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EXPERIMENTAL ARTICLES

Effect of *Bacillus pumilus* Ribonuclease on the Paramagnetic Centers of Microbial Cells

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Abstract—The potential clinical application of *Bacillus pumilus* cytotoxic ribonuclease (binase) for selectively inducing the death of tumor cells makes it imperative to investigate its effect on the normal human microflora. Flow cytometry was used to determine that binase concentration causing the apoptosis of cancer cells had no effect of the viability of *Escherichia coli* K12. The changes in the paramagnetic centers of *E. coli* K12 cells in the presence of nontoxic binase concentrations revealed by EPR spectroscopy included higher EPR signals from iron-containing proteins (including those from the Fe–S clusters) and of the Mn(II) hyperfine structure. The TMTH spin probe (*N*-(1-hydroxy-2,2,6,6-tetramethylpiperidine-4-il)-2-methylpropanamide hydrochloride) was used to reveal a twofold increase in the levels of reactive oxygen species (ROS) in the cells, which induced oxidative stress in the enzyme-treated bacteria. Inductively coupled plasma mass spectrometry revealed elevated contents of alkaline (Li, Na, K), alkali earth (Mg, Ca), transition (Cr, Mn, Fe, Cu, Zn), and post-transition metals (Bi, Pb) in the cells. Elevated levels of Cu and Zn (which impair the activity of the respiratory chain enzymes) and of Mn, which is known as a superoxide dismutase cofactor, confirmed development of the oxidative stress in bacteria.

Keywords: cytotoxic ribonucleases, *Bacillus pumilus*, binase, intestinal microflora, EPR, ROS, oxidative stress, transition metals

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Bacterial ribonucleases (RNases) possess a broad range of biological activity [1]. Secreted bacterial RNases may affect the metabolism of prokaryotes [2] and mediate certain reactions in eukaryotes [3]. Microbial RNases, and specifically *Bacillus pumilus* 3-19 RNase (binase), were reported to induce selective apoptotic death of malignant cells while not affecting non-malignant cells [4]. Potential clinical application of binase in chemotherapy of the tumors, including intestinal ones, requires better understanding of its biological effects toward the normal human intestinal microflora. It has been shown previously that binase in low concentrations stimulated microbial growth while high concentrations (about 1 mg/mL) of the enzyme resulted in growth inhibition [5]. The range of binase concentrations efficiently inducing malignant cell apoptosis varies from 0.1 to 0.75 mg/mL [6, 7]. These concentrations do not affect bacterial growth but may cause certain changes in the viability of microbial cells which may remain hidden for some time. The electron transport chain enzymatic complexes that are located in the cytoplasmic membrane may even respond to insignificant changes in the environment [8]. EPR analysis which detects the quantitative and qualitative composition of the paramagnetic centers within electron transport chains—both in the bacterial cytoplasmic membrane and in the membranes of the eukaryotic chloroplasts and mitochondria—makes it possible to characterize the state of these complexes [9].

The goal of the present work was to reveal the changes in the functional state of *Escherichia coli* K12 cells under the action of *Bacillus pumilus* 3-19 ribonuclease in concentrations not causing toxic and growth-stimulating effect by using a combination of the physicochemical analytical techniques.

MATERIALS AND METHODS

Enzyme. Guanyl-specific RNase from the wild type *Bacillus pumilus* 3-19 strain (binase) was used in the study (EC 3.1.27.3, molecular mass 12.3 kDa, 109 amino acid residues, pI = 9.5). The enzyme was isolated as a homogenous protein from the culture liquid of the *Escherichia coli* BL21 recombinant strain bearing the pGEMGX1/ent/Bi plasmid [10].

Culture media and cultivation conditions. Streptomycin-resistant *E. coli* K12 strain (VKPM-3254) was used as the test object. The cells were cultivated in LB broth with 100 μg/mL of streptomycin at 37°C for 18 h. The obtained culture was used for inoculation; it was transferred into the fresh culture medium (final cell concentration 1×10^6), incubated at 37°C for 24 h in the presence (100 or 300 μg/mL) of binase or without the enzyme, then the cells were centrifuged

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(2600 *g*, 20 min), resuspended in sodium phosphate buffer (PBS, Sigma, United States) at the concentration of 3×10^9 CFU/mL, and were used for the preparation of EPR samples.

