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EXPERIMENTAL ARTICLES

Putrescine as a Factor Protecting *Escherichia coli* against Oxidative Stress

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Abstract—The level of expression of *oxyR*, the gene that protects *Escherichia coli* against oxidative stress, was enhanced by physiological concentrations of the biogenic amine putrescine. This level was directly proportional to the degree of negative DNA supercoiling. 1,4-Diamino-2-butanone (DAB), a specific inhibitor of ornithine decarboxylase, the key enzyme of polyamine synthesis, produced an inhibitory effect on the level of *oxyR* expression under oxidative stress, which was relieved by the addition of putrescine. The direct relationship between the putrescine concentration and the degree of negative DNA supercoiling suggests that the mechanism of action of putrescine as the modulator of *oxyR* transcription activity is based on both its direct influence on the gene expression level and its indirect effect mediated by topological DNA changes. Putrescine was shown to produce a protective effect if the DNA is damaged by reactive oxygen species.

Key words: *Escherichia coli*, oxidative stress, putrescine, DNA supercoiling.

Oxidative stress is one of the most widely spread kinds of stress in microorganisms. It results from the effects of reactive oxygen species (ROS), predominantly superoxide radicals (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radicals ($OH^\bullet$). Their constant presence in the cell as by products of the respiratory chain operation has promoted the formation (in microorganisms) of protective systems aiming at the removal of ROS or the elimination of their destructive effects on cell components. The genetic structures of the systems that protect against oxidative stress include a number of regulons. Of paramount importance are *oxyR* and *soxRS*, which are responsible for protecting the cell against hydrogen peroxide and superoxide radicals, respectively [1]. Due to the high reactivity of ROS, they interact with diverse biological molecules, with the nucleic acids DNA and RNA being the most sensitive target molecules. These interactions cause the formation of oxidized forms of DNA bases, resulting in an increase in the frequency of mutations inducing GC $\longrightarrow$ AT and AT $\longrightarrow$ GC transversions, the rupture of one or both polynucleotide chain(s), and other kinds of damage [2].

Based on a number of recent works, polyamines, along with the above-mentioned protective systems, are of considerable importance in terms of preventing DNA damage by ROS [3]. It was shown earlier that polyamines, due to their polycationic structure, can neutralize anionic groups of DNA, thereby causing its condensation and compactization [4]. Some researchers believe that these properties of polyamines underlie the mechanism of their protective action, based on a decrease in the accessibility of the target sites in the condensed DNA structures [5]. In addition, some polyamine species, such as spermidine, can function as free radical traps, thereby providing for DNA protection against the destructive effects of ROS [6]. However, the interactions of DNA with polyamines in terms of the operation of the protective mechanisms are apparently not confined to this function. Data have been presented to show that there is a relationship between the polyamine content in the cell and the topological state of DNA under oxidative stress [7].

The involvement of the DNA topology in the regulation of gene promoter activity (including the regulation under stress in microorganisms) has been understood well. The change in the degree of DNA supercoiling, which is characteristic of many kinds of stress, is one of the factors causing an increase in the activity of adaptive genes [8]. However, virtually no data have so far been obtained that concerns the regulatory influence of changes in the DNA topology on the expression of genes involved in adaptation to oxidative stress, such as the *oxyR* gene responsible for the protection of microorganisms from hydrogen peroxide. Plausibly, the topological activity of polyamine is involved in the regulation of the gene expression in the relevant regulon at the transcription level.

Based on this suggestion, we investigated the role of the topological activity of putrescine in the regulation of the level of *oxyR* expression. This was the goal of this work, in addition to the elucidation of the putrescine properties involved in DNA protection.

