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Mechanisms of Protective Functions of *Escherichia coli* Polyamines Against Toxic Effect of Paraquat, Which Causes Superoxide Stress

A. G. Tkachenko

Institute of Ecology and Genetics of Microorganisms, Ural Branch, Russian Academy of Sciences, ul. Goleva 13, Perm 614081, Russia; fax: (3422) 646-711; E-mail: agtkachenko@ecology.psu.ru

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Abstract—The toxic effect of paraquat, mainly caused by production of superoxide radicals, results in the induction of polyamine synthesizing enzymes and their products in cells of exponentially growing *E. coli* cultures. The activity of ornithine decarboxylase increases approximately twofold and the activity of lysine decarboxylase increases 1.4-fold. Unlike cadaverine, putrescine and spermidine stimulate expression of the *soxRS* regulon genes, and this depends on the polyamine concentration and is specific for different genes of the regulon. Of six genes studied, the maximum (to 130%), minimum (about 40%), and average (60-80%) stimulation was observed for the stress induction of *nfo* (endonuclease IV), *sodA* (superoxide dismutase), and the *soxS* gene of the transcriptional regulator, respectively. Addition of paraquat to exponentially growing *E. coli* culture results in oscillations of the topological state of DNA. Putrescine prevents the drop in the extent of DNA supercoiling caused by the damaging effect of free radicals during the first minutes of stress and increases it during the restoration (the peak of the transcriptional activity of the *soxRS* regulon genes). These effects are due to properties of putrescine as a DNA protector and modulator of its topological state. The ability of putrescine to decrease the mutation rate under conditions of superoxide stress induced by addition of paraquat is shown by the example of rifampicin resistance.

Key words: polyamines, oxidative stress, superoxide radicals, *soxRS* regulon, gene expression, stimulators, DNA topology, mutation rate

Reactive oxygen species, mainly superoxide radical, hydrogen peroxide, and free hydroxyl radical, are generated in aerobic organisms as byproducts of the respiratory chain and can cause endogenous oxidative stress [1]. During both exponential growth and stationary phase, this stress can be escaped due to the constantly active antioxidant systems of cell defense organized as regulons, or groups of genes controlled by the same transcriptional regulator [2].

Maintaining of a sufficiently low and stable concentration of superoxide radical (about 10^{-10} M) in exponentially growing *E. coli* cells and also defense of different systems of the cell against its damaging effect is provided for by enzymes encoded by genes organized in the *soxRS* regulon [1]. This regulon includes more than ten genes whose products are responsible for different mechanisms of antioxidant defense of the cell. The level of superoxide radical is decreased by superoxide dismutase, the inducible manganese-containing form of which is encoded by the *sodA* gene and also by the aconitase (*acnA*) system for trapping O_2^- [3]. The abovementioned aconitase isoenzyme together with fumarase (*fumC*) are induced in the course of superoxide stress and are isoenzymes of dehydratases resistant to destruction by O_2^-. The reducing state of the cytoplasm under superoxide stress is maintained due to increased expression of the gene *zwf* which encodes glucose-6-phosphate dehydrogenase whose product NADPH is used not only for glutaredoxin- and thioredoxin-oxidoreductase systems of protein defense [4] but also to ensure functioning of flavodoxin (*fldA*) and ferredoxin reductases (*fpr*), which are thought to be responsible for maintaining of the reduced state of Fe-S enzyme clusters [1]. The transport of iron, which can be a source of hydroxyl radical under oxidative stress, is controlled by the repressor of iron utilization (*fur*), which is also a component of the *soxRS* regulon. During superoxide stress not only the availability of iron is limited, but also systems are activated which limit the entrance into the cell of xenobiotics and also the expulsion of them, including redox-cycling agents which can be a source of superoxide radicals in the cytoplasm. These agents include the antisense small RNA (*micF*), which is respon-

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sible for suppression of translation of mRNA of porin proteins OmpF of the outer membrane and also multidrug efflux pumps encoded by the *acrAB* gene. Under conditions of superoxide stress, repairing functions of the cell are provided for by endonuclease IV (*nfo*), which is involved in the repair of DNA damaged by reactive oxygen species.

