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EXPERIMENTAL ARTICLES

Soil Drying As a Model for the Action of Stress Factors on Natural Bacterial Populations

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Abstract—The drying of soil samples reduced the abundance (especially of predominant species) and the diversity of bacteria isolated from these samples, making easier the isolation of rare bacterial species. Some bacterial species that were minor before soil drying became dominant in dried soil samples. In general, soil drying allowed the diversity of soil bacteria to be determined more adequately. The bacteria that were isolated from dried soil samples turned out to be resistant to gamma radiation (with LD_{90} = 2.8–4.6 kGy) and desiccation. It is concluded that soil drying may serve as a model for the action of stress factors on natural bacterial populations. The hypothesis that periodic desiccation was the primary cause of formation of bacterial radioresistance in nature is discussed.

Key words: soil bacteria, dehydration, gamma radiation, resistance.

It is known that some highly radioresistant bacteria isolated from soil are also resistant to desiccation [1, 2]. Microbial diversity in soil is most frequently estimated by the agar plate technique [3], which was employed, for instance, for the study of the bacterial communities of swamps, deserts, and forest soils [4, 5].

In the present work, we employed this technique for a comparative study of bacterial diversity (with primary emphasis on mesophilic chemoorganotrophs) in soil samples before and after drying. The aim of this work was to create a model for the investigation of the action of stress factors on natural bacterial populations. The bacteria isolated from dried soil samples were tested for their resistance to such DNA-damaging factors as gamma radiation and desiccation.

MATERIALS AND METHODS

Bacterial isolates and their cultivation. Experiments were carried out with bacterial strains isolated from the samples of soddy podzolic soil collected in the environs of Kiev. Soil suspensions were plated onto glucose–potato agar (GPA) and mineral agar with 0.5% methanol (MMA) [6]. To suppress the growth of fungi, the media were supplemented with 50 mg/l nystatin. Pure bacterial cultures were cultivated on a shaker (220 rpm) at 30°C in a liquid mineral medium (MM) [6] with 0.5% methanol (for the growth of *Methylobacterium*) or 0.5% glucose and 0.1% yeast extract (for the growth of other bacteria). The effect of gamma radiation and desiccation on bacterial survival was studied using 18-h-old spore-free pure bacterial cultures containing from 10^8 to 10^9 cells/ml. The presence of spores in the cultures was controlled microscopically.

Plating of control (undried) soil samples. At each soil sampling site, samples were taken from two soil horizons, from the 0–2 cm surface layer and from the 3–4 cm subsurface layer. The aliquots (10 g) of soil samples were ground in a mortar to a powder state, suspended in 100 ml of sterile tap water, incubated on a shaker (220 rpm) for 45 min, and placed in a graduate cylinder. Samples (1 ml) of the aqueous soil suspensions taken from the middle layer were serially diluted tenfold, and aliquots (0.1 ml) of the appropriate dilutions (10^{-1} to 10^{-7}) were plated onto the aforementioned agar media.

The study of the effect of soil sample drying on bacterial survival. This effect was studied by the method of Mattimore and Battista [1], except that soil samples not bacterial suspensions were dried. For this purpose, the 1-ml samples of the soil suspensions (see the previous paragraph) were placed in sterile petri dishes and dried at room temperature in a desiccator above calcinated $CaSO_4$ [1] for 42 days (relative humidity in the desiccator was controlled with a membrane hygrometer). Then, the dried soil samples were suspended in a sterile 0.5% solution of NaCl and plated in triplicate onto GPA and MMA. The plates were incu-

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bated at 30°C for 7 days, after which bacterial colonies were enumerated on the plates on which their number did not exceed 50. The results were expressed in colony-forming units (CFU). Along with the total number of colonies, the numbers of colonies of particular colonial morphotypes were determined. From three to five colonies of each morphotype were isolated in pure cultures and identified to a genus or species level.

The survival rate of bacteria that tolerated soil drying was evaluated as the ratio (expressed as a percent) of the numbers of colonies of particular morphotype grown from a soil sample before and after its drying.