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Properties of the bacterial strains used in this work

E. coli strain	Genotype
BGF930	AraD139(argF-lac)205flbB5301 non-9 gyrA219 relAl rpsl150 metE70 btuB::Tn10 λ[oxyR'::lacZ)]
BGF940	Δ(lac)U169rpsL λ[F(oxyR'::lacZ)]

MATERIALS AND METHODS

Research subjects. The *Escherichia coli* strains used in this work were generous gifts of B. Demple and B. Gonsalez-Flecha from the Department of Molecular and Cellular Toxicology, Harvard Health School, Boston, Massachusetts (USA). The level of expression of the *oxyR* gene was determined using the oxyR'::lacZ fusion introduced into the chromosome DNA of the *E. coli* strains RK4936 and MC4100 by means of the λ phage [9, 10]. This made possible the determination of the expression level of the tested gene from β-galactosidase activity (see below).

The properties of the strains studied are given in the table. A variant of strain BGF930 transformed with plasmid pBR322 was used to investigate DNA topology. The transformation was performed by the conventional method using $CaCl_2$ [11].

Cultivation of microorganisms. The strains used in the studies (maintained on LB agar) were inoculated before the experiments into LB broth containing 50 μg/ml streptomycin. The transformed variants were inoculated into LB broth with 50 μg/ml streptomycin, 12.5 μg/ml tetracycline, and 100 μg/ml ampicillin. After 6 h of cultivation in a thermostat at 37°C, the cells were transferred to M9 medium with the antibiotics at indicated concentrations and L-methionine (100 μg/ml) and grown for 16 h in 500-ml flasks with 200 ml of M9 medium on a shaker at 37°C. The resulting culture was used as inoculum for four to six 250-ml flasks with 100 ml of M9 medium containing the antibiotics and methionine. The experimental cultures were grown under the same conditions.

The cultivation in the ANKUM-2 fermentor (Russia) was carried out aerobically (pO_2 = 80–100%) at pH 7.0 maintained by titration with 2 N NH_4OH. Consecutive additions of glucose (1–2 g/l) were made before the medium became completely glucose-depleted. Cell biomass was estimated from the optical density (OD_{600}) measured with a SF-46 spectrophotometer (LOMO, Russia) after diluting the culture with physiological saline.

β-Galactosidase activity was measured in cells pretreated with a sodium dodecyl sulfate–chloroform mixture by the Miller method [12].

The topological state of DNA was estimated from the degree of negative supercoiling of the plasmid DNA of transformed strains. The isolation of plasmid DNA from the bacterial cells was performed by the conventional alkaline hydrolysis method [13]. Topoisomer separation was carried out using horizontal electrophoresis in 1% agarose gels in TBE buffer (89 mM Tris–borate, pH 8.0, 89 mM boric acid, 2 mM EDTA) supplemented with 15 μg of chloroxine at a 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 in passing UV light. The luminescence intensity of plasmid DNA bands was measured using photometry of negatives with an MD-100 microdensitometer (Carl Zeiss). To estimate the degree of DNA supercoiling, mean Lk values were determined [14].

Polyamine content. The concentrations of putrescine and other polyamines (spermidine, cadaverine, and spermine) in the cells and in the medium were determined fluorometrically. Culture aliquots (500 μl) were centrifuged for 1 min at 16000 *g*. The cells were extracted with 0.4 N $HClO_4$ for 1 h with vigorous agitation. The supernatant was used to determine the polyamine content in the medium. 100 μl of $HClO_4$ extract (whose pH was adjusted to 9.0 with a 2 M K_2CO_3 solution) were supplemented with 100 μl of a solution of 1-dimethylaminonaphthalene-5-sulfonyl-chloride (dansyl chloride) in acetone (2.7 mg/ml) and incubated for 2 h at 37°C in the dark. The mixture was evaporated under a flow of cold air and extracted with benzene. The benzene extracts were quantitatively transferred to 100 × 100 mm silica gel plates for thin-layer chromatography (Sorbfil, Russia) and consecutively separated in solvent systems I (benzene–triethylamine, 20 : 2) and II (benzene–methanol, 10 : 0.45). The chromatograms were dried and photographed in UV light, which induced blue–green luminescence in dansyl-polyamine spots, whose size and brightness were proportional to the polyamine concentrations. Polyamine concentrations were determined by photometry of negatives with an MD-100 microdensitometer (Carl Zeiss).