The above-listed genes are target genes of the *soxRS* regulon and are controlled by two transcriptional regulators, SoxR and SoxS, encoded by two genes of the same names which are located "head to head" in the *E. coli* genetic map and are transcribed in opposite directions [5]. SoxR is bound near the promotor regions of both genes and in the active oxidized state activates the transcription of *soxS*. The product of the latter is a direct transcriptional activator of the target genes. The transition of SoxR into its active state occurs by direct oxidation with superoxide radical of [4Fe-4S] clusters of the homodimeric molecule of the protein during oxidative stress.

Normal products of the cell metabolism are known to be involved in the cell response to stress together with specific transcriptional regulators, and this ensures the fine adjustment of the gene expression which adapts the cells to environmental conditions most adequately and economically [6].

Among these metabolites polyamines, in particular putrescine, which modulates expression of the *oxyR* regulon genes, have been shown to play an important role in the regulation of the *E. coli* response to oxidative stress caused by hydrogen peroxide [7]. Polyamines of *E. coli* include putrescine, cadaverine, and spermidine, the first of these being significantly predominant quantitatively. These compounds are biogenic polycations due to protonation of their amino groups at physiological values of pH. The number of amino groups and the distance between them vary depending on the length of the carbon chain in molecules of different polyamines, and this determines the specificity of their interaction with negatively charged groups of cellular biopolymers, such as nucleic acids and phospholipids [8]. These interactions result in the influence of polyamines on various processes in the cell; therefore, they are considered to be universal cellular regulators [9]. The amount of data on the significant role of these compounds in adaptation of microorganisms to various stresses has recently increased [10, 11]. The response of *E. coli* to oxidative stress caused by addition of hydrogen peroxide has shown a significant increase in the activity of the system responsible for synthesis of polyamines, which provide for different protective functions during this stress [12]. However, studies on toxic effects of paraquat on polyamine-deficient *E. coli* mutants have shown a significant role of polyamines in defense of the microorganisms against superoxide stress, but mechanisms underlying the protective functions of polyamines during this stress remained unclear [13].

On this basis, the purpose of the present work was to study the activity of the system of synthesis of polyamines, their influence on the expression level of the *soxRS* regulon genes, topological properties of DNA, and mutation rate of *E. coli* cells under conditions of superoxide stress and, thus, to elucidate the role of these compounds in defense of the microorganisms against superoxide radicals.

MATERIALS AND METHODS

Objects of the study. *E. coli* strains used in the present work are listed in the table, with data on their genotypic features and sources.

To determine the expression level of the *soxRS* regulon genes, the principle of gene fusion was used when the promotor of the gene under study was fused with the promotor-free region of the reporter gene, with *lacZ* encoding β-galactosidase in this role. The activity of this enzyme in the cells is believed to be proportional to the rate of initiation of the studied promotor. Gene fusions were obtained by lysogeny using the bacteriophage λ as a vector [14]. To study topological changes in DNA under stress conditions, strains with gene fusions were transformed conventionally with the pBR 322 plasmid using $CaCl_2$ [15].

Culture of microorganisms. Before the experiment, *E. coli* strains stored on a slant LB-agar were inoculated into LB-broth which contained (μg/ml): for EH40, streptomycin (50); for N9210, N9211, N9212, and N9213,

Bacterial strains used in the present work

E. coli strains	Genotype	Source or reference
K-12	wild type	VKM (All-Russian Collection of Microorganisms)
EH40	derivative of GC4468 with a gene fusion λ EH40 (soxS′::lacZ)SoxRS$^+$	B. Demple
GC4468	K12 rpsL thi soxR$^+$ soxS$^+$	R.G. Martin
N9210	8452, but fpr::lacZ	
N9211	8452, but fumC-lacZ	
N9212	8452, but micF-lacZ	
N9213	8452, but nfo-lacZ	
N9086	7840, but sodA-lacZ	
N8452	rob$^-$, mar$^-$, sox$^+$, resistant to kanamycin	
N7840	mar$^-$, rob$^+$, sox$^+$, sensitive to kanamycin	

kanamycin (50); for the same strains transformed with the pBR 322 plasmid, additionally ampicillin (100) and tetracycline (12.5). After growth for 6 h in a thermostat at 37°C, the cells were transferred onto M-9 medium containing antibiotics in the concentrations mentioned and grown for 16 h in a 500-ml flask with 300 ml of M-9 medium on a thermostatted shaker (100 rpm) at the same temperature. The resulting culture was used as inoculate for transfer into 250-ml flasks containing 100 ml of M-9 medium with antibiotics and grown under the same conditions.

