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Dependence of Protective Functions of *Escherichia coli* Polyamines on Strength of Stress Caused by Superoxide Radicals

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Abstract—Mechanisms of antioxidant effect of polyamines were studied in dependence on the strength of superoxide stress. Under conditions of weak stress, polyamines from *Escherichia coli* cultures were shown to function mainly as a scavenger of free superoxide radicals, whereas under conditions of strong stress they mainly acted as positive modulators of antioxidant genes. Spectrofluorimetry was used to show that both polyamine-dependent mutants and wild type cells treated with inhibitors of polyamine synthesis contained an elevated amount of free oxygen radicals, which could be decreased to the normal level by addition of exogenous polyamines. Under conditions of strong stress, polyamines positively influenced expression of the *soxRS* regulon genes of antioxidant defense, which was accompanied by an increase in the quantity (activity) of their gene products, such as glucose-6-phosphate dehydrogenase (Zwf) and fumarase (FumC). These effects led to an increase in the number of live cells in the cultures subjected to superoxide stress.

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Key words: polyamines, oxidative stress, superoxide radicals, gene expression, antioxidant enzymes

At present, the problem of oxidative stress seems universal because resulting damage is now more frequently considered to be a consequence of a nonspecific cell response to any stress, a kind of the "suicidal" reaction of the microorganism population to unfavorable environmental factors [1, 2], be it heating or antibiotics [3]. In this sense, oxidative stress is a result of uncoupling of the energy and constructive cell metabolism and "overflow" of excess reducing equivalents in the respiratory chain. Byproducts of the respiratory chain include reactive oxygen species, which are products of one-electron chemical reduction, especially superoxide radicals generated in amount proportional to the rate of the respiratory chain functioning [4]. To deal with the damaging effects of superoxide radicals, microorganisms have developed means for defense organized as the *soxRS* regulon, which is a complex of genes encoding antioxidant proteins induced under the control of the transcriptional regulators SoxR and SoxS [5]. The oxidative stress signal represented by a superoxide radical is initially received by the protein SoxR, which contains [2Fe–2S] clusters. On oxidation of the iron–sulfur clusters, SoxR is activated and manifests features of a transcriptional activator for *soxS*. In turn, SoxS functions as a positive transcriptional regulator of expression of the *soxRS* regulon target genes. Multiple copies of this protein recognize specific sequences of target genes encoding antioxidant proteins and activate their transcription. For normal response to oxidative stress, it is necessary to maintain the integrity of iron–sulfur clusters that can be destroyed under the influence of factors, in particular glutathione, which are accumulated in the cell in response to oxidative stress [6].

The uncoupling of energy and constructive metabolism in *E. coli* is, in particular, displayed by an increase in the activity of key enzymes of polyamine synthesis [7]. Studies on the role of polyamines in the microorganism's defense against oxidative stress have revealed some properties of this class compounds responsible for their functions in the protection of microorganisms against stress-induced damage. A significant elevation of the intracellular level of polyamines followed by their accumulation in the culture medium is one of early reactions of cells to various kinds of oxidative stress [8]. Studies on protective functions of polyamines have shown that the transcrip-

Abbreviations: DAB) 1,4-diamino-2-butanone; G6PDH) glucose-6-phosphate dehydrogenase; PMS) phenazine methosulfate.

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tional modulation of the genes in the antioxidant defense regulons *oxyR* and *soxRS* is a mechanism underlying these functions [9, 10]. Moreover, these compounds can neutralize free oxygen radicals, and this determines their protective effect against the damage of DNA by reactive oxygen species [9, 11, 12]. Based on these studies, polyamines can be considered as compounds responsible for dual functions and capable of mutual influence. It was reasonable to suppose that antioxidant properties of polyamines would weaken the effect of stress due to neutralization of free radicals, which, in turn, should weaken the response to stress and, as a result, decrease expression of the antioxidant genes. However, the possibility must not be ruled out that, under conditions of elevated generation of free radicals during strong stress, polyamines would more distinctly act as positive transcriptional modulators.

Therefore, the purpose of the present work was to study and compare antioxidant functions of polyamines depending on the strength of oxidative stress.

MATERIALS AND METHODS

Objects of the study. *Escherichia coli* strains used in this work are presented in the table, and their genotype and source are indicated. The level of gene expression was determined using the principle of gene *lacZ* fusions prepared by transduction with bacteriophage λ as a vector [13].

