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THE EFFECTS OF LOW DOSES OF GAMMA RADIATION ON CELL SIZE PARAMETERS OF SOIL BACTERIA

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The changes of cell size parameters of Gram-negative *Pseudomonas aeruginosa* GRP3 and Gram-positive *Bacillus subtilis* AK3 soil bacteria during 30 *min* irradiation with low 7.2 *mGy* dose of Cs^{137} γ-rays with 2.56 $\mu W \cdot m^{-2} \cdot s^{-1}$ intensity were investigated. The obtained results have shown that the first 15 *min* irradiation was very stressful, and bacterial size parameters in both cases were increased, but during the next 15 *min* irradiation the size parameters were decreased approaching their initial values. Therefore, even though only very high doses of gamma radiation could be lethal for these bacteria, they are also sensitive to low doses. The results obtained allow to develop a new method for monitoring the level of radioactive contamination, based on membranous alterations in soil bacteria.

Keywords: gamma radiation, *Bacillus subtilis* and *Pseudomonas aeruginosa*, cell size.

Introduction. Today, in spite of the presence of huge amount of literature data about the dangers of radioactivity, the world continues to use radioactive materials as a powerful source of energy, and these operations contain risks for environmental pollution and following consequences. Solution of practical environmental objectives is not possible without a strong scientific basis for assessing the biological effects.

Prokaryotes are an interesting group of microorganisms. They possess intrinsic properties, such as reduced generation time and low cost of culture and maintenance, so they can be used as a tool for the scientific investigations to obtain important parameters [1], for example, the use of bacterial cells as biosensors for monitoring ionizing radiation. *Pseudomonas aeruginosa* and *Bacillus subtilis* are part of natural microbiota of different environments, particularly, of soils [2, 3], and could be used as sensors for monitoring the environmental radioactivity levels resulting from the release of radioisotopes into the environment from the nuclear wastes, mining activity, etc [1]. It is interesting to observe the effects of low doses of radiation, since in biology the effects of low doses are important, because even a

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small dose can cause alterations in cells at membranous levels, as shown for low intensity non-ionizing electromagnetic radiation [4].

The bacterial cell envelope consists of the cellular structures which surround the cytoplasm; cell membrane is a part of the cell envelope and, in addition of DNA, can be a target for ionizing radiation [5]. The Gram-negative bacterial envelope consists of inner and outer membranes with the periplasmic space between the membranes and of a thin layer of peptidoglycan in the periplasm. The Gram-positive cell envelope consists of the cytoplasmic membrane and tightly associated thick peptidoglycan layer. In the peptidoglycan layer there are interspersed teichoic acids, which are highly charged molecules and contribute to the overall net negative charge of the envelope. They may also be important for structural integrity, although this role has not yet been fully described. Bacterial envelope, in addition to serving as barrier, is intimately involved in critical cellular processes, including energy generation, cell division, assembly of macromolecular complexes, transport of nutrients into the cell, and export of molecules out of the cell. The cell envelope is also actively remodeled in response to the environment, during development. Stress responses of the cell envelope ensure the proper functioning of the envelope components and facilitate adaptation to changing environments. As shown in literature, common stressors, such as heat, ethanol, oxidative stress, and starvation, affect whole cells and often activate both cytoplasmic and cell envelope stress responses. Meanwhile, more specific stresses, such as treatment with chemicals that inhibit cell envelope processes, activate cell envelope and non-cytoplasmic stress responses, because the envelope is physically separated from the transcriptional machinery by the cytoplasmic membrane [6]. But all living beings are built up of molecules, all biological reactions have to be molecular, making life to be a molecular phenomenon [7].

Ionizing radiation has sufficient energy to remove electrons from atoms and molecules and convert them into ions. Further reactions of ions and electrons give rise to the formation of free radicals that are usually highly reactive, which eventually leads to changes in the system [8], such as envelope stresses in case of bacteria. The bacteria have different stress response mechanisms. Cell envelope stress responses, generally, fall into one of two major signaling modules: extra-cytoplasmic function σ factor / transmembrane anti-σ factor units and two component signaling systems. The central place in these signal transduction cascades is occupied by cytoplasmic membrane proteins that span both compartments and, therefore, are able to interact with inducing signals from the envelope and transcription factors in the cytoplasm [6]. As a result, changes occur in the activity of membrane enzymes and, therefore, in the membrane permeability for different ions and molecules, which, in turn, leads to changes in the size of bacteria.