The cells were grown in an ANKUM-2 cultivator (Russia) under aerobic conditions (pO_2 was 80-100%) at pH 7.0 (titration with 2 M NH_4OH). Glucose was added fractionally (1-2 g/liter), not awaiting its complete consumption.

The cell biomass was estimated by optical density (OD_{600}) after the culture had been diluted in saline with an SF-46 spectrophotometer (LOMO, Russia) or in values of absolutely dry biomass (ADB, mg/liter) by the previously calibrated optical density.

Activity of β-galactosidase. Activity of β-galactosidase was determined in cells pretreated with a mixture of SDS (Sigma, USA) and chloroform by the method of Miller [16].

Contents of polyamines. The concentration of polyamines was determined fluorometrically. The culture aliquots (500 μl) were centrifuged for 1 min at 16,000*g*. The cells were extracted with 0.4 M $HClO_4$ or with 7% butanol with vigorous shaking for 1 h. The supernatant fluid was used for determination of polyamine contents in the medium. The $HClO_4$ extract (100 μl) adjusted to pH 9.0 with 2 M Na_2CO_3 was supplemented with 100 μl of 1-dimethylaminonaphthalene-5-sulfonyl chloride (dansyl chloride) (Sigma) in acetone (2.7 mg/ml) and incubated in the dark for 2 h at 37°C. The mixture was evaporated in a flow of cold air and extracted with benzene. The benzene extracts were quantitatively placed onto Sorbfil silica gel plates (100 × 100 mm) (Russia) for thin layer chromatography and separated successively in two systems of solvents: I) benzene–triethylamine (20 : 2); II) benzene–methanol (10 : 0.45). The dried chromatograms were photographed with an Olympus C-3040 digital camera (Japan) under ultraviolet light exciting blue-green luminescence of dansyl-polyamine spots, the size and brightness of which were proportional to their concentration. Polyamine concentrations were calculated by results of computerized densitometry of the negatives using the standard Adobe Photoshop 5.0 program.

Activity of enzymes of polyamine synthesis. The culture of microorganisms was rapidly cooled to 0-4°C, the cells were precipitated by centrifugation (5 min at 10,000*g*), washed once in saline, resuspended in 0.1 M Tris-HCl buffer (pH 8.25), and treated ultrasonically at the frequency of 22 kHz twice for 15 sec at 0-4°C. The broken cells were centrifuged (20 min at 16,000*g*), and the supernatant fluid was decanted and, after determination of the protein content in it by the Lowry method, was used for the enzymatic reaction. The incubation medium (final volume 0.5 ml of 0.1 M Tris-HCl buffer (pH 8.5)) contained 1 mM dithiothreitol (Sigma), 40 μM pyridoxal phosphate (Sigma), 10 mM L-ornithine or L-lysine (for determination of ornithine- or lysine decarboxylase, respectively), and 100 μg of the supernatant fluid protein. The reaction was performed at 37°C for 30 min and stopped by addition of $HClO_4$ to the final concentration of 0.4 M. Activities of the enzymes were calculated by production of putrescine and cadaverine using the linear region of the accumulation curve of the reaction products.

The topological state of DNA was determined by the extent of negative supercoiling of the plasmid DNA of transformed strains. The plasmid DNA was isolated from bacterial cells routinely by conventional alkaline hydrolysis [17]. Topoisomers were separated by horizontal electrophoresis in 1% agarose gels in TBE buffer (89 mM Tris-borate (pH 8.0), 89 mM boric acid, 2 mM EDTA) with addition of 15 μg chloroquin at voltage of 1 V/cm for 48 h at room temperature. The gel was stained with ethidium bromide (1.0 μg/ml) for 30 min and photographed by transmitted ultraviolet with the Olympus C-3040 digital camera. The luminescence intensity of bands of DNA topoisomers was determined by computerized densitometry of negatives using the Adobe Photoshop 5.0 program. The extent of DNA supercoiling was evaluated by mean values of Lk [18].