Cultivation of microorganisms. Before the experiment, the *E. coli* strains maintained on LB-agar slants were inoculated into LB broth containing streptomycin (25 μg/ml). The cells were cultured for 11 h in a thermostat at 37°C and then transferred into M-9 medium containing streptomycin in the same concentration and cultured at 37°C for 13 h in a shaker at 120 rpm. The resulting culture was used as inoculate for seeding into 250-ml flasks containing 100 ml of M-9 medium with the antibiotic and grown under the same conditions. The *E. coli* strain HT306 was grown on M-9 medium supplemented with thiamine (1 μg/ml), proline (100 μg/ml), and pantothenate (1 μg/ml) [14].

The cell biomass was determined after preliminary dilution of the culture in saline monitored by the optical density (OD_{600}) with an SF-46 spectrophotometer (LOMO, Russia).

Activity of β-galactosidase. The activity of β-galactosidase was determined using Miller's method [15] in cells pretreated with a mixture of SDS (Sigma, USA) and chloroform.

Content of polyamines. The concentration of polyamines was determined by two methods: thin-layer chromatography [16] and HPLC using an LC-10Avp chromatograph (Shimadzu, Japan). The preparation of specimens before the analysis included extraction with subsequent derivatization of polyamines by dansylation [16]. Polyamines were separated on a Luna C18(2) column (250 × 4.6 mm) with particles 5 μm in size (Phenomenex, USA) at 25°C. Water and acetonitrile were flowed through the column at the rate of 1 ml/min with a linear gradient of acetonitrile concentration from 40 to 100% during 35 min, with subsequent equilibration for 10 min with 40% acetonitrile. The dansylated derivatives were detected with an RF-10AXL flow fluorometric detector (Shimadzu) with excitation wavelength of 400 nm and emission at 516 nm. The polyamine concentration was calculated using previously obtained calibration coefficients and compared with the biomass of microorganisms used for extraction of the specimens.

Determination of contents of free oxygen radicals in *E. coli* cells. The level of reactive oxygen species was evaluated by a modified spectrofluorimetric method [17]. The cells ($5 \cdot 10^5$) of exponentially grown *E. coli* culture on M-9 medium were rapidly transferred into 5 ml of the same medium and maintained for 20 min in the dark at 37°C. Then each tube was supplemented with 5 μM 2,7-dichlorofluoresceine diacetate (Sigma), and 30 min later the fluorescence was measured in quartz cuvettes with the optical path length of 10 mm at the excitation wavelength of 480 nm and emission at 521 nm with an RF-1501 spectrofluorimeter (Shimadzu).

Determination of *in vitro* antioxidant properties of polyamines. To determine the properties of polyamines as compounds involved in suppression of free radical reactions, a system of *in vitro* generation of superoxide radicals was used which contained phenazine methosulfate (PMS)–NADH [18]. The reaction was performed at room temperature. The reaction mixture contained

Bacterial strains used in the present work

E. coli strain	Genotype	Source or reference
EH40	GC4468 lysogenised with λEH40 soxS'::*lacZ* (SoxRS+)	Demple, B. [13]
GC4468	DE(*argF-lac*)169 Lam-IN(*rrnD-rrnE*)1 *rps*L1799(*strR*)	–"–
HT306	*thr-1*, *araC14*, Δ*speD98*, Δ(*gpt-proA*)*62*, *lacY1*, *gln V44*(AS), *galK2*(Oc), λ^-, Δ(*SpeB-SpeA*)*97*, Δ(*SpeC-glcB*)*63*, *rpsL25*(strR), *xylA5*, *mtl-1*, *thi-1*, *ampCp-1*, *cadA2*	Tabor, H. [14]

2.5 ml of 21 mM phosphate buffer (pH 8.3), 4 μM PMS (Sigma), 0.7 mM NADH, 17 μM nitro blue tetrazolium, and the corresponding quantity of polyamine. The reaction was initiated by addition of PMS. In 7 min, the amount of produced formazan was determined with a spectrophotometer at 560 nm. The control reaction without polyamine was performed in parallel. The inhibition of the formazan production (in %) was calculated by the formula: $I = 100 - (K_1/K_0 \cdot 100)$, where I is inhibition of the reaction (%), K_1 is the spectrophotometer reading for the experimental specimen, and K_0 is the spectrophotometer reading for the control specimen.