Cytofluorimetric analysis of the viability of *E. coli* K12. The cultures of *E. coli* K12 grown for 24 h in LB broth with or without binase (as described above) were centrifuged (2600 *g*, 20 min), washed with cool sodium phosphate buffer (Sigma, United States), and the changes in the viability of *E. coli* K12 cells stained with propidium iodide (PI) were studied on a flow cytofluorimeter (FACSCanto II, BD, United States) [11].

EPR characteristics of the paramagnetic centers in *E. coli* K12 cells. EPR characteristics of the cells incubated in the presence of binase were determined using an ESP-300 stationary spectrometer (Bruker, Germany) with the operating frequency of 9.4–9.9 GHz, at magnetic field strength of 20–1600 mT (error not exceeding 0.01 mT), and modulation frequency of 100 kHz at the microwave emission intensity of 2–20 mW. The capillaries with the volume of 25 μL were used (Sigma, United States). The samples for the experiment were prepared in two forms: frozen at 77 K and lyophilized. The cells were lyophilized to complete drying using a Martin Christ dryer (Germany) at –76°C and the pressure of 0.001 bar. Bacterial cells grown without binase, supernatant, and sodium phosphate buffer (Sigma, United States) were used as the controls. The spectra of the frozen samples were registered at 77 K. The lyophilized samples were studied at 15 K using an Oxford-9 helium flow cryostat (United Kingdom); the temperature was controlled using ITC 4 Oxford (United Kingdom).

The changes in cellular ROS content were detected using the cyclic hydroxylamine *N*-1-hydroxy-2,2,6,6-tetramethylpiperedine-4-il)-2-methylpropanamide hydrochloride (TMTH, Institute of Organic Chemistry, Novosibirsk, Russia) as a spin probe. This probe is analogous to those widely used for detection of superoxide by generation of the stable nitroxyl radical [12]. TMTH (1 mM) was added to 1 mL of *E. coli* K12 cell suspension (1×10^7 cell/mL), EPR spectra were registered at room temperature (25°C), at the microwave emission intensity of 1 mW, using 9.72 GHz, high-frequency modulation amplitude of 0.3 G, and 25 μL capillaries.

Content of the metals in *E. coli* K12 cells. Quantitative determination of the metals was performed according to the RF standards and using the relevant methodical recommendations [13]. The measurements were carried out using an Elan DRC II mass spectrometer (Perkin Elmer, United States). The samples were subjected to microwave treatment using MWS-3 (Berghof, Germany) at 150°C, in the presence of nitric acid and double-distilled water.

RESULTS

Cytofluometric assessment of *E. coli* K12 cell viability. Analysis of *E. coli* K12 viability revealed that binase in concentrations toxic for malignant eukaryotic cells did not cause bacterial cell death. The share of nonviable cells with the compromised membrane was 5.9 and 5.4% at RNase concentrations of 100 and 300 μg/mL, respectively, and did not show significant differences with the variant not exposed to binase.

EPR analysis of *E. coli* K12 cell samples. In *E. coli* K12 preparations, several different types of EPR signals were detected. The first type was registered in the frozen samples at g = 2.0. The intensity of this signal in the cells exposed to binase was higher than that in the cells without enzyme exposure (Fig. 1, I, II). The same signal, albeit at a higher intensity, was registered in the lyophilized samples; it increased similarly after binase exposure (Fig. 1, III). The line form of the signal and the g values suggested the source of the signal to be a sulfur-containing radical [14].

The second type of the signal was detected at g = 3.0, and its intensity did not depend on the binase exposure (Fig. 1, I).

The third type of the signal had g = 1.93 and was well distinguished at small sweep range of magnetic field (Fig. 1, II). Its intensity did not correlate with the first type of the signal, although it also increased in the samples exposed to binase. The source of this signal was probably an iron-containing protein [14]. It was not detected in the lyophilized samples.

The fourth type of the signal was represented by six lines of equal intensity in the area of g = 2.0 (Fig. 1, II). These lines corresponded to the EPR spectrum of a Mn^{2+} ion hyperfine structure (HFS) [15]. As the magnetic field increased, the components of the spectral lines widened, and three strong field HFS lines became difficult to detect. The manganese spectrum was well-distinguished in the samples exposed to binase, while in the cells not treated with binase its intensity was significantly lower (Fig. 1, II).

Apart from these signals, two more types of the signals with g factors of 4.27 and 5.84, respectively, were detected. These signals were observed only in the lyophilized samples (Fig. 1, III). Similar signals that had been registered previously in biological systems were referred to as paramagnetic complexes of the iron-containing proteins [14].