Determination of mutation rate. The mutation rate was counted by the resistance to rifampicin [19].

Results were processed statistically using the Statistica for Windows 5.0 computer program (StatSoft, Inc., 1995) in StatsGraph mode. Statistical figures present mean data from series of similar experiments (not less than three); vertical segments show values of mean square deviations.

RESULTS AND DISCUSSION

Unlike the electroneutral hydrogen peroxide molecule, which can easily diffuse across the cytoplasmic membrane, the negatively charged superoxide radical is unable to penetrate into the cell. Therefore, superoxide stress is usually induced by redox-cycling compounds, such as paraquat, menadione, and plumbagin, which penetrate into the cells easily and induce a cyclic one-electron reduction of molecular oxygen to superoxide radical by the oxidation of redox enzymes of the cell [1]. The absence of toxic effect of these compounds under anaerobic conditions confirms that their damaging effect is mainly caused by superoxide stress [13].

Increased sensitivity to paraquat of *E. coli* mutants with disturbed synthesis of polyamines [13] suggests a role

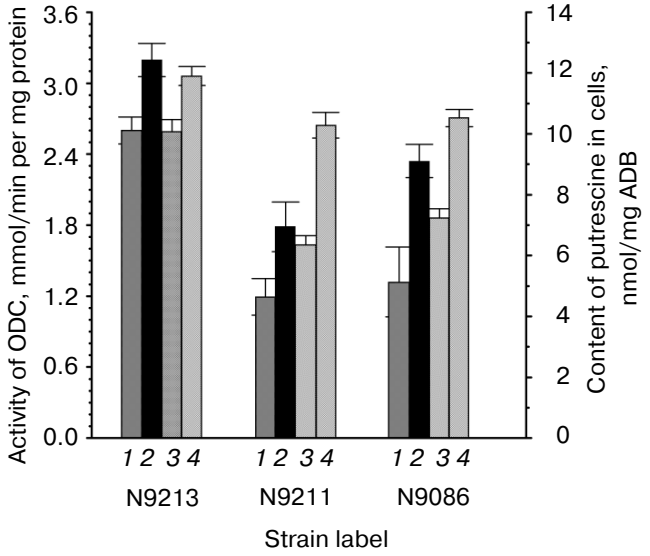

Fig. 1. Effect of superoxide stress on activity of ornithine decarboxylase (*1*, *2*) and content of putrescine (*3*, *4*) in *E. coli* cells. Cultures of different *E. coli* strains were grown on a thermostatted shaker (see "Materials and Methods") to optical density of 0.3 (OD_{600}), then one of two identical portions of each strain was left intact (*1*, *3*), whereas in the other portion superoxide stress was induced by addition of 50 μM paraquat (*2*, *4*). After incubation for 2 h, the activity of ODC and content of putrescine were determined in the cells of each culture.

of these compounds in mechanisms of adaptation of the microorganisms to superoxide stress. However, it remains unknown if polyamines are involved in the stress response of wild type cells and what the mechanisms of their protective functions are.

The response of the system of polyamine synthesis to addition of paraquat into the exponentially growing *E. coli* culture was used in the present work as a primary parameter for evaluation of involvement of polyamines in the cellular response to superoxide stress.

Ornithine decarboxylase (ODC) producing putrescine is a key enzyme whose activity is conventionally used to characterize the activity of polyamine synthesis in *E. coli* [8]. The activity of this enzyme was studied in different strains of *E. coli*, and it was found to be significantly induced in response to superoxide stress that significantly increased the level of putrescine in the cells (Fig. 1). Unlike spermidine, which is a derivative of putrescine and is quantitatively associated with its level in the cell, cadaverine in *E. coli* is synthesized through an independent pathway catalyzed by lysine decarboxylase [8]. Like ODC, this enzyme was shown to be significantly induced under conditions of superoxide stress (Fig. 2). The pool of cadaverine, which is the product of this reaction and is virtually absent in the cells under normal conditions, sharply increased.