Determination of enzyme activities. *The activity of glucose-6-phosphate dehydrogenase* (G6PDH) was determined by spectrophotometry. The culture of microorganisms was rapidly cooled to 0-4°C, the cells were harvested by centrifugation for 5 min at 10,000*g* using a 5804R centrifuge (Eppendorf, Germany), washed once in saline, resuspended in 0.3 M Tris-HCl buffer (pH 7.5) containing 4 mM $MgSO_4$, and broken by triple ultrasonication for 10 sec with frequency of 22 kHz at 0-4°C. The broken cells were centrifuged for 20 min at 16,000*g*, the supernatant fluid was collected, and, after determination of the protein content by the Lowry method, it was used to perform the enzymatic reaction. The incubation medium contained 100 μl of 15 mM NADP, 50 μl of 1 M glucose-6-phosphate, and 200 μg protein of the supernatant. The final volume (3 ml) was adjusted with buffer. The enzyme activity (mmol/mg protein per min) was determined by the rate of NADPH production, which was recorded with a spectrophotometer at the wavelength of 340 nm.

Activity of fumarase C was determined spectrophotometrically [19]. The culture of microorganisms was rapidly cooled to 0-4°C, the cells were harvested by centrifugation for 5 min at 10,000*g* using a 5804R centrifuge, washed once in saline, resuspended in 50 mM phosphate buffer (pH 7.3), and broken by triple ultrasonication for 10 sec with the frequency of 22 kHz at 0-4°C. The broken cells were centrifuged for 20 min at 16,000*g*, the supernatant fluid was collected, and, after determination of the protein content by the Lowry method, it was used to perform the enzymatic reaction. To differentiate the activity of FumC from other type fumarases, the protein extracts of the cells were preheated at 37°C for 45 min. The incubation medium contained 100 μl of 0.1 M solution of D,L-malate and 200 μg the protein extract. The final volume (3 ml) was adjusted with buffer. The enzyme activity (mmol/mg protein per min) was determined by the rate of malate transformation to succinate, which was recorded with a spectrophotometer at the wavelength of 250 nm.

Results were processed statistically using standard programs Statistica for Windows 5.0 (StatSoft, Inc., 1995) in the StatsGraph regimen. Figures present the mean results from a series of at least three similar experiments and the standard errors.

RESULTS

Polyamines as regulators of free radical content in *E. coli* cells. To study the possible role of polyamines in the regulation of free radical content during aerobic growth of *E. coli*, the cells were treated with 1,4-diamino-2-butanone (DAB), an inhibitor of ornithine decarboxylase, which is a key enzyme of the polyamine synthesis, to decrease the content of these compounds (Fig. 1). The addition of DAB (5 μg/ml) caused an about 1.2-fold increase in the level of free radicals in the cells. Approximately the same E/E_0 ratio was observed when the emission of the mutant *E. coli* strain HT306 unable to synthesize polyamines was compared with the emission of the wild type strain (*E. coli* EH40).

A decrease in the putrescine content in the cells of the exponentially growing aerobic *E. coli* culture with an increase in the DAB concentration was accompanied by a proportional increase in the expression of the *soxS* gene encoding the transcriptional activator of the antioxidant defense genes (Fig. 2). This suggested the progress of oxidative stress as a result of the decrease in the putrescine concentration and confirmed its functions as a compound scavenging reactive oxygen species. These data have shown that the level of free oxygen radicals in the cell culture under study depends inversely on the content of polyamines.

