacterial population was determined in the phylo- and rhizosphere. In 24 h, the excrements were collected one time. The number of colony-forming units (CFU/g of excreta) was counted. The sowing was conducted in 3 replicates, as well as the tests themselves.

RESULTS AND DISCUSSION

The population dynamics of the *S.* Typhimurium and *E coli* O157:H7 survival in the CE under the conditions of the short-term experiments were rather similar and independent of the initial inoculation dose. Both in the highest (9.5×10^8 cell/g of dry CE) dose (for *S.* Typhimurium and *E coli* O157:H7) and the lowest (7.1×10^4 and 6.8×10^4 CFU/g of dry CE for *S.* Typhimurium and *E coli* O157:H7, respectively) inoculation doses, 10 days after, their population remained almost the same (5.5×10^8 cell/g and 5.8×10^4 CFU/g of dry CE). On the whole, the population dynamics of the *P. fluorescens* 32 *gfp* were similar to those of enterobacteria. However, under the highest inoculation doses, the decrease in its population was more pronounced during the 10 days of the experiment (Fig. 1).

Naturally, the mixing of the CE containing the introduced bacteria with the soil immediately led to a drastic decrease in their number as the soil *diluted* their concentration. However, their *dilution* was not even in the substrate, especially at the low inoculation doses, probably, due to the less homogenous distribution of the population with a low density over the substrate.

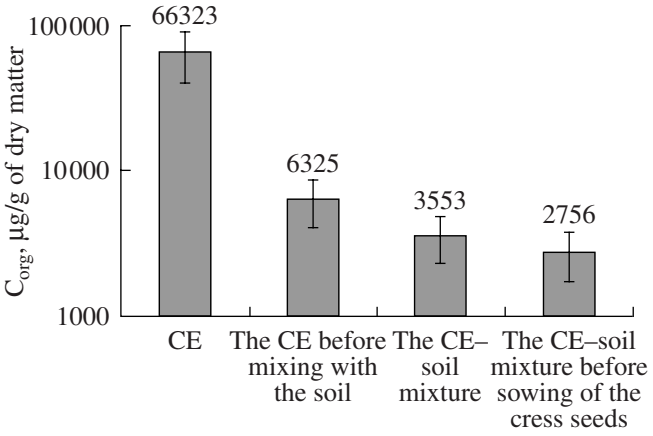

Fig. 2. Concentration of water-soluble organic carbon in the CE and the CE–soil mixture at different stages of the experiments.

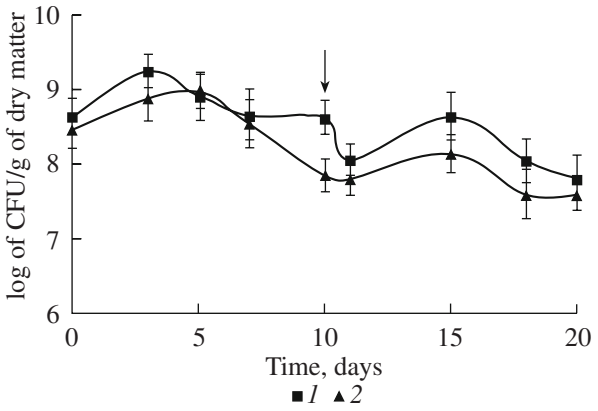

Fig. 3. The dynamics of the number of aborigines in the CE and the CE–soil mixture. Designations: *1*—copiotrophs, *2*—oligotrophs.

The dynamics of the enterobacteria in the CE–soil mixture were mainly similar to those in the CE, although the number of the introduced bacteria decreased to a greater extent than was observed in the CE. In addition, the number of enterobacteria decreased more distinctly in the inoculation of the highest and lowest doses. The number of *S*. Typhimurium and *E coli* O157:H7 decreased, on the average, by an order of magnitude at the highest initial inoculation dose (10^9 cell/g of dry CE) and the lowest one (10^5 CFU/g of dry CE). The relative stability of the survival of the bacteria was observed upon their inoculation at intermediate doses. For *P. fluorescens* 32 *gfp*, this regularity manifested itself only partially: the best survival was observed at the minimal inoculation dose—the population decreased only by 20.8% (Fig. 1).

The stable survival of the soil saprotrophic bacteria, *P. fluorescens* 32 *gfp*, and the enterobacteria in the CE and CE–soil mixture may be mainly explained by the quality (composition) of the CE, which in turn depends on the diet of the animals. It is known that the greater the amount of coarse food in the animal's diet and the older the animals, the quicker the introducents die in such feces [14, 15]. The ration of the animals contained much protein food and little vegetative food. At the same time, the determination of the water-soluble organic carbon content (WSC) at the different stages of introducing the bacteria to the CE and the CE–soil mixture revealed a significant decrease in this parameter (Fig. 2). Surely, the WSC carbon content weakly reflects the concentration of real substrates consumed by the bacteria. However, the scope of the changes in the WSC and the character of the dynamics of the introducers may testify to the unique trophic situation in the CE and CE–soil mixture. Probably, during the whole experiment, the amount of the substrate necessary for the stable maintenance of the introduced bacteria population was sufficient, but it was insufficient for increasing their biomass or they had limitations with respect to a substance or a factor. The dynamics of the CFU of the aborigenous copiotrophic and oligotrophic bacteria determined in the CE and CE–soil mixture evidenced this fact to some degree (Fig. 3). If in the CE and the CE–soil mixture the dynamics of the introducents—the soil saprotrophic bacteria, *P. fluorescens* 32 *gfp*, and enterobacteria—were rather smooth, the dynamics of the aboriginal species were wavy as in natural populations [7, 11, 25]. Thus, the introduced microorganisms (independently of their initial number) were both in the CE and the CE–soil mixture in the state of passive survival rather than in the active form.