The significant induction of the system of polyamine synthesis presented in Figs. 1 and 2 convincingly shows its involvement in the stress response, and this was the reason for further studies on the role of polyamines during the exposure of *E. coli* cells to superoxide radicals.

Genes encoding the enzymes of *E. coli* defense against superoxide stress are organized as the *soxRS* regulon, and the level of their expression is directly controlled by the transcriptional activator SoxS. The known properties of polyamines as transcriptional modulators under conditions of another oxidative stress caused by hydrogen peroxide [7, 11] became the reason for studies of similar polyamine properties relative to the *soxRS* regulon genes.

The expression level of *soxS* encoding the transcriptional activator was significantly stimulated by exogenous putrescine, the effect of which depended on its concentration and was maximal at 5 mM (Fig. 3). Further increase in the concentration of putrescine was accompanied by a decrease in the stimulating activity due to the homeostatic regulation of the stress response adequate to increase the protective functions, including those responsible for limitation of the entrance into the cell of paraquat type xenobiotics. In *E. coli* these functions are realized by qualitative and quantitative regulation of porin channel proteins of the outer membrane (OmpC and OmpF), which provide for the transport into the periplasmic space of relatively small hydrophilic molecules [20]. This process involves a product of the *micF* gene (a component of the *soxRS* regulon), which is a small regulatory RNA complementarily binding to mRNA of *ompF* and preventing its translation [21]. Along with regulation of the quantity of OmpF, in *E. coli* cells a mechanism functions which limits the duration of the open state of the

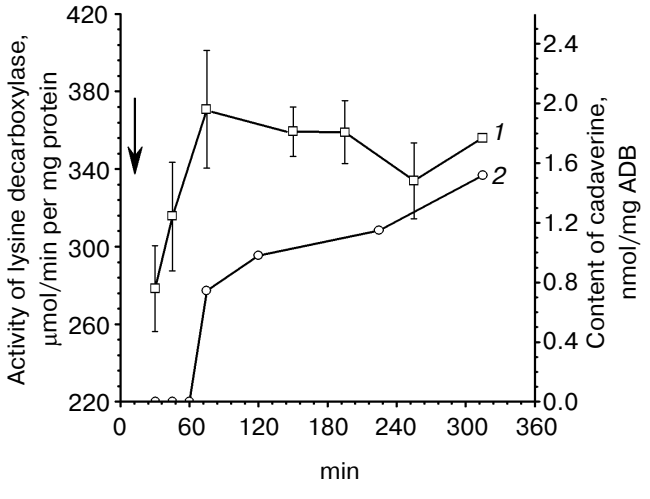

Fig. 2. Changes in activity of lysine decarboxylase (*1*) and content of cadaverine (*2*) *in E. coli* K-12 cells (VKM) under conditions of superoxide stress. The arrow indicates the introduction of paraquat. The culture was grown in an ANKUM apparatus (see "Materials and Methods") to the density of 1-1.5 g ADB per liter. Superoxide stress was obtained by addition of 5 μM paraquat.