The protective properties of putrescine displayed upon DNA damage by ROS were investigated using the DNA of plasmid pBR322 that had been preamplified in *E. coli* cells and isolated by alkaline hydrolysis. Plasmid DNA was dissolved in 1 M PBS buffer (NaH_2PO_4 and Na_2HPO_4, pH 7.4) containing putrescine at concentrations of 0 to 20 mM. A mixture of 500 μM Cu(II) and 1500 μM H_2O_2 was employed as the system generating free hydroxyl radicals ($OH^{\bullet}$). The samples were incubated for 1 h at 37°C, and the DNA fractions were thereupon separated by horizontal electrophoresis in

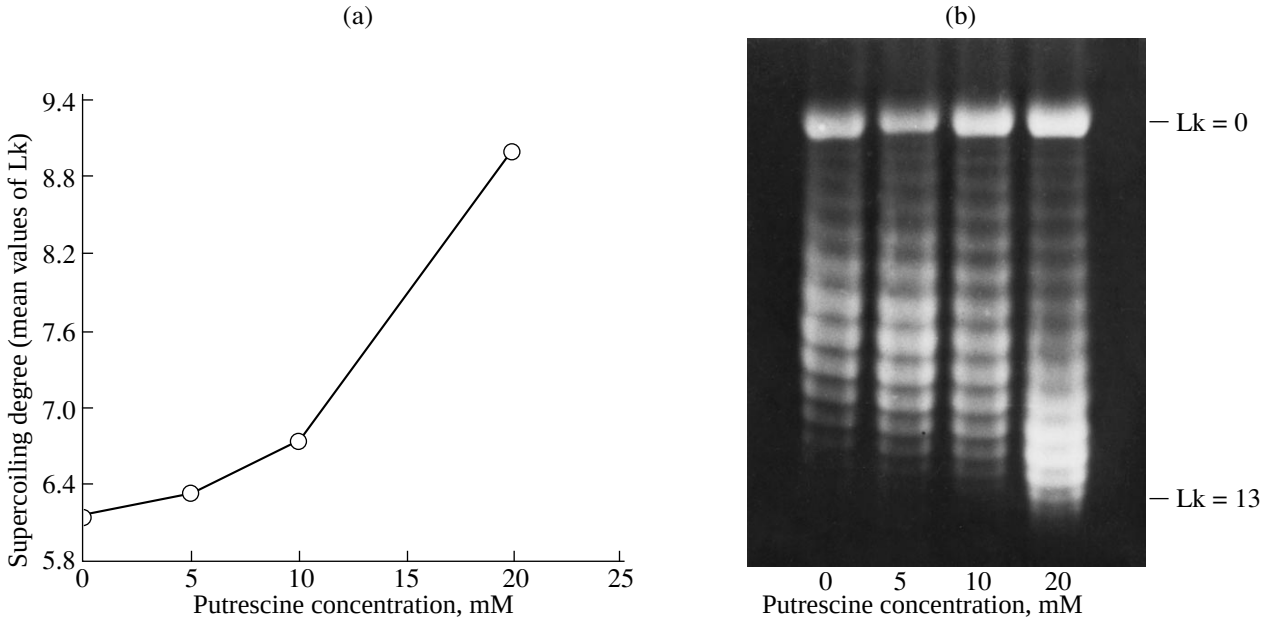

Fig. 1. Dependence of the topological state of the DNA of *E. coli* BGF930 (pBR322) on the putrescine content in the medium (a). The mean values of Lk were plotted based on the results of densitometric measurements of topoisomer contents (electrophoregram, b). Cells were cultivated in M9 medium supplemented with different putrescine concentrations, on a shaker (100 rpm) at 37°C for 18 h; thereupon, the cells were quickly cooled, the plasmid was isolated, and its topology was determined.

1% agarose gel without chlorochine for 6 h at a voltage of 2 V/cm. The gel was stained with ethidium bromide (1.0 μg/ml) for 30 min and photographed in passing UV light. The luminescence intensity of the bands of the relaxed and supercoiled fractions of plasmid DNA was measured using photometry of negatives with an MD-100 microdensitometer (Carl Zeiss). Based on the densitometric data, the peak areas were calculated, which provided estimates of the contents of the relaxed and supercoiled plasmid fractions.