The identification of bacterial isolates. Cell morphology (shape, size, motility, and the presence of spores) and Gram staining were studied by conventional methods. The morphology of colonies was studied by taking into account their pigmentation, consistence, size, the excretion of water-soluble pigment, and the formation of extracellular slime. The physiological properties of bacterial isolates were studied according to the handbook [7]. The ability of the isolates to assimilate carbon sources (glucose, fructose, sucrose, lactose, arabinose, galactose, acetate, citrate, lactate, succinate, oxalate, methanol, ethanol, dulcitol, mannitol, and glycerol) was studied by growing them in liquid MM medium and on MMA. The taxonomic position of the isolates was determined to a genus or species level using the identification criteria of Bergey's Manual [8] or, in the case of methylotrophic bacteria, according to Romanovskaya *et al.* [6].

The study of the effect of gamma radiation on the survival rate of pure bacterial cultures. Aliquots (1 ml) of the cell suspensions of soil bacteria were placed in 2-ml Eppendorf tubes and exposed to gamma radiation in a device manufactured by Minsredmash (USSR). The source of gamma radiation was ^{60}Co. The radiation dose was varied from 0.5 to 9 kGy at a dose rate of 0.05 Gy/s.

The survival rate of bacteria exposed to gamma radiation was determined in the same way as during the study of the effect of soil drying. The radioresistance of bacterial cells was characterized by the following parameters: D_q (the threshold dose, which characterizes the capability of cells for repair), LD_{90} (the radiation dose that kills 90% of exposed cells), and $LD_{99.99}$ (the radiation dose that kills 99.99% of exposed cells). D_q was determined from the quasishoulder value on the dose–response curve [2].

The study of the effect of desiccation on the survival rate of pure bacterial cultures. This effect was studied by the method of Mattimore and Battista [1].

RESULTS

The structure of soil microbial communities. The total number of bacteria isolated from soil samples on GPA medium varied from 0.8×10^7 to 6×10^7 CFU/g soil, whereas the number of methanotrophic bacteria detected on MMA medium varied from 0.02×10^4 to 3×10^4 CFU/g soil. The major bacterial taxa revealed in the soddy podzolic soil are listed in Table 1. Soil samples taken from different sampling sites virtually did not differ in bacterial diversity and the abundance of particular bacterial taxa. For this reason, this paper presents only the results of the microbiological analysis of two soil samples taken from the 0–2 and 3–4 cm soil horizons of one sampling site (Fig. 1).

Table 1. Chemoorganotrophic bacteria detected in the undried and dried soil samples collected in the environs of Kiev from the 0–2 and 3–4 cm soil horizons

Chemoorganotrophic bacteria		
detected in undried soil samples	detected in dried soil samples	not detected after soil drying
The class Proteobacteria		
Pseudomonas sp.		*Pseudomonas* sp.
Enterobacter sp.		*Enterobacter* sp.
	Myxococcus sp.	
Bacillus brevis		
B. subtilis		
B. polymyxa		
B. cereus		
	Bacillus sp.	
*Methylobacterium extorquens**		
The class Actinobacteria		
Streptomyces sp.		
	Nocardioforms	
	Coryneforms	

Note: *M. extorquens* was detected on MMA medium. All other chemoorganotrophic bacteria were detected on GPA medium.

As can be seen from the data presented in Fig. 1, the major genera of soil bacteria were *Enterobacter*, *Bacillus*, *Pseudomonas*, and *Streptomyces.* The use of the selective MMA medium allowed us to detect the minor methylotrophic species *Methylobacterium extorquens*, whose abundance in the soil studied did not exceed 10^4 CFU/g soil. The detection of minor bacterial species in soil can also be facilitated by exposing soil samples to various stress factors, such as desiccation.

