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Polyamines as Modulators of Gene Expression under Oxidative Stress in *Escherichia coli*

A. G. Tkachenko* and L. Yu. Nesterova

Institute of Ecology and Genetics of Microoragnisms, Ural Division of the 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—Activity of enzymes of polyamine synthesis and contents of their products increased in *E. coli* cells in response to oxidative stress caused by addition of hydrogen peroxide to an exponentially growing culture. Putrescine and spermidine added to the culture medium in physiological concentrations significantly increased expression of genes *oxyR* and *katG* responsible for defense against oxidative stress, whereas cadaverine had no effect. The role of polyamines as modulators of the gene expression was confirmed by experiments with an inhibitor of polyamine synthesis, 1,3-diaminopropane, which decreased the level of cell polyamines and thus abolished the ability of the cell to induce *oxyR* expression under oxidative stress. A genetic method gave similar results: under oxidative stress mutants with disorders in polyamine synthesis displayed a significantly decreased level of induction of the *oxyR* and *katG* genes, and this level was recovered on addition of putrescine. In the presence of inhibitors of DNA-gyrase, nalidixic acid and novobiocin, the *oxyR* expression depended on the extent of DNA supercoiling. Putrescine decreased the inhibitory effects of nalidixic acid and novobiocin, and this confirmed its properties of a stimulator of DNA supercoiling. Resistance to rifampicin was studied to exemplify the mutation rate under oxidative stress. Putrescine decreased twofold the level of mutations and increased the number of viable cells in the culture exposed to oxidative stress.

Key words: polyamines, oxidative stress, *oxyR*, modulators, gene expression, DNA topology, survival, mutation frequency

Oxidative stress, which is widely distributed in both pro- and eucaryotes, appears in response to cell exposure to reactive oxygen species (ROS) mainly represented by hydrogen peroxide, superoxide radical, and free hydroxyl radical. Aerobic organisms have in their respiratory chain a constant source of ROS generation due to one-electron reduction of oxygen [1], and this promotes the formation of systems for defense against oxidative stress. Enzymes of these systems are encoded by gene groups combined into regulons due to a common regulator of transcription. For *E. coli* two main regulons of oxidative stress have been described, *oxyR* and *soxRS*, and their genes encode defense enzymes against hydrogen peroxide and superoxide radical and are controlled by transcription activating proteins OxyR and SoxR, respectively [2]. On exposure of *E. coli* cells to H_2O_2 eight of 30 to 40 inducible proteins are products of the *oxyR* regulon genes. The main products are hydroperoxidase I (catalase I, *katG*), alkyl hydroperoxide reductase (*ahpCF*), glutathione reductase (*gorA*), glutaredoxin 1 (*grxA*), a nonspecific DNA-binding protein DPS (*dps*), a regulatory RNA of OxyS (*oxyS*), and a repressor of iron transfer (*fur*). The proteins of defense against hydrogen peroxide are responsible for cleavage of ROS (*katG*) and of lipid peroxidation products (*ahpCF*), for maintaining of redox homeostasis of the cell (*gorA*) and SH-groups of proteins (*grxA*), for protection of DNA against the damaging effect of free radicals (*dps*, *oxyS*), for limitation of the transport of iron as a substrate for generation of free hydroxyl radicals (*fur*), etc. Catalase I (*katG*) cleaving H_2O_2 to water and molecular oxygen is a vanguard in the anti-peroxide defense of *E. coli*. Thus, *katG* is one of main gene targets for the transcriptional regulator OxyR.

Recently it was reported that expression of the *oxyR* and *katG* genes is increased in the presence of putrescine, which is a polyamine of *E. coli*, and this suggests that putrescine is a transcriptional modulator of these genes [3]. Polyamines, which are present in all biological materials, are aliphatic hydrocarbons with a chain from four to ten atoms in length. These compounds have from two to four amino groups protonated at physiological pH values that make them polycations [4]. Polyamines of *E. coli* include putrescine, cadaverine, and spermidine, and the first of these is significantly paramount. Although the role

* To whom correspondence should be addressed.