The aim of this study was to investigate the changes of cell size parameters of Gram-negative *P. aeruginosa* and Gram-positive *B. subtilis* during 30 *min* irradiation with low 7.2 *mGy* dose of Cs^{137} γ-rays.

Materials and Methods.

Bacteria and Growth Conditions. P. aeruginosa GRP3 *and B. subtilis* AK3 wild type strains were used for experiments. The bacteria were grown aerobically in 13% Nutrient broth ("Hi-Media", India,) with shaking (150 *rpm*) at 37º*C* (*B. subtilis*) and 30º*C* (*P. aeruginosa*) until stationary growth phase during 18–20 *h*.

Bacterial growth was monitored by measuring the light absorbance of the cells suspension (optical density). The optical density was determined by UV-Vis Auto spectrophotometer ("Labomed Co.", USA). The pH of the nutrient broth was 7.2, as measured by a pH-potentiometer with an ion selective electrode (HJ1131B, "HANNA Instruments", Portugal) [9].

Irradiation and Estimation of Bacterial Sizes by Observing Cells under the Microscope. The biomass was harvested by centrifugation (15 *min*, 6000 *rpm*), washed and diluted in distilled water. Then a thin film of bacterial suspension (density was $\sim 2 \cdot 10^8$ cell), the so-called smear, was spread over the surface of a slide (after dilution in 2 times), but did not dried (alive preparation), and covered with a thin coverslip. Then the slide was fixed under microscope and covered with radioactive source Cs^{137} with 2.56 $mW \cdot m^{-2} \cdot s^{-1}$ intensity [10]. They were subjected only to γ rays and irradiated for 5, 10, 15, 20, 25 and 30 *min*. The photo-pictures of irradiated living cells were taken every 5 *min*. During irradiation, the microscope chamber was covered with a lead slide.

Computational Analysis. Computational analysis was performed based on the grain analysis method using images obtained using a light microscope with a digital camera Jame (Japan). The obtained images were analyzed using LabView and NOVA computer software programs. The grain analysis mode comprised a source image, a section of the source image, a table of geometrical parameters of the bacterial cell (area, average size, perimeter, length, volume, etc.) and a histogram of the distribution density of one of the parameters of grains [9].

To calculate a three-dimensional parameter, the bacterial cells were considered to be cylinders with two hemispherical caps, and the volume was then calculated based on the two-dimensional parameters obtained by image analysis. Assuming this model, three sets of equations (algorithms) were presented to calculate the area, perimeter, and average size, as described by Massana et al. [11].

Determination of Cell Size Parameter. The bacterial size parameters (area, perimeter, average size) were calculated by computational analysis (Lab View and NOVA), as described above. These changes were very sensitive and statistically valid. The general parameter of size – the shape of bacteria (α) was computed as: $\alpha = S/P^2$, where S is the surface area of individual bacterial cell and P is its perimeter (i.e. 2D projection of bacterial cell perimeter in photo document) [8].

Data Processing. Each experiment was repeated at least three times, and the resulting error bars were presented. Standard error was calculated using Microsoft Excel 2013. Student's t-test (p) was employed to validate the difference between the average data from independent series of experiments (radiated cells) and the appropriate controls (nonradiated cells), as described previously [12, 13].

Results and Discussion. During the experiments the photos of samples were taken every 5 *min* of irradiation for 30 *min*. The irradiated samples were compared with non-irradiated control. As shown in Fig. 1, the bacterial size parameters of *P. aeruginosa* were changed during irradiation. Particularly after 15 *min* irradiation the area, average size and perimeter were increased by ~13%, ~8% and ~10%, respectively ($p<0.05$), but α was decreased by ~10%. After treatment with γ-rays for 30 *min*, the bacterial size parameters did not differ significantly from those of non-irradiated controls (see Fig. 1).