A somewhat different regularity in the behavior of the introduced bacteria, at least of the enterobacteria, was observed in the counting of them in the phyllosphere and rhizosphere of the cress plants. The fate of the enterobacteria and saprotrophic *Pseudomonas* at different inoculation doses was monitored on the plants and in the animal excreta after the feeding of the invertebrates on these plants (Fig. 4). In the CE and the CE–soil mixture, some insignificant decrease in their number took place. However, on the plants, both in the rhizosphere and phyllosphere, the population of all the bacteria studied increased. Consequently, the ecological niche of the plants was favorable both for the enterobacteria and the saprotrophic pseudomonades and promoted their growth and concentration. The facts revealed are very important from the practical standpoint. Cress germs are known to be widely used in the food industry as "green" vitamins. In this connection, the use of incompletely composted organic mixtures and, moreover, of fresh manure in growing of such green vitamins can lead to serious adverse consequences (food intoxication and even epidemics). Another not less important fact that was revealed is the survival of the bacteria in their passing through the gastroenteric tract of the invertebrates. Although the bacterial population somewhat decreased even in the lowest inoculation dose, the bacteria were able to overcome

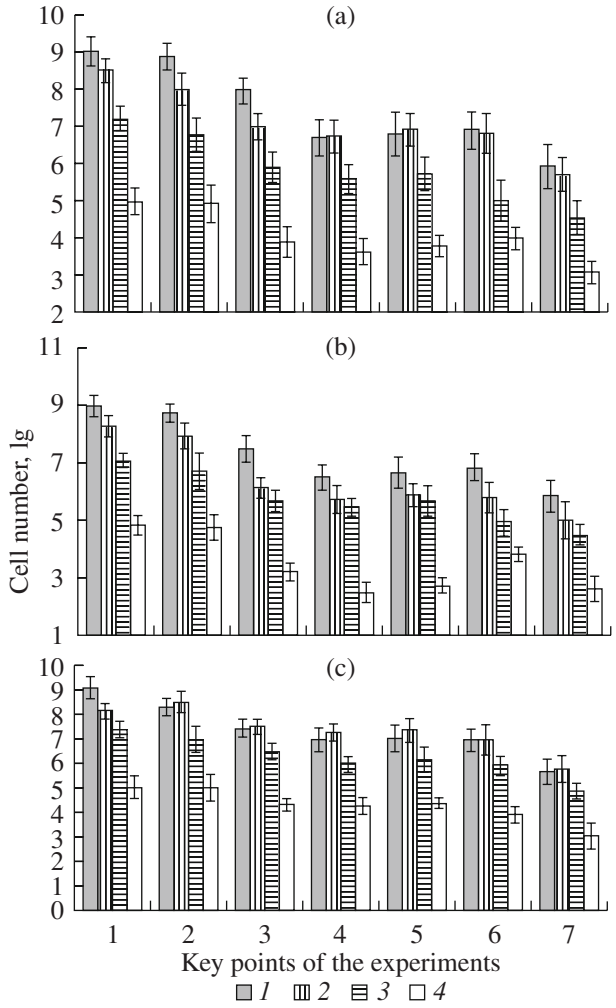

Fig. 4. The dynamics of the survival of (a) *S. Typhimurium* MAE 110 *gfp*, (b) *E. coli* O157:H7 *gpf*, and (c) *P. fluorescens* 32 *gfp* 25°C *P. introduced* to the CE in different initial numbers and at key points of their movement to the soil, on the cress plants, and through the gastroenteric tract of the snails after their feeding on the cress.

The initial concentration (cell/g of dry CE): *1*—10^9, *2*—10^8, *3*—10^7, and *4*—10^5 CFU/g. Key points of the experiments: 1—cell/g of dry CE, 2—cell/g of dry CE before mixing with the soil, 3—cell/g of dry CE–soil, 4—cell/g of the dry mixture before sowing of the cress seeds, 5—cell/g of the dry cress roots, 6—cell/g of the dry cress stalks, and 7—CFU/g of the dry excrements of the French snails.

this serious ecological barrier. In addition, it is quite evident that the invertebrates studied are active distributors of microorganisms in nature, playing the role of vectors in the epidemiological sense.

CONCLUSIONS

Our investigations showed that both the saprotrophic and enterobacteria—potential enteropathogens—are capable of surviving successfully under their different number in different natural substrates. Even under the lowest initial population density, the bacteria pass through a long and complicated chain, thus confirming the validity of the existence of multistep introduction in nature. The gastroenteric tract in many mammals can be an incubator for the reproduction of both saprotrophic and enterobacteria. Our studies showed that plants can also be considered as incubators.

Attempts to study the translocation of microorganisms through several natural substrates were made earlier, but the number of links in such chains was limited. In our work, a long chain of natural substrates was analyzed. In the case when plants infected with some enteropathogens and, moreover, with the remains of small animals' excrements on their surface are consumed by other animals, one can tell about the cycle or turnover of the microorganisms in ecosystems.

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