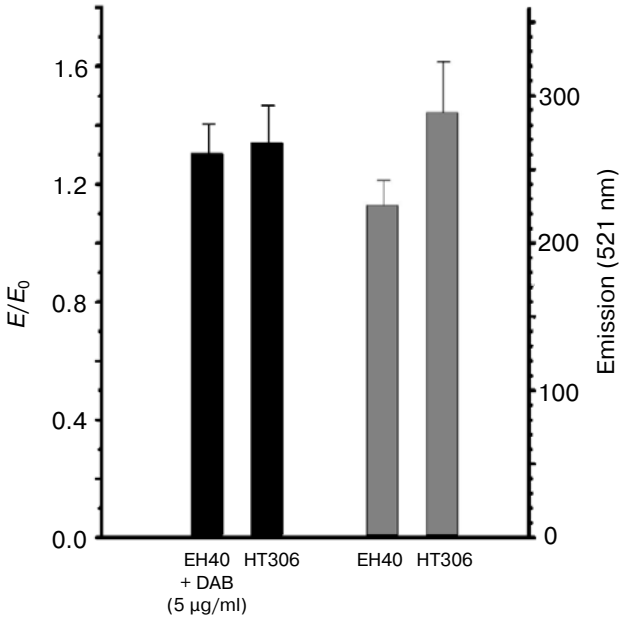

Fig. 1. Effect of the polyamine synthesis inhibitor (DAB) on the free radical ratio in cells of *E. coli* wild type strain EH40 as compared with this ratio in cells of polyamine-dependent mutant HT306. Black columns, E/E_0 ratio; gray columns, emission value. E/E_0 is the ratio of the emission of the EH40 strain cells treated with the inhibitor or the HT306 strain cells in the absence of putrescine (E) to the emission value of strain EH40 intact cells (E_0) stained with the fluorescent dye (see "Materials and Methods").

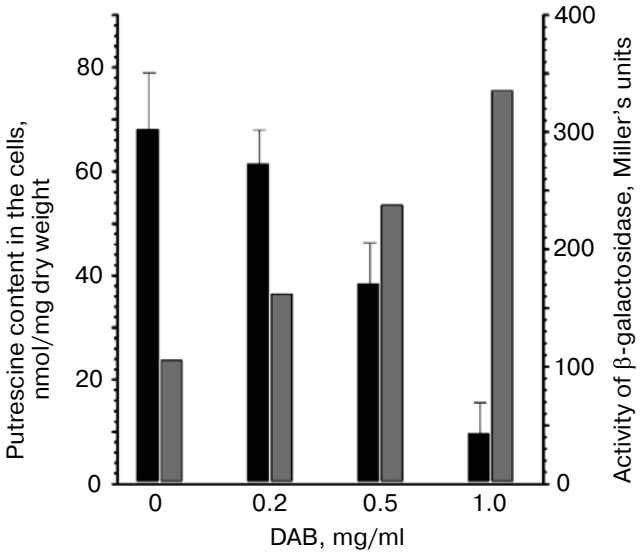

Fig. 2. Dependence of *soxS* expression on polyamine content in *E. coli* EH40 cells treated with the inhibitor of polyamine synthesis (DAB). Black columns, intracellular concentration of putrescine; gray columns, *soxS* expression level.

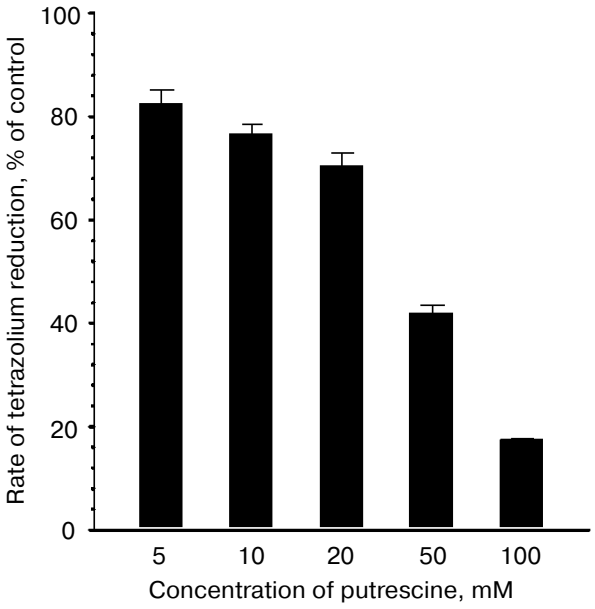

Fig. 3. Dependence of the rate of nitro blue tetrazolium *in vitro* reduction on putrescine concentration in the PMS–NADH system.