To confirm the intracellular localization of the EPR signal sources, we analyzed the buffer used in the study and the supernatants of the experimental and control cultures. All the above types of the signals were shown to be localized exceptionally within the cells (Fig. 1, IV).

Element analysis of *E. coli* K12 cells. The treatment of *E. coli* K12 with sub-bactericidal binase concentration affected the functional state of bacteria and changed their biochemical parameters. To assess the changes in the elemental composition of binase-

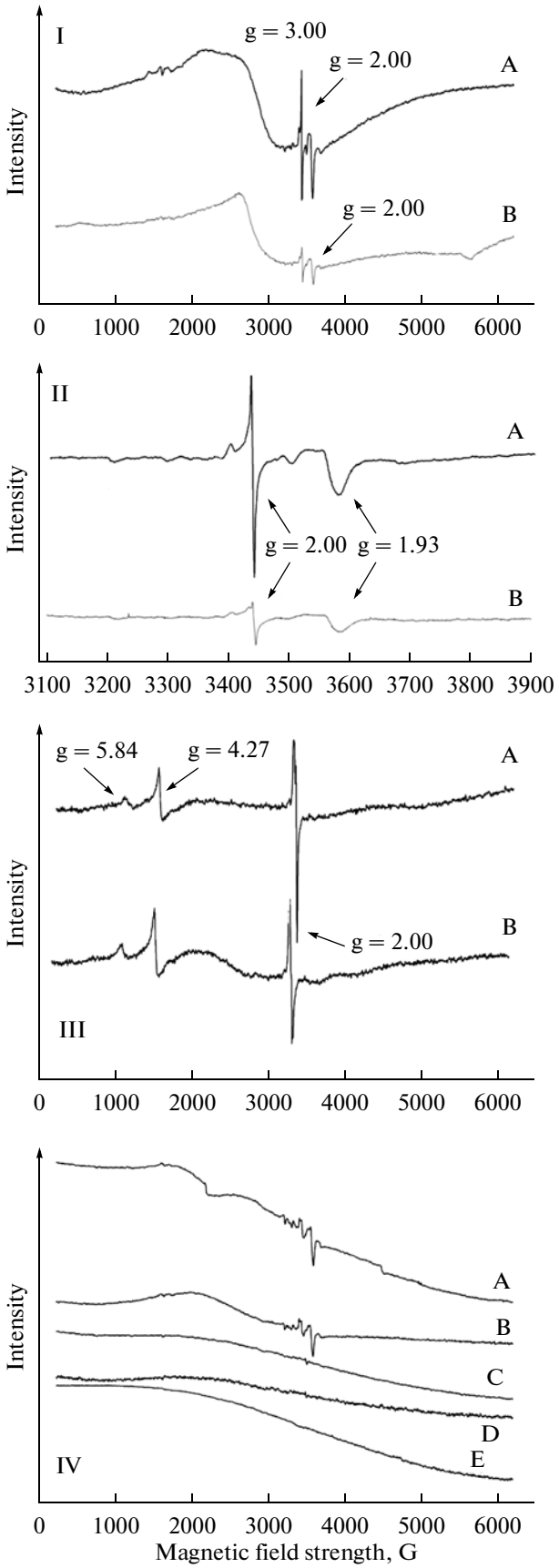

Fig. 1. EPR spectra of *E. coli* K12 cells (operating frequency, 9.63 GHz, microwave emission intensity, 20 mW): I. EPR spectra of the frozen cells in a wide range of magnetic fields at 77 K; II, EPR spectra of the frozen cells in the area of magnetic fields of g = 2 at 77 K; III, EPR spectra of the lyophilized cells in a wide range of magnetic fields at 15 K; IV, EPR spectra of the cells from the negative control and of the supernatant. Bacterial cells after binase treatment (300 μg/mL, 24 h) (A), bacterial cells untreated with binase (B), sodium phosphate buffer (C), supernatant after precipitation of the cells incubated with binase (D), and supernatant after precipitation of the cells untreated with binase (E).

treated *E. coli* K12, we used inductively coupled plasma mass spectrometry. Treatment of bacteria with 100 and 300 μg/mL binase caused an increased levels of alkali (Li, Na, K), alkali earth (Mg, Ca), transition (Cr, Mn, Fe, Cu, Zn), and post-transition (Bi, Pb) metals (table). The most significant increase in the content of transition metals was observed at binase concentration of 300 μg/mL: the quantities of Mn, Cu, and Zn increased 5.9, 4.8, 2.9 times, respectively.