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of polyamines as universal regulators of cellular processes has relatively long been known [5], mechanisms of their involvement in these processes are still unclear in detail. Studies on functions of polyamines in adaptation of microorganisms to various stress exposures seem to be promising for elucidation of this problem. Studies along this line suggested that the system of polyamine synthesis could be considered as a peculiar mechanism of conjugation of energy and structure metabolism [6], as an alternative system of cation transport [7], and revealed the stimulatory effect of putrescine on the extent of negative supercoiling of DNA [8] and also its role as a transcriptional modulator of expression of defense genes against oxidative stress [3].

However, it is still unknown how activities of the enzymes of polyamine synthesis change under oxidative stress and what is the role of other polyamines in *E. coli*, in particular, of spermidine and cadaverine, in the regulation of expression of anti-peroxide defense genes, and how polyamines influence the level of spontaneous mutations and cell survival under these conditions. Study on these questions along with the more careful study on the features of polyamines as modulators of the expression under oxidative stress and their effect on DNA supercoiling was the purpose of the present work.

For this purpose genetic and biochemical methods were combined, including the use of inhibitors affecting the activities of enzymes responsible for synthesis of polyamines and gyrase, which regulates the extent of DNA supercoiling.

MATERIALS AND METHODS

Biological materials. Strains and plasmids used in the present work are listed in Table 1 with data on their genotypic features and sources.

To determine the expression level of the gene *oxyR*, a gene fusion *oxyR*′:: *lacZ* was used inserted into the chromosomal DNA of *E. coli* strains RK4936 and MC4100 with bacteriophage λ [9]. In addition, with the same purpose plasmid gene fusions *oxyR*′:: *lacZ* and *katG*′:: *lacZ* were used inserted into strains TA4477 and TA4479, respectively [10]. To obtain polyamine-dependent *E. coli* strains BE0101 and BE0102 with similar gene fusions, an *E. coli* strain TI60 [11] was transformed with plasmids isolated from TA4477 and TA4479. The transformation was performed routinely using $CaCl_2$ [12]. The gene fusions used allowed us to determine the level of expression of the genes under study by determination of activity of β-galactosidase (see below).

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 BGF930 and BGF940, 50 streptomycin; for TA4477 and TA4479, 50 streptomycin and 100 ampicillin; for BE0101 and BE0102, 100 ampicillin. After culture for 6 h in a thermostat at 37°C, the cells were transferred onto M-9 medium containing antibiotics in the same concentrations and grown for 16 h on a shaker (100 rpm) in a 500-ml flask with 300 ml of M-9 medium at the same temperature. The grown culture was used as inoculate 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 at PO_2 of 80-100% and pH value of 7.0 (titration with 2 N NH_4OH). Glucose was

Table 1. Bacterial strains and plasmids used in the present work

E. coli strains or plasmids	Genotype	Source or reference
Strains		
K-12	wild type	VKM
BGF930	derivative of RK4936 with a gene fusion λ[Φ(oxyR′::lacZ)]	B. Demple
BGF940	derivative of MC4100 with a gene fusion λ[Φ(oxyR′::lacZ)]	B. Demple
RK4936	AraD139(argF-lac)205flbB5301 non-9 gyrA219 relA1 rpsl150 metE70 btuB::Tn10	*E. coli* Genetic Stock Center
MC4100	Δ(lac)U169 rpsL	*E. coli* Genetic Stock Center
TA4477	RK4936/pAQ23	G. Storz
TA4479	RK4936/pAQ24	G. Storz
TI60	derivative of BGA8, but leu^+ thr^+ Δ(gpt-lac)5	I. G. Kim, T. J. Oh
BGA8	$λ^-$speB speC thi leu thr	[6]
BE0101	TI60/ pAQ23	present work
BE0102	TI60/ pAQ24	present work
Plasmids		
pAQ23	pRS415 containing oxyR′::lacZ	TA4477
pAQ24	pRS415 containing katG′::lacZ	TA4479

added fractionally (1-2 g/liter), not awaiting its complete exhaustion.

The cell biomass was estimated after a preliminary dilution of the culture in saline by optical density (OD_{600}) using 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 [13].