The role of polyamines in neutralizing free radical reactions was manifested by their effect on the *in vitro* reduction rate of nitro blue tetrazolium in the system of NADH–phenazine methosulfate generating superoxide radicals (Fig. 3). The reaction rate was slowed down proportionally to the increase in the concentration of putrescine added into the system, which prevented the electron transfer from the superoxide anion onto tetrazolium. Cadaverine and spermidine demonstrated the same effect (data not presented). Considering the low expression of the *soxS* gene of antioxidant defense, which was three-to-fourfold lower than the maximum level, endogenous oxidative stress caused by a decrease in the polyamine content in the cell could be classified as weak. The stress strength evaluated by the deceleration of the specific rate of the microorganism's growth was preset by changes in the concentration of paraquat added into the culture, which induced the intracellular formation of superoxide radicals (Fig. 4).

Addition of DAB to the cells subjected to weak stress slightly increased *soxS* induction, whereas introduction of putrescine significantly decreased it, and this suggested that, under these conditions, polyamines predominantly acted as a scavenger of superoxide radicals. Under conditions of strong stress, the *soxS* induction was significantly lowered, which could be caused by destruction of [2Fe–2S] clusters of the SoxR protein responsible for activation of the *soxS* expression [6]. On this background, the addition of DAB markedly decreased *soxS* induction, and the introduction of putrescine considerably stimulated the expression of this gene, indicating that under these conditions putrescine mainly functioned as a positive transcriptional modulator.

Polyamines as positive modulators of *soxS* regulon expression under conditions of strong stress. Functions of polyamines as transcriptional modulators during oxidative

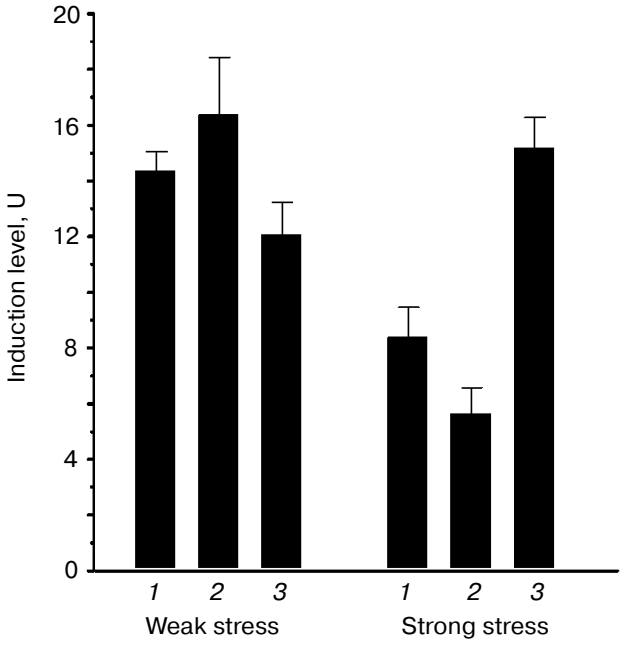

Fig. 4. Influence of putrescine on the *soxS* induction in *E. coli* EH40 cells depending on stress strength: *1*) culture grown without additions; *2*) culture grown in the presence of DAB (300 μg/ml); *3*) culture grown in the presence of DAB (300 μg/ml) and putrescine (10 mM).

stress are known to be associated with changes in the topological state of DNA [10, 20]. To more clearly reveal the properties of polyamines as transcriptional modulators, their activities could be studied under conditions of DNA gyrase suppression by inhibitors, in particular, nalidixic acid. The addition of nalidixic acid (70 μg/ml) into the culture of *E. coli* cells subjected to strong oxidative stress was accompanied by a twofold decrease in *soxS* expression (Fig. 5) that suggested a significant role of the DNA supercoiling for realizing the activity of the SoxR transcriptional modulator under these conditions. Addition of 10 mM putrescine into the medium not only restored the control level of the expression in the presence of paraquat but even doubled it which indicates that under these conditions putrescine behaves as positive transcription modulator.

Along with the regulatory gene *soxS*, polyamines are involved in the positive modulation and fine tuning of expression of the *soxRS* regulon target genes, which encode the antioxidant defense enzymes [20]. Such a role of polyamines was confirmed by data on activities in the cells of the regulon gene products in response to addition of polyamines under stress conditions. Strong oxidative stress was accompanied by a pronounced enhancement of the activities of glucose-6-phosphate dehydrogenase and fumarase C (Fig. 6), in coordination with the induction of the regulatory gene *soxS* (Fig. 5). Inhibition of DNA gyrase with nalidixic acid under stress conditions strongly decreased and addition of putrescine stimulated the enzyme activities in the cell, and this fully reproduced a similar dependence in relation to the gene expression and