Generation of ROS by *E. coli* K12 cells under binase action. ROS play one of the most important roles in the cell activity. The treatment of bacterial cells with binase caused a significant increase (by 100%) of the EPR signal of the TMTH probe if compared with that of untreated cells (Fig. 2). This suggests an enhancement of ROS generation. Thus, the enzyme may provoke an oxidative stress in *E. coli* K12.

DISCUSSION

Binase in concentrations lethal for malignant eukaryotic cells does not cause the death of bacterial cells. However, analysis of the EPR spectra of lyo-

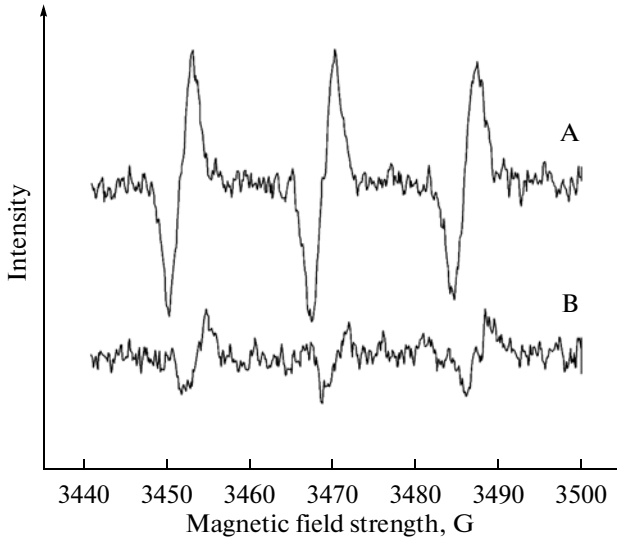

Fig. 2. Spectra of the TMTH spin trap EPR-adducts in *E. coli* K12 cells treated with binase (300 μg/mL, A) and of untreated cells (B).

Alterations in *E. coli* K12 intracellular metal concentrations after binase treatment (100 and 300 μg/mL)

Elements	Metal concentration, ng/1000 cells		
	without binase treatment	binase, 100 μg/mL	binase, 300 μg/mL
Li	0.003 ± 0.0002	0.008 ± 0.0009	0.006 ± 0.0006
Na	0.13 ± 0.0005	0.69 ± 0.001	0.78 ± 0.02
K	0.36 ± 0.007	0.61 ± 0.02	0.63 ± 0.007
Mg	0.414 ± 0.001	1.03 ± 0.07	0.48 ± 0.05
Ca	13.87 ± 0.02	19.97 ± 0.1	21.55 ± 0.06
Cr	0.015 ± 0.0006	0.024 ± 0.001	0.032 ± 0.002
Mn	0.022 ± 0.001	0.11 ± 0.02	0.13 ± 0.005
Fe	0.98 ± 0.01	1.10 ± 0.09	1.19 ± 0.02
Cu	0.096 ± 0.0005	0.25 ± 0.06	0.47 ± 0.05
Zn	0.039 ± 0.0014	0.089 ± 0.007	0.115 ± 0.07
Bi	0.00024 ± 0.00005	0.001 ± 0.0001	0.00099 ± 0.0001
Pb	0.013 ± 0.002	0.16 ± 0.005	0.18 ± 0.009

philized and frozen samples of *E. coli* K12 cells exposed to binase suggested that binase affected the energetic processes in the cell. EPR spectra detected around g = 2 (Fig. 1) belonged to sulfur-containing radicals, and those with the g factor values of 1.93, 4.27, and 5.84 belonged to iron-containing proteins [14]. Therefore, the source of the detected signals could be Fe–S proteins, the major components of prokaryotic electron transport chains and of the electron-transport chains of chloroplasts and mitochondria.

Normally, several paramagnetic Fe–S clusters are detected in the cell, and quantitative and qualitative variations in these clusters reflect the energetic state of a biological system [16]. For example, superoxide anions cause a significant increase in Fe EPR signals intensity in *E. coli* K12 cells resulting from Fe released from the [4Fe–4S]-clusters [17]. In eukaryotic cells a similar process occurs: in *Saccharomyces cerevisiae* cells under superoxide-induced stress conditions, the level of the free Fe increases [18].