Contents of polyamines. The concentration of polyamines was determined fluorimetrically. The culture aliquots (500 μl) were centrifuged for 1 min at 16,000*g*. The cells were extracted with 0.4 N $HClO_4$ or with 7% butanol for 1 h with vigorous shaking. The supernatant fluid was used for determination of polyamines in the medium. The $HClO_4$-prepared extract (100 μl) adjusted to pH of 9.0 with 2 M Na_2CO_3 was supplemented with 100 μl of dansyl chloride (1-dimethylamino-1-naphthalenesulfonyl 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 (Russia) silica gel plates (100 × 100 mm) 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 in ultraviolet light which excited blue-green luminescence of dansyl-polyamine spots, the size and brightness of which were proportional to the concentration. Densitometry of the negatives was performed with a MD100 microdensitometer (Karl Zeiss, Germany), and the concentration of polyamines was calculated.

Activities 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 ultrasonicated 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*), the supernatant fluid was decanted and, after determination of protein by the Lowry method, used for the enzymatic reaction. The incubation medium (final volume of 0.5 ml 0.1 M Tris HCl, pH 8.25) 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 protein. The reaction was performed at 37°C and stopped by addition of $HClO_4$ to the final concentration of 0.4 N. Activities of the enzymes were calculated by contents of putrescine and cadaverine in the $HClO_4$-extracts before and after the incubation.

Determination of mutation rate. The mutation rate was assessed by the character of resistance to rifampicin [9].

Counting of viable cells. The content of viable cells in the culture was determined by plating onto dishes with LB-agar and counting of colonies grown after 24 h of incubation at 37°C.

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

RESULTS AND DISCUSSION

Oxidative stress caused by the influence of hydrogen peroxide on the exponentially growing *E. coli* culture was earlier shown to significantly increase the level of putrescine in the cells and medium that indirectly suggested activation of the polyamine-synthesizing system [14]. This was the reason for studies on activities of enzymes of polyamine synthesis in *E. coli* under conditions of oxidative stress.

On addition of 3 mM H_2O_2 to the exponential culture of *E. coli*, the activity of ornithine decarboxylase, which is a key enzyme of polyamine synthesis, increased nearly threefold within the first 45-60 min (Fig. 1). The activity of lysine decarboxylase resulting in production of cadaverine increased still more, sixfold. The content of cadaverine in response to oxidative stress temporarily increased from zero values to 1-2 nmol/mg ADB, signif-

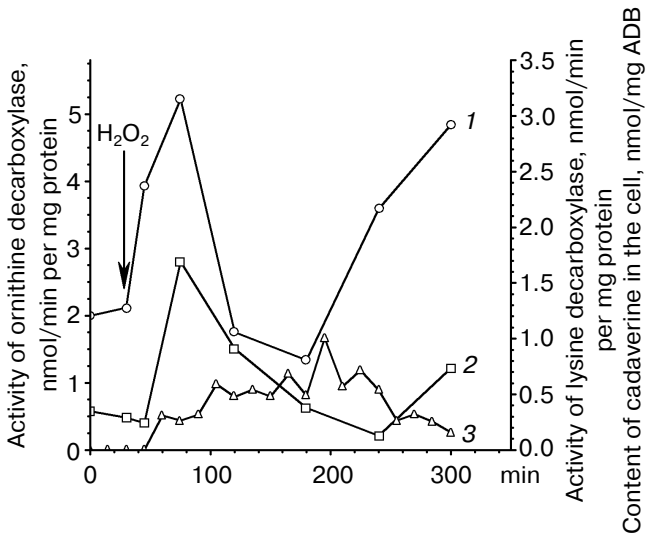

Fig. 1. Changes in activities of enzymes of polyamine synthesis and in the cellular pool of cadaverine in *E. coli* K-12 (VKM) under oxidative stress: *1*) ornithine decarboxylase; *2*) lysine decarboxylase; *3*) intracellular pool of cadaverine. The culture was grown in ANKUM culture apparatuses (see "Materials and Methods") to the density of 1-1.5 g ADB/liter. Oxidative stress was modeled by addition of 3 mM hydrogen peroxide.

icantly contributing to the total content of intracellular polyamines. Fluctuations in the enzyme activity were associated with accumulation in the cell of polyamines, which are end products of the enzymatic reaction and are involved in feedback-type regulation [15]. This is well illustrated by comparison of the activity of lysine decarboxylase and intracellular pool of cadaverine. Consequently, the activation of the enzyme synthesis is one of the primary responses of *E. coli* to oxidative stress, with a resulting intense production of polyamines.