The effect of soil sample drying on the survival rate of indigenous bacteria. The drying of soil samples for 42 days at $a_w = 0.09$ influenced both the qualitative and quantitative composition of the soil bacterial complex. In this case, the total number of bacteria decreased from $(0.8–6.0) \times 10^7$ to $(4–9) \times 10^7$CFU/g soil. Some bacterial genera that dominated in the undried soil samples (as *Pseudomonas* and *Enterobacter*) were not detected in the dried soil samples (Table 1, Fig. 1). On the other hand, the drying of soil

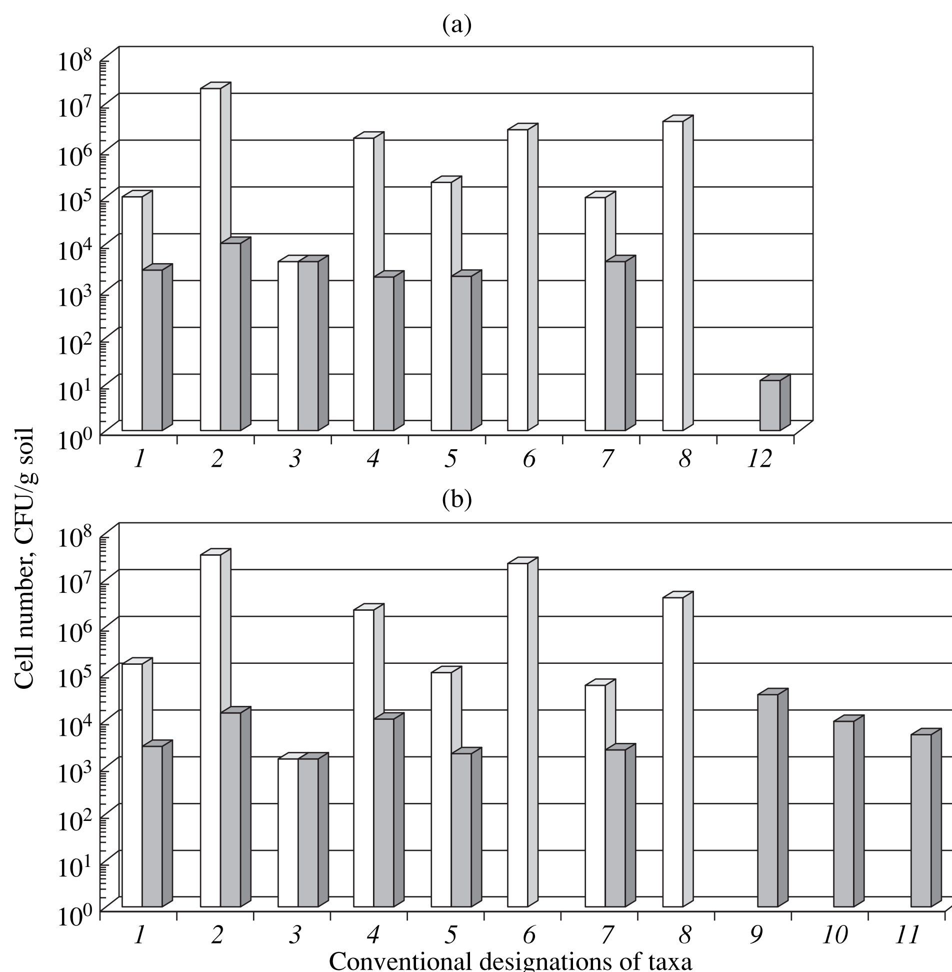

Fig. 1. The number of cells of particular bacterial species before (open bars) and after (shaded bars) drying of soil samples taken from (a) the 0–2 cm and (b) 3–4 cm soil horizons: (*1*) *B. subtilis*, (*2*) *B. brevis*, (*3*) *M. extorquens*, (*4*) *Streptomyces* sp., (*5*) *B. polymyxa*, (*6*) *Pseudomonas* sp., (*7*) *B. cereus*, (*8*) *Enterobacter* sp., (*9*) *Bacillus* sp., (*10*) coryneforms, (*11*) nocardioforms, and (*12*) *Myxococcus* sp.

samples allowed some minor bacteria to be easily detected in soil (*Myxococcus*, coryneforms, nocardioforms, and *Bacillus* sp.). This can be explained by the fact that cells of minor bacterial species are absent in soil suspension dilutions of 10^{-4} and 10^{-5}, whereas their growth in lower-level soil suspension dilutions is masked by the growth of abundant species. The drying-induced decrease in the total number of bacteria in soil by 2–3 orders favors the detection of minor species of soil bacteria.