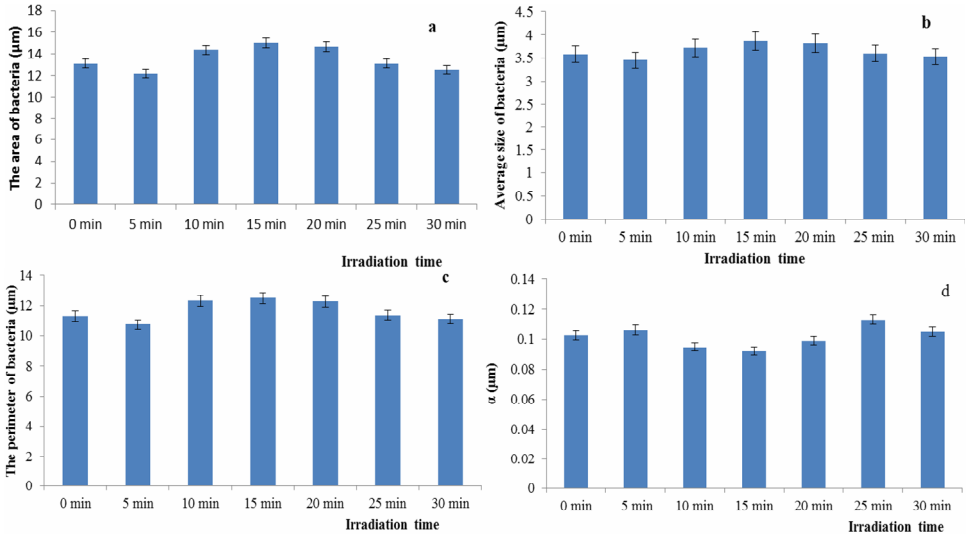

Fig. 1. The alterations of cell size parameters: area (a), average size (b), perimeter (c) and α (d) ($p<0.05$) of the Gram-negative bacteria *P. aeruginosa* GRP3 irradiated for 5, 10, 15, 20, 25 and 30 *min*. For details, see Materials and Methods.

As shown in Fig. 2, the bacterial parameters, viz. area, average size and perimeter were changed during irradiation. Particularly after 10 and 15 *min* irradiations, these parameters were increased by ~30%, ~20%, ~15% ($p<0.05$) and ~10%, ~30%, ~20%, respectively ($p<0.05$), but α was decreased by ~40% and ~30%, respectively. After 30 *min* irradiation, the same parameters were changed by ~16%, ~8% and ~15%, respectively, but α was decreased by ~20%.

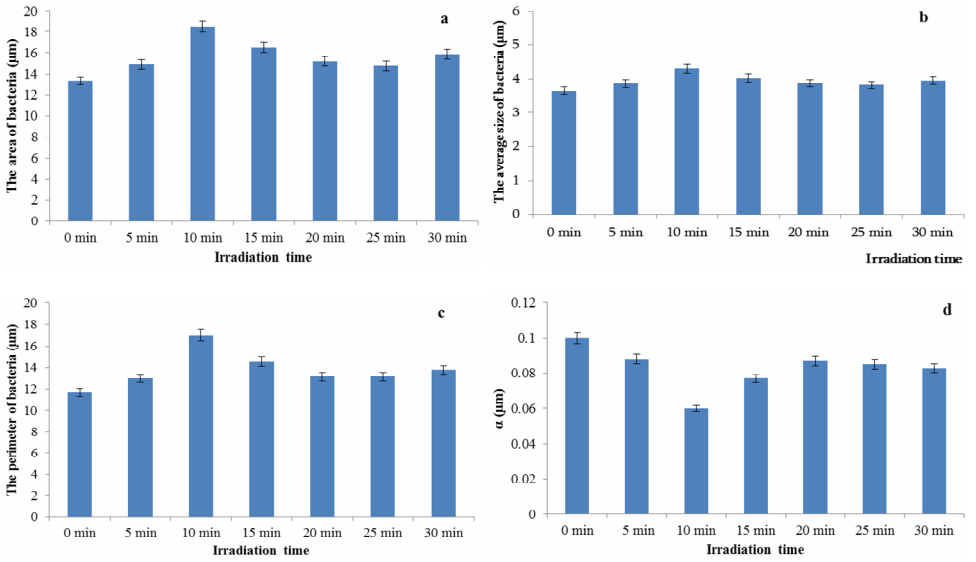

Fig. 2. The alteration of cell size parameters: area (a), average size (b), perimeter (c) and α (d) ($p<0.05$) of the Gram-positive bacteria *B. subtilis* AK3 irradiated for 5, 10, 15, 20, 25 and 30 *min*. For details, see Materials and Methods.