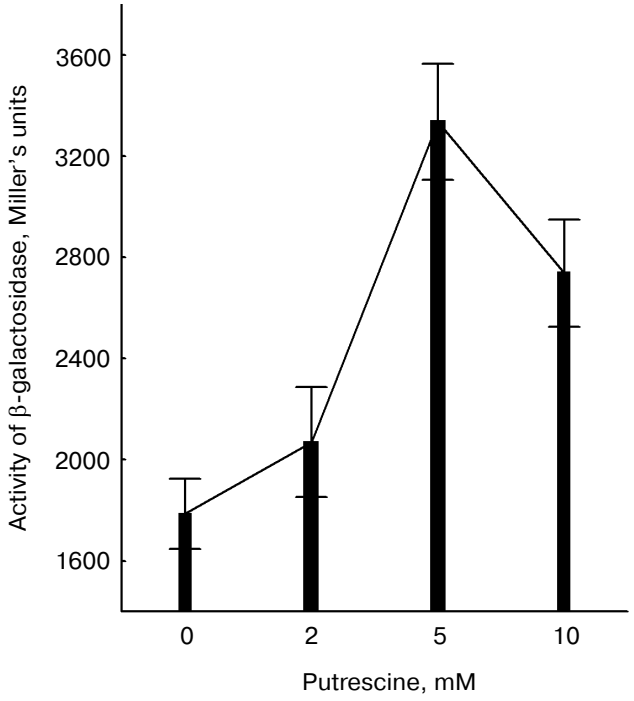

Fig. 3. Dependence of the *soxS* gene expression in *E. coli* EH40 on putrescine concentration under superoxide stress. The microorganisms were grown on a thermostatted shaker (see "Materials and Methods") to optical density of 0.3 (OD_{600}). Superoxide stress was induced by addition of 50 μM paraquat. Putrescine was added into the nutrition medium initially.

channel. Polyamines (putrescine, cadaverine) are inhibitors of the OmpF channel functions [22]. This confirms the earlier hypothesis that the limitation of the paraquat transport into the cell can be one of the protective functions of polyamines under conditions of superoxide stress [13]. The combined functioning of these mechanisms seems to be responsible for decrease in the transcriptional effect of high concentrations of putrescine.

Because putrescine has properties of a transcriptional modulator with respect to *soxS*, the question arises whether other polyamines (spermidine and cadaverine) of *E. coli* possess similar activities. The *soxS* expression was studied in the presence of physiological concentrations of these polyamines, and specificity of their regulatory effects was shown (Fig. 4): similarly to putrescine, spermidine significantly stimulated the expression, whereas in the presence of cadaverine the expression level was near the control. The molecular structure of polyamines is mainly specified by the presence of amino groups, which at physiological pH values impart polyamines properties of polycations and provide for the functional activity due to electrostatic interaction with anionic structures of the cell, such as biological membranes and DNA. Variations in the number of amino groups and the distance between them in different polyamines are responsible for their functional specificity when spermidine (N-[3-aminopropyl]-1,4-butanediamine) and putrescine (1,4-diaminobutane) optimally correspond to location of negative charges on DNA [23] and cadaverine (1,5-diaminopentane) is known as an effective regulator of functions of porin channels of the outer membrane [24].

Induction levels of the *soxRS* regulon genes under conditions of superoxide stress (Fig. 5) were significantly increased in the presence of exogenous putrescine, and the stimulation varied in different genes from 40-50% (*sodA*) to 120-130% (*nfo*, *fpr*). The persistent generation of superoxide radical as a byproduct of the respiratory chain functions during the aerobic growth resulted in a significantly higher, compared to other genes of the regulon, initial level of expression of the gene encoding superoxide dismutase (*sodA*) (1300-1400 Miller's units), which promoted maintaining a low level of superoxide radical in the cell [1]. It is known that besides the inducible SodA two other forms of the enzyme function in *E. coli*, the constitutive SodB and periplasmic SodC, which are also present in the cell under aerobic conditions and contribute to this process [25]. Under these conditions, paraquat caused a relatively low induction of the gene, whereas its induction on addition of putrescine was moderate. Another situation occurred with the *nfo* and *fpr* genes: their initially low expression levels (200-400 Miller's units) under superoxide stress were also induced insignificantly; however, the stimulation with putrescine was maximal. The effect of putrescine on the genes *soxS*, *micF*, and *fumC* was intermediate (60-80%).