It should be noted that undried soil samples from different horizons did not exhibit noticeable differences in the species diversity of soil bacteria, while dried soil samples did. As a rule, the lower soil layer (3–4 cm in depth) contained a greater number of minor bacterial species than did the surface soil horizon.

Drying virtually did not influence the number of viable *Methylobacterium* cells in soil (Fig. 1), which is likely due to the high resistance of bacteria of this genus to various DNA-damaging stress factors, such as gamma and UV radiation, hydrogen peroxide, and dehydration [2, 9]. Drying did not change the diversity of *Bacillus* species in soil but diminished the number of viable cells of this genus.

The estimation of the relative number of particular bacteria in soil samples before and after their drying showed that the percent of some bacteria (e.g., *M. extorquens* and *Bacillus cereus*) drastically increased as a result of drying (Fig. 2a). The percent of cells of *Myxococcus* and *Bacillus* sp., which were not detected in the undried soil samples at all, was as high as 25 and 42%, respectively, in the dried soil samples (Figs. 2a, 2b). In contrast, the percent of cells of the *Enterobacter* and *Pseudomonas* genera in soil decreased from 14–35% before drying to thousandths of a percent after drying (such content of cells is insufficient for detecting them by the conventional agar plate method). These data are in agreement with the observations of other researchers that soil drying leads to a decrease in the relative number of gram-negative bacte-

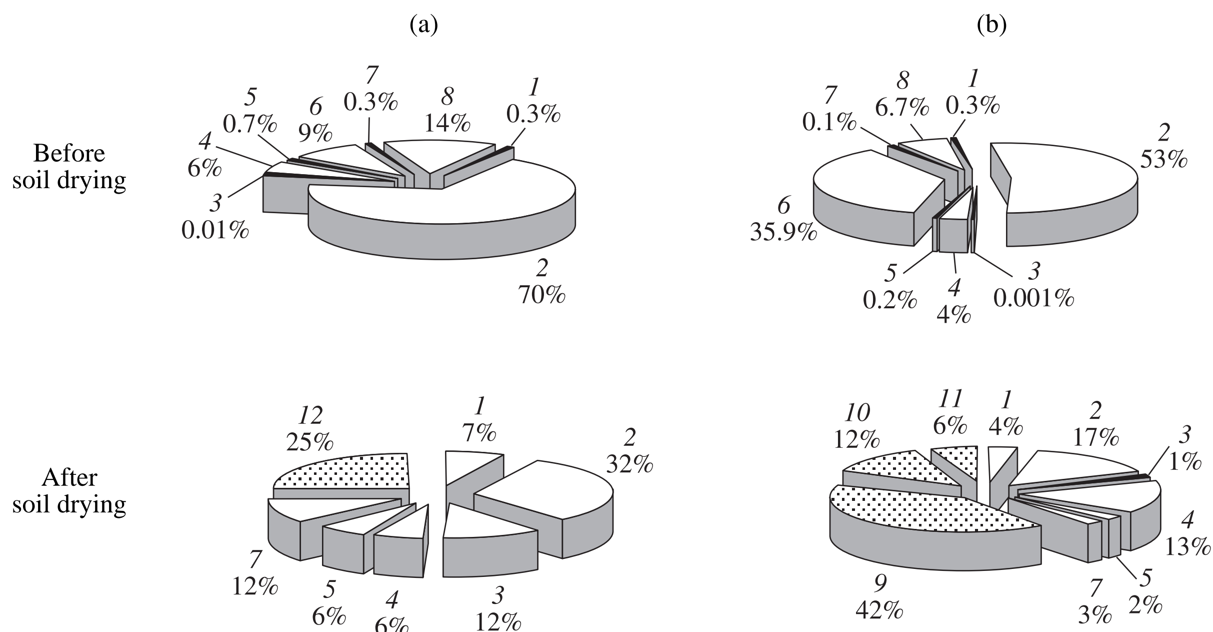

Fig. 2. Percent of cells of particular bacterial species that survived the drying of soil samples taken from (a) the 0–2 cm and (b) 3–4 cm soil horizons. Species designations are the same as in the legend to Fig. 1. The surface-shaded pie chart segments correspond to the bacterial species that were detected only after soil drying.

ria, to an increase in the relative number of gram-positive bacteria and in the relative abundances of actinobacteria and bacilli, and to a reduction of bacterial diversity [10]. In general, our experiments showed that soil drying diminished the percent of soil bacteria that were dominant in undried soil samples and increased the percent of cells of minor species. Moreover, after soil drying, some minor species became dominant.