Addition of putrescine into the medium was earlier shown to stimulate the concentration-dependent expression of the *oxyR* genes of the *E. coli* regulon of defense against oxidative stress [3]. However, until now nothing was known about the possible regulatory effects of other *E. coli* polyamines on the level of gene expression. A comparative study of polyamines with consideration of their approximate ratio in the cell revealed a significant difference in their effects on the gene expression (Fig. 2). While putrescine and spermidine provided for nearly 100% stimulation of the *oxyR* expression, cadaverine had no noticeable effect. This seems to be caused by specific features of molecular structure of various polyamines, in particular, the length of carbon chain and the distance between positively charged amino groups that determines their differential interaction with different cellular structures. The location of positive charges of spermidine (N-[3-aminopropyl]-1,4-butanediamine) and putrescine (1,4-diaminobutane) is optimal for interaction with negative charges of the phosphate skeleton of DNA [16], whereas 1,5-diaminopentane (cadaverine) with amino groups located more distantly is not prone to such interactions but is an optimal regulator of porin channels OmpF and OmpC [17]. Our previous fractionation of *E. coli* has shown lysine decarboxylase be mainly or only located in the membrane fraction of the cells [18] that ensures an approach of cadaverine to the place of its regulatory functions. The predominant binding of cadaverine to the cell membrane structures was also confirmed in our experiments by its almost complete extraction with a non-polar solvent (butanol), which influenced the phospholipid fraction of membranes more effectively. Functions of cadaverine as an inhibitor of opening of porin channels explain the biological reasonability of the significant increase in its free pool in response to oxidative stress described by us (Fig. 1), because these channels promote the cell defense by limitation of entrance of such xenobiotics as hydrogen peroxide. Inhibition of *ompF* mRNA by its complementary binding to the small antisense RNA of the *micF* gene which is a part of the *soxRS* regulon of superoxide stress [2] is another mechanism for regulation of porins. Thus, the functional specificity of polyamines seems to be due to their chemical structure and preferential location in the cell.

To confirm the role of polyamines as transcriptional modulators, the effect of an inhibitor of ornithine decarboxylase, 1,3-diaminopropane (DAP), on the expression of *oxyR* was studied (Fig. 3). Influencing the key enzyme of polyamine synthesis, DAP significantly decreases the level of cellular polyamines [19]. The inhibitor added to exponential culture of *E. coli* approximately 75% decreased the maximal level of *oxyR* expression under oxidative stress, whereas the presence of 5 mM putrescine in the medium provided for its 60% recovery. Similar results were earlier obtained with another inhibitor of ornithine decarboxylase, 1,4-diamino-2-butanone [3], which confirms the role of polyamines as modulators of gene expression.

We have also used a genetic approach to otherwise prove the role of polyamines as transcriptional modulators. A polyamine-dependent mutant strain *E. coli* TI60 deleted by the *lac*-operon [11] was used to construct two strains with *oxyR*′::*lacZ* and *katG*′::*lacZ* fusions by its transformation with plasmids pAQ23 and pAQ24, respectively [10]. The induction levels of *oxyR* and *katG* in polyamine-dependent *E. coli* mutants BE0101 and BE0102 under oxidative stress were significantly lower than in *E. coli* strains TA4477 and TA4479 with undisturbed synthesis of polyamines, and the addition of putrescine to the medium significantly stimulated the gene expression of the polyamine-dependent strain (Table 2). This confirms once more the role of putrescine as a transcriptional modulator of genes of the *oxyR* regulon.