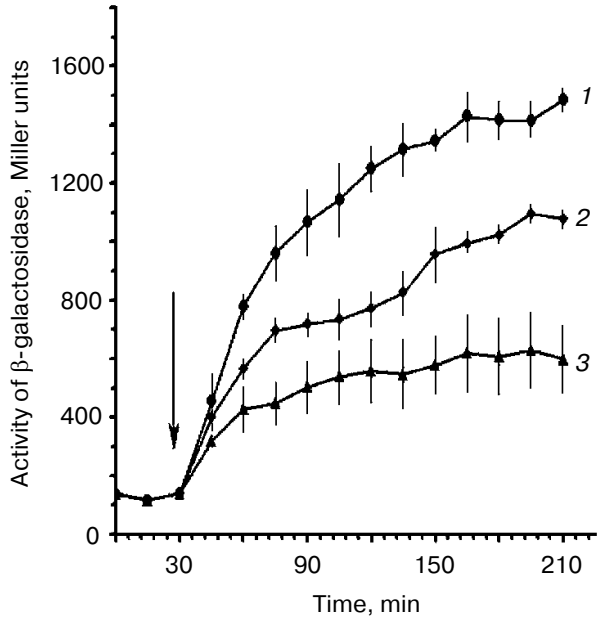

Fig. 5. Putrescine as an antagonist of nalidixic acid action on the level of *soxS* expression in *E. coli* EH40 cells under conditions of oxidative stress (50 μM paraquat) which was reproduced: *1*) in the presence of nalidixic acid (70 μg/ml) and putrescine (10 mM); *2*) without additions; *3*) in the presence of nalidixic acid (70 μg/ml).

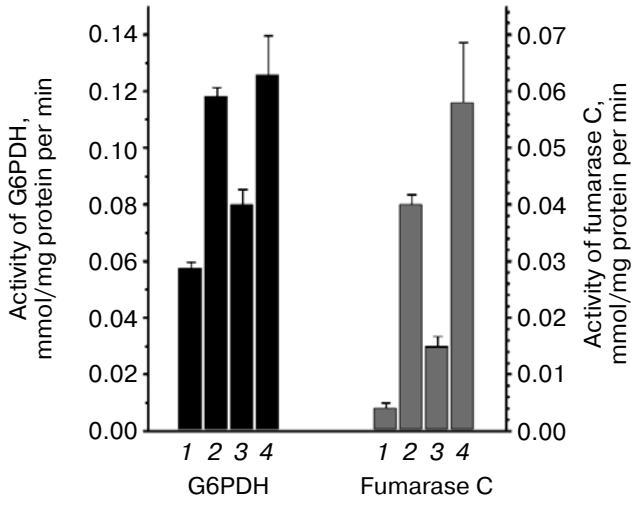

Fig. 6. Role of putrescine in modulation of quantity (activity) of antioxidant enzymes glucose-6-phosphate dehydrogenase (G6PDH) and fumarase C in *E. coli* EH40 cells under conditions of oxidative stress in the presence of nalidixic acid: *1*) culture without additions (control); *2*) culture with addition of 50 μM paraquat; *3*) culture with additions of paraquat (50 μM) and nalidixic acid (70 μg/ml); *4*) culture with addition of paraquat (50 μM), nalidixic acid (70 μg/ml), and putrescine (10 mM).