Cell viability highly depends on integrity of cellular membrane that is cooperatively associated with the initiation and regulation of cell processes that are related with cell surface. The bacterial cell envelope is the first and major line of defense against environmental threats. It is an essential and yet vulnerable structure that gives the cell its shape and counteracts the high internal osmotic pressure. It also provides an important sensory interface and molecular sieve, mediating both the information flow and the controlled transport of solutes [5–7, 14]. The cell envelope is also a target for different environmental physical factors, such as radioactive irradiation. As we know, the microorganisms are divided into two groups in response to Gram staining: Gram-positive and Gram-negative. We have investigated the effects of γ-rays on both Gram-positive (*B. subtilis*) and Gram-negative (*P. aeruginosa*) bacteria.

The bacteria were grown until stationary phase, and therefore, their membrane formation was completed. Although the cell size parameters changed during 15 *min* irradiation, but then these parameters after 20, 25 and 30 *min* irradiation were close to control values (see Fig. 1). As shown for *Thermococcus gammatolerance,* after exposure to high doses of *γ*-irradiation, the stationary phase bacteria reconstitute damaged chromosomes more rapidly than exponential phase bacteria [15]. Cytoplasmic membranes are essential for the cell integrity, providing a barrier between the inside and outside environments for the cell [16]. These barriers act as support for different proteins that are involved in several different cell functions, such as signal transduction, solute transport, protein targeting and trafficking, etc. [17]. A number of studies suggest that membranes can sense extreme environmental changes and, in particular, the presence of reactive oxygen species in the media, as reported in several works [18, 19]. Previous investigations have revealed that Gram-positive bacterial strains are more resistant to *γ*-radiation than Gram-negative strains. Importantly, pathogenic bacterial strains follow the following descending order pattern: *B. cereus* > *Staph. Aureus* > *Strept. facecalis* > > *Salmonella* sp. > *P. aeruginosa* > *E. coli*. The difference between Gram-positive and Gram-negative cells may be explained on the base of differences between them in the cell wall structure. Gram-positive bacteria have membrane, surrounding the cell, and cell wall, primarily made up of peptidoglycan layer. This cell wall is rich in sulfur compounds, they protect the cells from harmful *γ*-radiation and become resistant. Sulfur compounds found in the cell wall of Gram-positive bacterial cell make a scavenger for free radicals and protect the cells [20, 21].

It is known that exposure of bacterial cells to ionizing radiation creates an additional stress to the cells, which tends to disturb their organization. It should be considered that the bulk of the literature data were obtained for high doses of radiation, for example, the viable count of *Pseudomonas aleovorans* completely reduced by 3.0 *kGy γ*-radiation, and 10.0 *kGy* dose completely reduced the viable count of *Bacillus sp*. MAM-40; the main target for these high doses are nucleic acids [2]. In our experiments, very low dose (7.2 *mGy*) of *γ*-radiation was used, and it is interesting that these low doses affect the bacterial cells, particularly their morphological parameters. The obtained results have shown that first 15 *min* irradiation was very stressful, and bacterial size parameters in both cases were increased, but during next 15 *min* irradiation the size parameters were decreased close to their initial values. This can be explained by that during this short time of

irradiation, the cell membrane, in particular, membrane enzymes, is activated, with the subsequent activation of the stress response mechanisms, as mentioned above [6], which lead to the restoration of bacterial sizes.

Therefore, even though only very high doses of γ-radiation could be lethal to these bacteria, they are also sensitive to low doses. The results obtained allow to develop a new method for monitoring the level of radioactive contamination, based on membranous alterations in soil bacteria.

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