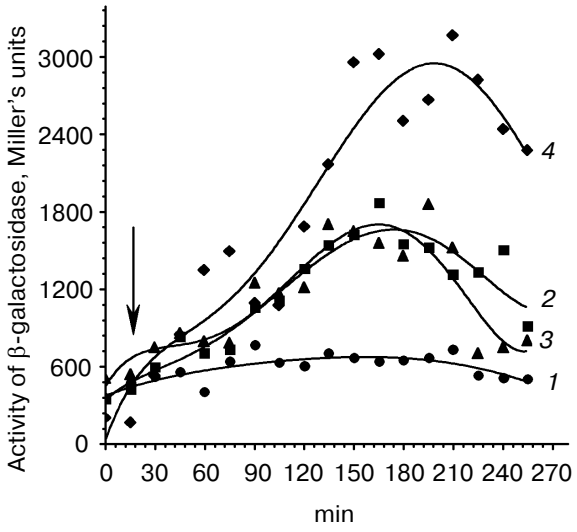

Fig. 4. Effects of different polyamines on *soxS* expression in *E. coli* EH40 under conditions of superoxide stress. *1*) Culture without additions (control); *2*) supplemented with 50 μM paraquat; *3*) supplemented with 50 μM paraquat and 5 mM cadaverine; *4*) supplemented with 50 μM paraquat and 1 mM spermidine. The arrow indicates the introduction of paraquat. The culture conditions were similar to those shown in Fig. 3.

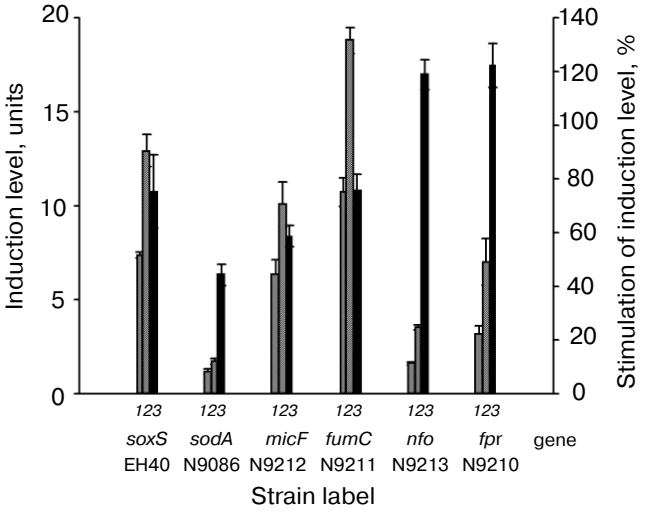

Fig. 5. Role of putrescine in the regulation of induction level of *soxRS* regulon genes in *E. coli* under superoxide stress: *1*) in the absence of putrescine; *2*) in the presence of 5 mM putrescine; *3*) stimulation of the induction level by putrescine. The culture conditions were similar to those shown in Fig. 3. The induction level is the ratio of the maximum gene expression under oxidative stress (Miller's units) to the expression in the absence of stress.

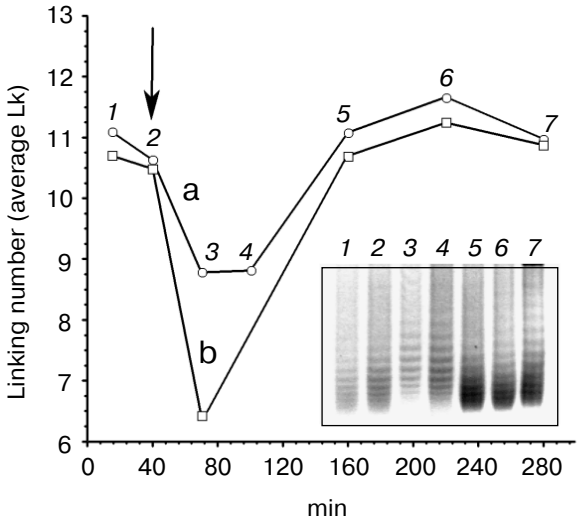

Fig. 6. Effect of putrescine on changes in the topology of DNA of *E. coli* N9211 under superoxide stress: a) in the presence of 5 mM putrescine; b) in the absence of putrescine. The insert presents the photo of the electrophoregram of the plasmid DNA from the culture stressed in the presence of putrescine. The numbers indicate samples of DNA in the electrophoregram and the calculated values of Lk shown as points on the curve. The culture conditions were similar to those shown in Fig. 3.

Thus, different genes of the regulon react differently to putrescine, and this suggests the involvement of polyamines as normal cellular metabolites in the fine adjustment on the genetic level of the stress response of the cell. This correlates well with data on the functional activity of polyamines with respect to DNA, in particular, with data on production of local bends and condensation of DNA [26] and also on involvement of polyamines in the regulation of topological features of DNA [12], which determine the rate of generation of open complexes of individual promotors with RNA-polymerase.