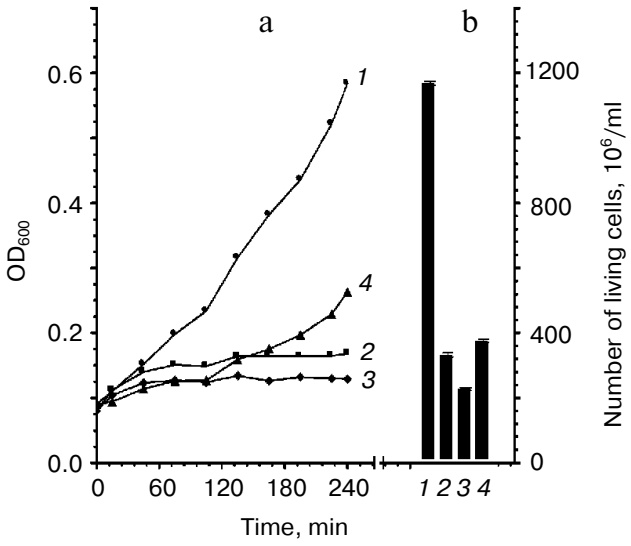

Fig. 7. Dependence of optical density OD_{600} (a) and number of viable cells (b) on putrescine presence in *E. coli* EH40 culture subjected to superoxide stress: *1*) culture without additions (control); *2*) culture with addition of paraquat (50 μM); *3*) culture with additions of paraquat (50 μM) and DAB (0.3 mg/ml); *4*) culture with additions of paraquat (50 μM), DAB (0.3 mg/ml), and putrescine (10 mM).

indicated that the positive transcriptional modulation by polyamines finally increased the amount of the gene products.

Positive functions of polyamines in the modulation of the *E. coli* stress response promoted increased survival of *E. coli* cells (Fig. 7). Addition of the polyamine synthe-

sis inhibitor significantly lowered the number of living cells in the stressed culture, whereas the introduction of putrescine considerably increased the cell survival.

DISCUSSION

In the course of normal functioning of the respiratory chain, reactive oxygen species are generated in *E. coli* cells as a result of side reactions of chemical one-electron reduction of molecular oxygen at the level of flavin-containing carriers [4]. The main antioxidant systems of the microorganism's defense against oxidative stress are organized as the *oxyR* and *soxRS* regulons, providing for adequate induction of the genes encoding antioxidant proteins [5]. Along with the main systems of the antioxidant defense, during oxidative stress enzymes of polyamine synthesis are induced, and this significantly increases the intracellular content of these compounds [10, 14], which are involved in the positive modulation of the antioxidant defense gene expression [21]. This effect of polyamines, similar to the effect of DNA-binding proteins [22], seems to depend on their interaction with DNA resulting in formation of bends which influences the DNA topology and changes gene expression. Polyamines are preferentially bound in the regions of DNA bends, which are often present in the promoter area of genes [23], and this, in particular, conditions their functioning as transcriptional modulators. At present, the number of genes with established polyamine-dependent expression is increased; therefore, some authors have combined this group into a polyamine modulon [24].

The content of polyamines, especially putrescine, is rather high in *E. coli* cells even under normal conditions without stress; therefore, they are suggested to play a role in the regulation of the intracellular level of free radicals. The reasonability of this suggestion is supported by earlier data on qualitative differences in the fluorescence intensities of wild type *E. coli* cells and the polyamine-dependent mutant treated with a fluorescent dye sensitive to free radicals [12]. Our findings described in the present paper allowed us to quantitatively characterize the dependence between the polyamine content in the cells, the level of free oxygen radicals, and the expression of the oxidative stress genes (Figs. 1 and 2), and also to confirm experimentally the role of polyamines as scavengers of free radicals (Fig. 3).

Our findings, as well as the literature data, have shown that polyamines play a double role in the defense of *E. coli* against oxidative stress: they are transcriptional modulators and scavengers of free radicals. Thus, on one hand, the quenching of free radicals by polyamines relieves stress and must lower the level of gene expression. On the other hand, polyamines are shown to act as positive transcriptional modulators. To resolve this contradiction, we have supposed that the predominance of one or the other function of polyamines should depend on the stress strength. We have shown that under conditions of weak stress the scavenging of free radicals is the predominant function of polyamines, while strong stress promotes the manifestation of their properties as transcriptional modulators.

The generalized results illustrating the effects of putrescine on *soxS* expression (Fig. 8) allowed us to characterize antioxidant functions of putrescine depending on superoxide stress strength, which was assessed by the paraquat-caused suppression of the specific growth rate (μ) of exponentially growing cultures of *E. coli*. Weak stress accompanied by a slight decrease in the μ value (from 5 to 15%) increased the *soxS* expression, which was approximately fivefold higher than the expression under conditions of endogenous stress induced by the decrease in the intracellular concentration of polyamines (Fig. 2). Under these conditions, exogenous putrescine caused a decrease in *soxS* expression at the cost of suppression of free radical reactions (hatched area *1* in Fig. 8). The maximum expression in the absence of putrescine (dotted line) was recorded under conditions of moderate stress, which slightly (on average, by 20%) decreased the μ value. Under these conditions, the addition of putrescine was accompanied by a considerable increase in the maximal level of *soxS* expression; therefore, we set aside the zone of the absolute positive modulation (zone *2*, Fig. 8). The further deceleration of the microorganism's growth on strengthening the stress action of superoxide radicals in the absence of putrescine was accompanied by a decrease in the expression, presumably associated with oxidative destruction of [2Fe–2S] clusters of SoxR [6], which was completely abolished by putrescine along with the 50% suppression of the specific growth rate and significantly