Statistical treatment of the results obtained was carried out using the Statistica for Windows 5.0 software (StatSoft, Inc., 1995) in the StatsGraph mode. The means calculated for a series of uniform experiments (at least three replicate experiments were performed) are shown in the figures. The mean square deviation values are shown as vertical bars.

RESULTS AND DISCUSSION

To test the topological activity of putrescine, *E. coli* BGF930 cells transformed with plasmid pBR322 were cultivated in the presence of various putrescine concentrations (Fig. 1). An increase in the putrescine concentration in the medium was accompanied by a proportional shift in the distribution pattern of plasmid DNA topoisomers towards higher Lk values, suggesting an increase in the degree of negative supercoiling of the *E. coli* DNA in response to the addition of putrescine. Hence, the results obtained point to a strong dose-dependent stimulatory effect of putrescine within the physiological concentration range on the mean Lk value. This is consistent with our suggestion concerning the topological activity of putrescine.

The question of whether or not topological changes in the DNA can produce a regulatory effect on the level of *oxyR* expression has not yet been clarified. Therefore, we studied the relationship between the level of *oxyR* expression and the degree of negative supercoiling of the *E. coli* DNA under oxidative stress (Fig. 2). If the nutrient medium contained putrescine (20 mM), the initial value of the degree of negative supercoiling was well above that observed in the usual medium (M9), which is in agreement with the data on the topological activity of putrescine (Fig. 1). A decrease in the supercoiling degree occurred at the first stage of oxidative stress, due to the destructive effect of hydrogen peroxide on the DNA. This stage was also accompanied by a decrease in the level of *oxyR* expression. The second stage was characterized by an increase in the supercoiling degree, and the highest *oxyR* activity occurred upon attaining the maximum level of supercoiling. The direct relationship between the supercoiling degree and *oxyR* expression supports the above suggestion concerning the regulatory role of the DNA topology in *E. coli* adaptation to oxidative stress.

The role of polyamines in maintaining the native conformation of nucleic acids has been understood well [15]. It manifests itself, for instance, in the tighter binding of the complementary DNA strands, which prevents their separation under the influence of a denaturing agent and results in the elevation of the melting point

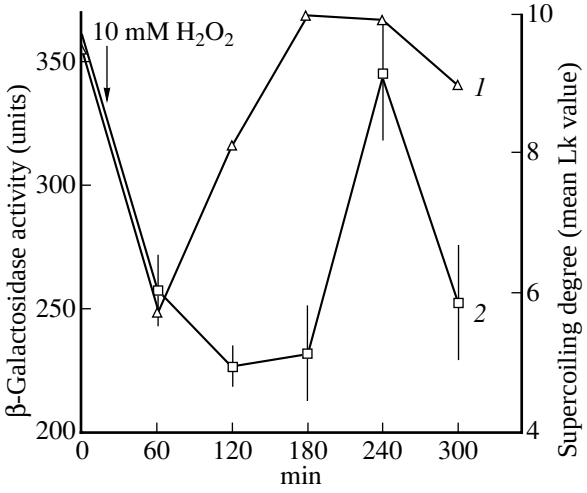

Fig. 2. Dependence of the level of *oxyR* expression on the DNA topology in the culture of *E. coli* BGF930 (pBR322) under oxidative stress. *1*, mean value of catenation site numbers; *2*, β-galactosidase activity (units). The culture was grown in a flask placed on a shaker (100 rpm) at 37°C and transferred to M9 medium with 20 mM putrescine 2 h before the beginning of the experiment.