Topological changes in DNA on addition of paraquat into the exponentially growing *E. coli* culture (Fig. 6) are characterized by a dramatic shift in the quantitative distribution of the plasmid topoisomers to lower values of Lk that reflects a decrease in the extent of negative supercoiling of DNA as a result of breaks in the polynucleotide chains and relaxation caused by damage by reactive oxygen species. Although the energy of superoxide radical itself is insufficient to cause the breaks, its reduction to hydrogen peroxide in the reaction catalyzed by superoxide dismutase or as a result of chemical transfer of an electron generates breaking hydroxyl radicals during the Fenton reaction [4]. And free bivalent iron as the electron source in this reaction can be generated endogenously as a result of the destructive effect of superoxide on Fe-S clusters of dehydratases (fumarase, aconitase) [1].

This period is characterized by increase in the activity of the polyamine synthesizing system (Fig. 2) and starting of the induction of the *soxRS* regulon genes (Fig. 4), the maximum of which (180-200 min) was concurrent with the return of the negative supercoiling of DNA and its increase over the pre-stress extent (Fig. 6). The decrease in the negative supercoiling of DNA during the initial stage of superoxide stress was significantly less pronounced in the presence of putrescine (Fig. 6). This effect was due to at least three properties of putrescine: 1) as a stimulator of expression of the antioxidant protection genes including those responsible for repair of DNA; 2) as a modulator of the topological state of DNA increasing the number of supercoils [12]; 3) as a protector of DNA acting as a scavenger of free radicals [12].

It is known that damage to DNA including that induced by reactive oxygen species is usually accompanied by increase in the mutation rate [25], and factors preventing such damage have antimutagenic properties. This was the reason for studies on the effect of putrescine on mutation rate under conditions of superoxide stress. These studies indicated (Fig. 7) that addition into *E. coli* culture of paraquat as a generator of superoxide radicals in the absence of putrescine approximately twofold increased the mutation rate, which decreased later and stabilized at a level slightly higher than before the stress. The decrease in the mutation rate was concurrent with manifestation of the maximum expression of the *soxRS* regulon genes (Fig. 4) including the *nfo* encoding endonuclease IV, an enzyme of repair. In the presence of putrescine which stimulated twofold the *nfo* induction the mutational changes were minimal, and this confirmed the

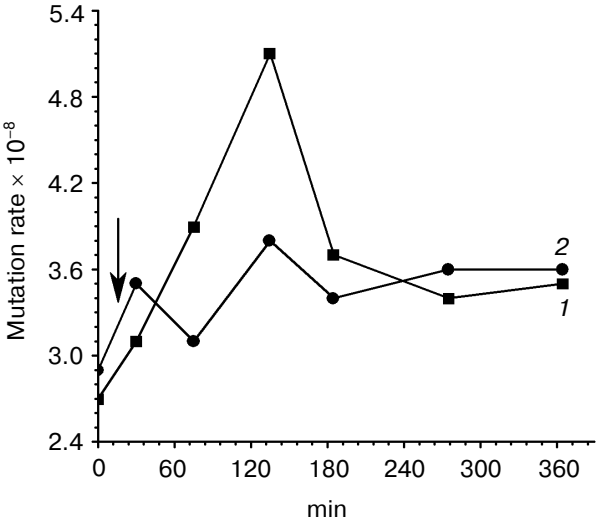

Fig. 7. Effect of putrescine on the mutation rate under superoxide stress: *1*) in the absence of putrescine; *2*) in the presence of 5 mM putrescine. The arrow indicates the introduction of putrescine. The culture conditions were similar to those shown in Fig. 3.

role of putrescine as an antimutagenic factor in the defense of *E. coli* against superoxide stress.

Thus, in response to superoxide stress the activity of enzymes of polyamine synthesis and polyamine contents are significantly induced in *E. coli* cells. Polyamines stimulate expression of the *soxRS* regulon genes, stabilize the topological state of DNA, and decrease the mutation rate. Thus, they increase the protective potential of cells under stress conditions.

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