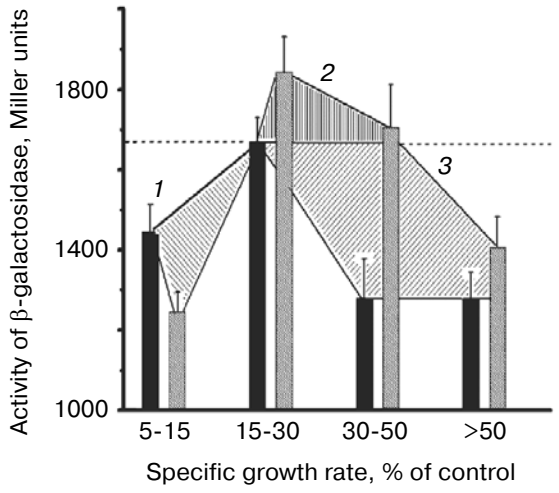

Fig. 8. Ratio of antioxidant action mechanisms of putrescine depending on superoxide stress strength. Explanations in the text.

decreased under conditions of stronger stress (zone *3*, Fig. 8).

The duality of polyamine functions is especially important for the genes expressed depending on specific proteins which are transcriptional activators containing iron–sulfur clusters. During interaction with reactive oxygen species, these clusters can not only transform the protein into the active state (SoxR), but also undergo oxidative destruction with production of the inactive apoprotein [6, 25]. It may be that just such a situation occurs under conditions of strong oxidative stress associated with a decrease in *soxS* expression (Fig. 8). Along with special systems directed to compensate for the lost iron–sulfur clusters [26], the cells of such microorganisms use natural reducers. However, some reducers, e.g. glutathione, can also display an opposite effect. The [2Fe–2S] clusters of SoxR in *E. coli* cells are destroyed by glutathione, which is in millimolar concentration in the cells [6]. Under conditions of strong superoxide stress (e.g., due to actively functioning superoxide dismutase), hydrogen peroxide can be accumulated with the resulting induction of the *oxyR* regulon [27], which as a target gene has *gor* encoding glutathione reductase. Reduced glutathione produced by this enzyme during interaction with reactive oxygen species can generate oxidized radicals, which actively destroy the [2F–2S] clusters of SoxR [6]. However, such reducers as L-cysteine can protect the iron–sulfur clusters.

We think that polyamines, in particular putrescine, can also act as protectors. In this case, maintaining the integrity of the SoxR [2F–2S] clusters at the cost of the polyamine functioning as a free radical scavenger can partially enhance their functions as positive transcriptional modulators of *soxS* expression, especially under conditions of strong stress. Another mechanism of the protective effect of polyamines associated with formation of covalent complexes with glutathione must not be ruled out, and this can markedly lower its content in the cell and decrease the destruction of the iron–sulfur clusters [28, 29].

The topological state of DNA determining the local bends in the signal area of the genes is one of the main factors which influence gene expression, especially of genes related with adaptation to stress [30, 31]. In many cases, the action mechanism of transcriptional regulators, including SoxR, is based on changes in the local topological DNA state in this area [32]. Considering the dependence of the DNA topological state on polyamines [10, 22], it was supposed that the functions of polyamines as transcriptional regulators should be more distinctly revealed under conditions of an artificial decrease in DNA supercoiling using inhibitors of DNA gyrase. The data obtained by this approach indicated that under strong stress conditions polyamines mainly acted as positive transcriptional modulators (Figs. 6 and 8), whereas under conditions of weak stress the main function of polyamines was neutralization of free radicals (Figs. 1 and 2). These finding were confirmed on the level of gene expression and also on the level of gene products, enzymes of antioxidant defense (Fig. 6). Functioning of polyamines during oxidative stress increased the viability of microorganisms in cultures subjected to oxidative stress (Fig. 7).

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