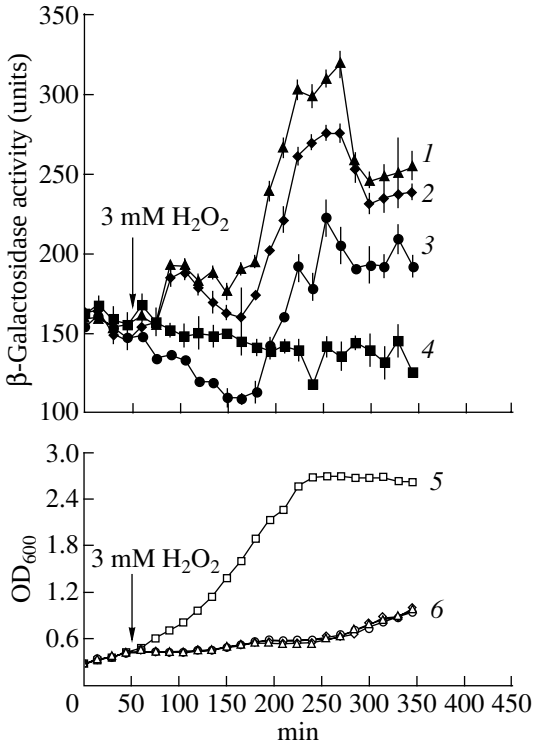

Fig. 3. Effect of putrescine (PT) on *oxyR* expression under oxidative stress in an exponential phase culture of *E. coli* BGF940. β-Galactosidase activity in culture under oxidative stress: (*1*) with 10 mM PT; (*2*) with 5 mM PT; (*3*) without PT; (*4*) in a control culture not exposed to the stress factor (grown without PT). Curve *5* shows optical density of the control culture grown without PT; curve *6* shows optical density of cultures under stress, grown at different PT concentrations. The cells were grown in M9 medium with glucose (0.3%) on a shaker. The control culture without the addition of putrescine was grown without stress, and the other three cultures were supplemented with 3 mM H_2O_2 after growing for 50 min.

[16]. This property of polyamines also seems to account for their effect on the DNA topology: tightening the bonds between DNA strands due to a change in the catenation site number (ΔLk) promotes formation of supercoils (Wr) with a minor change in the number of revolutions of one of the strands around the other strand (Tw) in accord with the well-known equation ΔLk = Tw + Wr [8]. In addition to the direct stimulatory influence on DNA supercoiling, the regulatory effect of putrescine can be due to inhibition of the activity of the DNA-relaxing enzyme DNA topoisomerase I [17].

The data obtained demonstrate for the first time the stimulatory effect of putrescine on the degree of supercoiling of *E. coli* DNA, which, in turn, may increase the promoter activity of the genes of the *oxyR* regulon under oxidative stress.

In order to confirm the putrescine role in regulating *oxyR* expression, we researched the influences of different putrescine concentrations on the level of β-galactosidase activity in *E. coli* BGF940 under oxidative stress (Fig. 4). The growth of this microorganism without oxidative stress in the synthetic M9 medium with glucose was characterized by a gradual decrease in the level of *oxyR* expression, which was apparently due to the low rate of endogenous H_2O_2 formation in the presence of glucose. The addition of hydrogen peroxide to the medium initiated biphasic changes in the β-galactosidase activity. They were similar to those described in *E. coli* cultures growing on LB broth without glucose [10]. The decrease in the *oxyR* expression that occurred immediately after the addition of H_2O_2 soon changed to an increase in the expression. The maximum value of β-galactosidase activity occurred prior to the resumption of growth of the stressed culture (Fig. 3). These data point to the stimulatory effect of putrescine on the level of *oxyR* expression under oxidative stress and, in conjunction with the results concerning the topological activity of putrescine, afford the conclusion that the mechanism of action of putrescine as a transcription activity modulator involves both its direct effect on the gene expression level and its indirect influence mediated by topological changes in the DNA.

Direct evidence of the involvement the putrescine in the regulation of the level of *oxyR* expression was obtained in studies using 1,4-diamino-2-butanone (DAB), the specific inhibitor of ornithine decarboxylase (Fig. 4) that is known to drastically decrease the putrescine level in the cell [18].