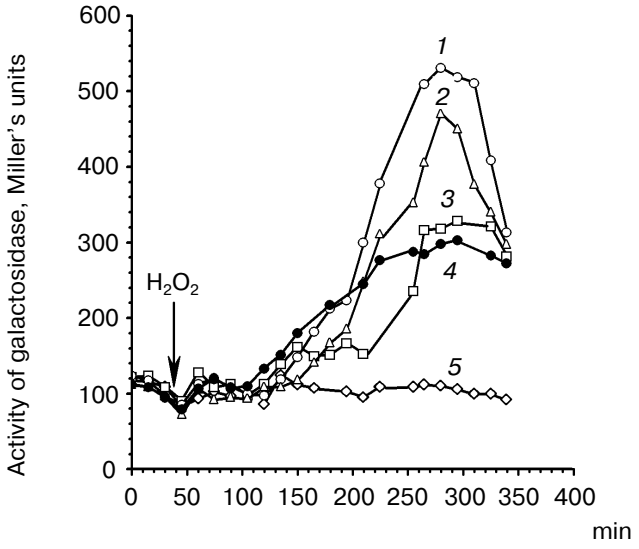

Fig. 2. Effects of polyamines on the expression level of the *oxyR* gene of *E. coli* BGF940 under oxidative stress: *1*) 5 mM putrescine; *2*) 2 mM spermidine; *3*) without additions (control); *4*) 5 mM cadaverine; *5*) without additions in the absence of oxidative stress (control). Microorganisms were grown on a shaker (see "Materials and Methods"). On reaching optical density of 0.3 (OD_{600}), the culture was supplemented with 3 mM H_2O_2. Polyamines were initially added to the nutritional medium.

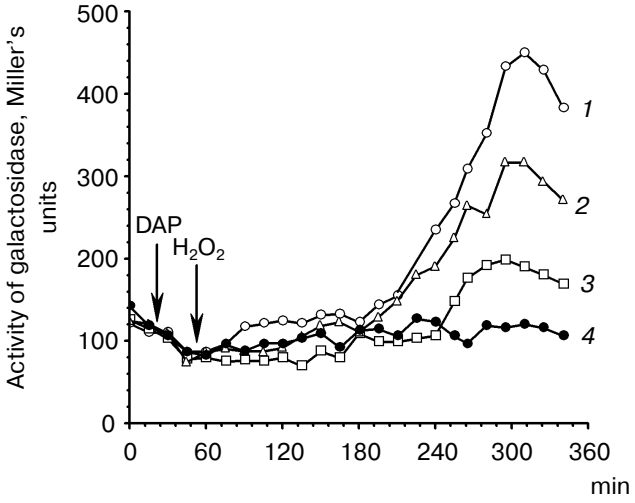

Fig. 3. Effects of an inhibitor of ornithine decarboxylase, 1,3-diaminopropane (DAP), and putrescine on the level of *oxyR* of *E. coli* BGF940 expression under oxidative stress: *1*) without additions; *2*) in the presence of DAP (50 μg/ml) and 5 mM putrescine; *3*) in the presence of DAP (50 μg/ml); *4*) without oxidative stress and additions (control). The culture conditions were similar to those shown in Fig. 2. Putrescine and DAP were initially added to the nutritional medium.

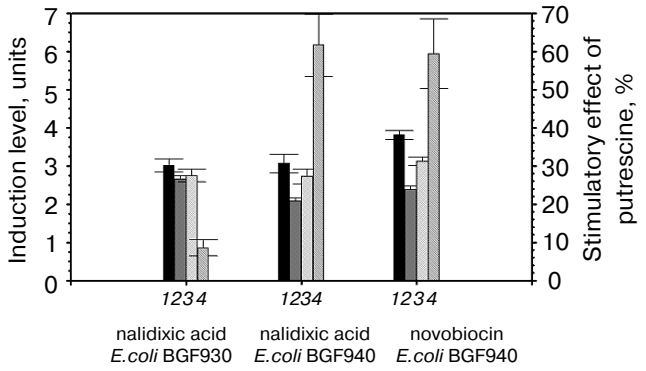

Fig. 4. Effects of inhibitors of DNA-gyrase on the expression of *oxyR* of *E. coli* BGF940 (inhibitor-sensitive) and BGF930 (resistant to nalidixic acid) strains under oxidative stress. The induction level: *1*) in the culture without additions (control); *2*) in the presence of inhibitors (nalidixic acid (50 μg/ml), novobiocin (100 μg/ml)); *3*) in the presence of inhibitor and 5 mM putrescine; *4*) the stimulatory effect of putrescine on the gene expression in the presence of inhibitor. The induction level is the ratio of the maximal gene expression under oxidative stress (Miller's units) to the expression level without stress. The culture conditions were similar to those shown in Figs. 2 and 3.