Binase in concentrations causing malignant cells death caused a twofold increase in the ROS level in *E. coli* K12 (Fig. 2) and the subsequent development of oxidative stress. Some antitumor agents are known to mediate ROS formation in mitochondria. Thus, enhanced ROS production in the cells of human lung carcinoma A549 in the presence of 5-fluorouracil initiates the release of cytochrome *c* from mitochondria and triggers caspase-dependent apoptosis [19]. Another antitumor agent, apigenin (5,7,4-trihydroxy flavone), also demonstrates cytotoxic action towards A549 carcinoma. Directly affecting the mitochondria, it triggers DNA fragmentation, ROS accumulation, cytochrome *c* release, mitochondrion membrane depolarization, and apoptosis [20].

We have shown previously that binase caused a decrease in the membrane potential of the mitochondria of malignant cells [21]. Taking into account the accepted theory of the origin of eukaryotic mitochondria by endosymbiosis with aerobic bacteria [22], one could suggest that binase affects the electron-transport chain of prokaryotic membranes in a similar. The mechanism of such action probably involves the damaged structure of the Fe–S proteins, components of the electron transport chain, as was confirmed by our results (Fig. 1).

The mitochondrial electron transport chain is the main source of ROS in mammalian cells [23]. It includes four enzymatic complexes, each of which is capable of superoxide generation. NADH dehydrogenase and ubiquinone–cytochrome oxidoreductase generate most of superoxide; the first complex releases superoxide only into the mitochondrial matrix, while the superoxide formed in the second complex is located at both sides of the inner mitochondrial membrane [24]. The study of molecular mechanisms of superoxide production by prokaryotic and mitochondrial enzyme complexes indicated that these processes are significantly similar in these evolutionarily related systems [25].

It may be suggested that oxidative stress provoked by the action of binase on *E. coli* K12 cells resulted from the damage to the electron-transport chain functions. Moreover, enhanced levels of transition metals in *E. coli* K12 cells after binase exposure also contributes to the oxidative stress by activation of the Fenton reaction, by which hydrogen peroxide and transition metals interact [26].

Copper is present in the enzymes catalyzing one-electron transitions and, similar to other transition metals, plays an important role in cell metabolism. The ability of the copper atoms to undergo redox

changes [Cu(I) ⟷ Cu(II)] and to provoke ROS formation in reactions similar to the Fenton reaction are the reasons for the danger associated with the enhanced intracellular concentration of this metal. The release of Fe and Fe–S proteins attacked by Cu(I) may also contribute to the development of oxidative stress [27]. In gram-negative bacteria, Cu-containing proteins are localized in the cytoplasmic membrane at the side of the periplasm or are secreted into the medium, thus limiting the accumulation of this metal into the cytoplasm. Moreover, to avoid the damage caused by copper excess, bacteria and archaea evolved specific detoxication systems [27]. Elevation of the copper content in *E. coli* K12 after binase exposure (table) may be associated with the dysfunction of such systems and with subsequent copper accumulation in the cell. Elevation of the intracellular zinc content is also potentially dangerous for the cell. While Zn(II) is a very important microelement involved in many physiological processes, its excess may damage the structure of biological macromolecules and their complexes [28].

Homeostasis of one more transition metal, Mn, is maintained in bacteria by regulation of ionic transport [29]. Prokaryotes use manganese in the Mn-containing superoxide dismutase involved in ROS detoxication. Some groups of microorganisms can use enhanced concentrations of intracellular Mn not included in the superoxide dismutase in order to protect themselves from the superoxide anion, although the underlying mechanisms are poorly studied [29, 30]. Thus, elevation of Mn content in *E. coli* K12 cells after binase exposure, which was revealed by both EPR (table) and element analysis (Fig. 1, II), is associated with the activation of the systems protecting the cell from oxidative stress caused by elevated levels of Cu and Zn.

Thus, an effect of *B. pumilus* 3-13 ribonuclease on the paramagnetic centers of microbial cells was revealed in this study. Binase in the range of concentrations that cause tumor cells apoptosis was demonstrated to alter the EPR signals in *E. coli* K12, enhancing the intensity of the signals from the iron- and sulphur-containing proteins. This indicates the structural changes in the electron transport chain enzymes and their impaired functioning. Twofold increase in the ROS generation level after binase exposure was associated with a significant increase of the intracellular content of transition metals (Zn and Cu) that may induce oxidative stress, and by elevated content of intracellular Mn(II) which, in contrast, participates in the oxidative stress protective systems.

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