Bacterial resistance to gamma radiation. This set of experiments was carried out taking into account two earlier observations: (1) some radioresistant bacteria are resistant to desiccation as well [9] and (2) dehydration and gamma radiation cause the same DNA lesions in the exposed bacteria [1, 11]. Experiments were performed with the bacterial species isolated from dried soil samples. The LD_{90} and $LD_{99.99}$ of gamma radiation for *M. extorquens, Bacillus subtilis*, and *B. cereus* reached 2.8–4.6 and 6.1–9.0 kGy, respectively (Table 2). *Myxococcus* sp. was less resistant to gamma radiation. The analysis of the dose–response survival curves presented in Fig. 3 showed that the threshold doses of gamma radiation for *Methylobacterium* and *Bacillus* were high (D_q = 1.7–2.0 kGy) (Table 2), indicating the functioning of active DNA repair systems in these bacteria. In spite of the fact that the threshold doses of gamma radiation for *Methylobacterium* and *Bacillus* were the same, bacteria of the latter genus were more radioresistant than bacteria of the former genus, as is evident from the higher $LD_{99.99}$ values of bacilli (Fig. 3, Table 2). It is likely that bacilli have

Table 2. Resistance of bacteria isolated from dried soil samples to gamma radiation and desiccation

Species	Laboratory strain designation	Gamma radiation, kGy			Percent of cells that survived desiccation
		D_q	LD_{90}	$LD_{99.99}$	
Methylobacterium extorquens	19P	1.7	2.8	6.1	60
	M8	2.0	3.1	6.5	94
Bacillus cereus	5D	1.4	3.3	9.0	80
Bacillus sp.	18K	1.4	3.6	>9	64
B. subtilis	P21	1.7	4.2	>9	95
	11A	2.0	4.6	>9	73
Nocardioforms	9-5H	0.2	0.6	2.0	13
Myxococcus sp.	24K	0.3	0.8	2.4	33

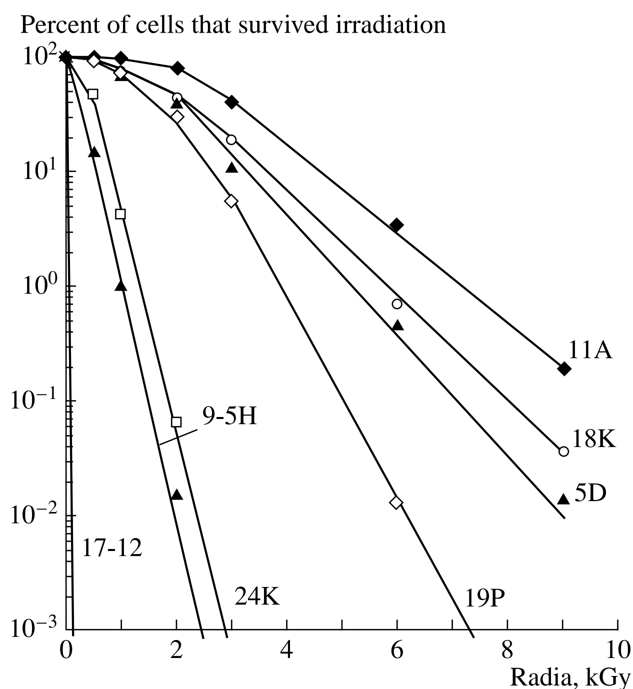

Fig. 3. The effect of gamma radiation on the survival rate of bacteria isolated in pure cultures from dried soil samples. For the designation of bacterial strains, see Table 2. 17-12 is the radiosensitive laboratory *Pseudomonas* sp. strain that was investigated earlier.

evolved more efficient mechanisms of DNA repair than methylobacteria have. The experimental data show that the bacteria that are resistant to desiccation are resistant to gamma radiation as well.