The level of gene expression depends not only on functioning of transcriptional regulators which are responsible for local changes in regions adjacent to promotors but is significantly determined by the topological state of DNA as a whole (the density of supercoiling) which can be strongly changed under various stresses [20]. The earlier described properties of putrescine as a modulator of the topological state of DNA [8] have become a basis for studies of the effect of putrescine on the expression of *oxyR* in the presence of inhibitors of DNA-gyrase responsible for formation of supercoils in DNA of *E. coli* (Fig. 4).

Nalidixic acid and novobiocin were used as inhibitors of DNA-gyrase to selectively inhibit the topoisomerase activity of A subunits and the ATPase activity of B units, respectively [21]. The *E. coli* BGF930 strain resistant to nalidixic acid insignificantly decreased the level of *oxyR* expression under oxidative stress in response to addition of inhibitor and weakly reacted to addition of putrescine (Fig. 4). However, the addition of nalidixic acid into the culture of the sensitive strain *E. coli* BGF940 exposed to oxidative stress was accompanied by a significant (about 30%) decrease in the level of *oxyR* induction. An addition of putrescine that stimulated the supercoiling

Table 2. Effect of putrescine on the induction level of *oxyR* and *katG* genes under oxidative stress in the polyamine-dependent BE0101 and BE0102 (+) and polyamine-independent TA4477 and TA4479 (–) *E. coli* strains

E. coli strain	Dependence on polyamines	Type of lacZ fusion	Induction level ± *m*		Stimulatory effect of putrescine ± *m*, %
			in the absence of putrescine	in the presence of putrescine	
BE0101 (pAQ23)	+	*oxyR::lacZ*	1.55 ± 0.07	2.50 ± 0.08	62.21 ± 8.10
TA4477(pAQ23)	—	*oxyR::lacZ*	2.50 ± 0.08	2.93 ± 0.00	17.49 ± 3.86
BE0102 (pAQ24)	+	*katG::lacZ*	1.79 ± 0.09	2.53 ± 0.12	42.15 ± 9.82
TA4479(pAQ24)	—	*katG::lacZ*	3.57 ± 0.22	4.80 ± 0.32	34.68 ± 5.80

Note: The induction level is the ratio of the maximal gene expression under oxidative stress (Miller's units) to the expression level in the absence of stress.

approximately 60% recovered the expression of *oxyR* of the inhibited culture. This suggested that the transcription of *oxyR* directly depended on the supercoiling extent of DNA, and the effect of putrescine on the gene expression was partially due to its stimulatory effect on the formation of supercoils. The qualitative and quantitative effects of novobiocin and putrescine on the culture of the sensitive strain *E. coli* BGF940 were very similar to the effect of nalidixic acid.

The similarity of effects of inhibitors of DNA-gyrase different in chemical structure and action mechanism shows that the changes in the expression level of *oxyR* are due to topological changes in DNA. The role of polyamines as antagonists of inhibitors of DNA-gyrase seems to be a result of their influence on topological parameters of DNA [8], although in this case their possible functions as blockers of porin channels which limit the entrance of antibiotics into the cell should not be ruled out [17]. Note that even very high concentrations of inhibitors, in particular, the concentration of novobiocin up to 1000 μg/ml suppressed the *oxyR* expression no more than by 50% (data not presented), and this suggests that the extent of DNA supercoiling is an important but not the only regulatory factor of the gene expression, along with transcriptional activators and modulators. In this connection, putrescine should be given attention because its effect on the gene expression can be due to both its influence on the density of supercoiling of the whole DNA molecule and its properties as a local transcriptional modulator. Therefore, it is interesting which of the above-mentioned properties is dominating. This problem could be somewhat elucidated by the finding that, unlike inhibitors of DNA-gyrase, even very small concentrations of inhibitors of polyamine synthesis, in particular, DAP at the concentration of 50 μg/ml (Fig. 3) and higher suppressed, respectively, by about 80% and completely the *oxyR* expression. This can be interpreted with caution in favor of the prevalence in polyamines of properties of transcriptional modulators. However, whatever properties were prevalent in polyamines, their effects were manifested by increase in the expression of the *oxyR* regulon genes, and, as a result, this suggested an increase in the cellular functions of defense against the damaging effect of reactive oxygen species.

Nucleic acids are known to be most vulnerable under oxidative stress, and their damage results in the increase in mutation rate and cell death [1]. Therefore, to assess the effect of polyamines on the defense functions of *E. coli*, the effect of putrescine on the mutation rate was studied by the example of the cell resistance to rifampicin and cell survival under conditions of oxidative stress.

The mutation rate was maximal 1 h after oxidative stress and then decreased due to the functioning of the defense mechanisms (Fig. 5). Putrescine added to the culture medium at the concentration of 5 mM decreased approximately twofold the rate of spontaneous mutations

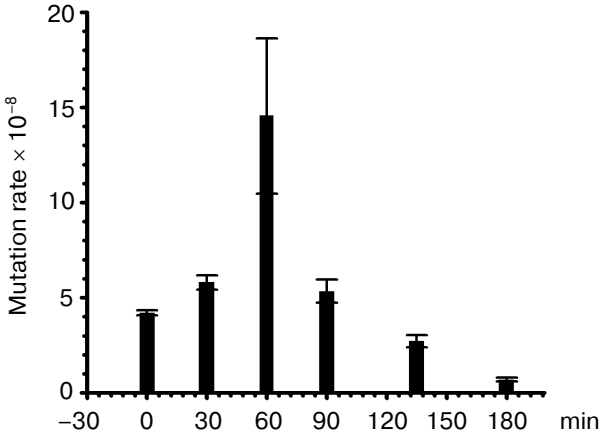

Fig. 5. Changes in the mutation rate of *E. coli* K-12 (VKM) by resistance to rifampicin under oxidative stress. The culture was grown in ANKUM culture apparatuses (see "Materials and Methods") to the density of 1-1.5 g ADB/liter. Oxidative stress was obtained by addition of 3 mM H_2O_2.

(Fig. 6), which is in agreement with its activity as a transcriptional stimulator of *oxyR* of the anti-peroxide defense regulon and properties of DNA protector [8].

Study on the effect of putrescine on number of viable cells in the culture exposed to oxidative stress showed the greatest result 1.5-2 h after the stress (Fig. 6). At this time,

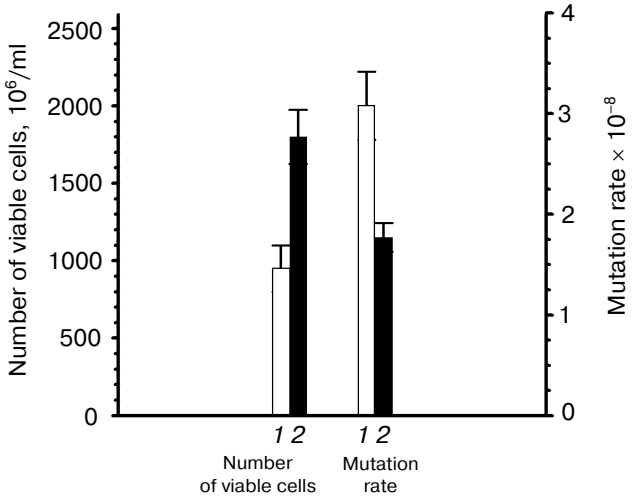

Fig. 6. Effect of putrescine on the cell survival and mutation rate of *E. coli* BGF940 under oxidative stress: *1*) oxidative stress in the absence of putrescine; *2*) the same in the presence of 5 mM putrescine in the nutritional medium. Microorganisms were grown on a shaker (see "Materials and Methods"). On reaching optical density of 0.3 (OD_{600}), the culture was supplemented with 3 mM H_2O_2. Putrescine was added into the nutritional medium initially. The data shown in the figure correspond to 2 h after oxidative stress.

5 mM putrescine increased the number of viable cells in the culture nearly twofold compared to the control. The time of appearance and multiplicity of this effect approximately corresponded to parameters of the gene expression level and rate of spontaneous mutations. Thus, it was reasonably to consider these events to be links of the same chain: oxidative stress is accompanied by increase in the activity of enzymes of polyamine synthesis and in the amount of cellular polyamines, which stimulate the expression of the defense genes of the *oxyR* regulon; the increased cellular content of gene products represented by enzymes of antioxidant defense responsible for cleavage of reactive oxygen species results in decrease in the mutation rate and increase in the number of viable cells.

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