Furthermore, soil drying diminishes the total number and diversity of bacteria detected in soil samples and makes easier the detection of minor bacterial species, which allows soil bacterial diversity to be determined more adequately. Bacteria isolated from dried soil samples are resistant to gamma radiation, suggesting that the drying of soil samples may simulate the effect of various DNA-damaging stress factors on natural bacterial populations.

DISCUSSION

The survival strategy of bacterial cells exposed to the action of gamma radiation, desiccation, and other DNA-damaging factors lies in the activation of cellular mechanisms that either minimize the damaging effect of these factors or repair the induced DNA lesions. The damaging effect of stress factors is minimized, for instance, through the development of pigmentation or the formation of resting cell forms, such as spores and akinetes. *B. subtilis* spores are protected from the action of hydrogen peroxide, artificial UV-B, UV-C, and solar UV light by the protein layer of the spore coat [12]. These spores also contain some DNA-bound proteins, which protect spore DNA from lesions induced by desiccation, UV light, and hydrogen peroxide, but not by ionizing radiation [13]. Mal'tsev *et al.* [14] showed that the sublethal dose of gamma radiation for *B. subtilis* spores is 8.9–9.0 kGy, which is of the same order of magnitude as the sublethal dose of gamma radiation for the vegetative cells of this species (Table 2). Consequently, spore-forming bacteria are likely protected from the action of gamma radiation by the mechanisms that not only minimize cell damage but also repair the induced DNA lesions.

As for the protective function of pigments (particularly carotenoids), this is beyond doubt, as is evident from the fact that pigmented bacteria are fairly resistant to artificial and solar UV radiation. Carotenoids are very efficient antioxidants scavenging reactive oxygen species. All of the known radioresistant bacteria are highly pigmented (*Deinococcus* and *Methylobacterium* strains contain carotenoids [15, 16], whereas *Halobacterium salinarium* and *Rubrobacter radiotolerans* contain bacterioruberin [17, 18]). The nonpigmented *H. salinarium* mutant that is unable to synthesize bacterioruberin is more sensitive to gamma radiation, UV light, and hydrogen peroxide than is the wild-type strain [17]. On the other hand, a comparative study of the wild-type and the mutant *Deinococcus radiodurans* and *Sarcina lutea* strains defective in carotenoid synthesis showed that carotenoid pigments likely do not protect bacterial cells from gamma radiation [19]. Thus, there is no agreement in the literature as to the protective role of carotenoid pigments against gamma radiation.

The DNA repair system is a more efficient mechanism of cell protection from gamma radiation than pigmentation. This survival strategy is used by extremely radioresistant bacteria (as *D. radiodurans*) [20] and pink-pigmented facultative methylobacteria. *Methylobacterium* strains are characterized by high threshold doses of gamma radiation, which implies that the activity of the DNA repair system in these strains is high. The analysis of the dose–response curves of *Methylobacterium* strains exposed to gamma radiation also shows that their radioresistance is due to the functioning of the active DNA repair system. The same is likely true for all non-spore-forming extremely radioresistant bacteria (as *D. radiodurans*) [20], whereas spore-forming bacteria accomplish the survival strategy of cell damage minimization.

The evolution of the mechanisms of bacterial radioresistance has long attracted researchers' interest. The existence of extremely radioresistant bacteria seems to be evolutionally unjustified, because these bacteria can withstand radiation levels that have never existed on Earth, even at the early stage of its development. In this work, we proceeded from the fact that there is a correlation between the resistances of bacterial cells to dehydration and ionizing radiation. It should, however, be noted that there is no direct evidence that the high resistance of bacteria to desiccation

is beneficial for their survival at high radiation levels and favors the selection of radioresistant bacteria in nature, although in fact many radioresistant bacteria were isolated from very dry environments with intense insolation, such as rocks in the Mohave Desert and weathered granite in the Dry Valleys in Antarctica. Like gamma radiation, desiccation causes DNA fragmentation in some bacteria [1, 11]. As was already mentioned, there is a correlation between the resistances of bacterial cells to radiation and desiccation [1]. The bacterial strains isolated from dried soil samples during this work, as a rule, were resistant to both desiccation and gamma radiation. All this confirms the hypothesis [1, 20] that desiccation is the primary mechanism of formation of bacterial resistance to radiation in nature.

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