The addition of DAB to the medium resulted in a marked decrease in the β-galactosidase activity in both stages of oxidative stress (as compared to the control data in a culture without additions). Apart from completely removing the inhibitory effect of DAB,

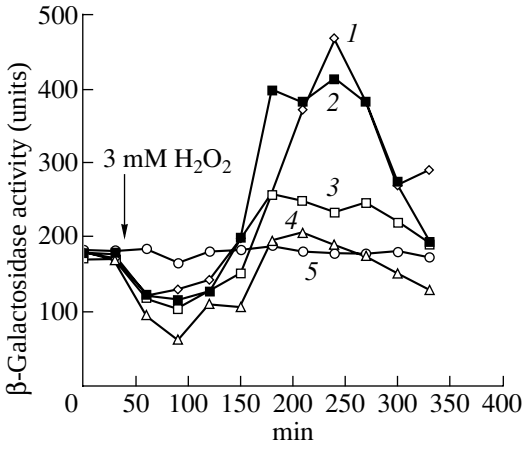

Fig. 4. Dependence of the level of *oxyR* expression in *E. coli* BGF930 on the ornithine decarboxylase inhibitor 1,4-diamino-2-butanone (DAB) under oxidative stress in the presence of (*1*) 5 mM putrescine; (*2*) 20 mM DAB and 5 mM putrescine; (*4*) 20 mM DAB. Curve *3* is control without additions; curve *5* is control without oxidative stress and without additions.

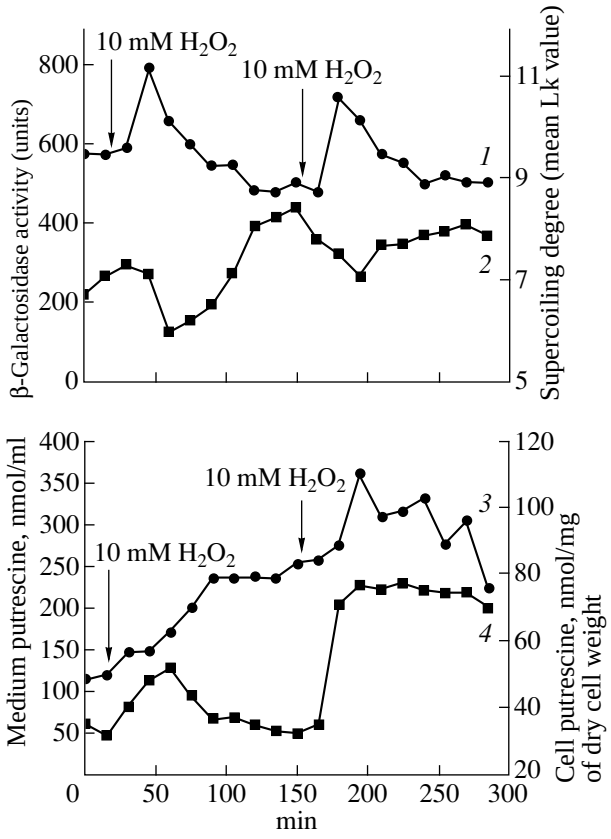

Fig. 5. Dependence of the level of *oxyR* expression in *E. coli* BGF930 (pBR322) on the supercoiling degree and on the putrescine content in the cell and the medium under oxidative stress (the culture was grown in a fermentor): (*1*) β-galactosidase activity (units); (*2*) degree of DNA supercoiling (mean Lk values); (*3*) putrescine content in the medium (nmol/ml); (*4*) putrescine content in the cells (nmol/mg of dry cell weight).

putrescine at a concentration of 5 mM caused a twofold stimulation of the level of *oxyR* expression, which approached that in an uninhibited culture stimulated with 5 mM putrescine (Fig. 4). The results of these experiments confirm the idea that putrescine is involved in the protection of cells against oxidative stress as one of the cell modulators of *oxyR* expression.

The above experiments demonstrated the regulatory effects of exogenous putrescine added to the medium. In order to prove or disprove functioning of this mechanism in vivo, we investigated the relationship between the polyamine content, *oxyR* activity, and the level of DNA supercoiling in *E. coli* BGF930 (pBR322) cells experiencing oxidative stress during controlled growth in a cultivator (Fig. 5). Growing bacteria in a cultivator enables maintaining the main medium parameters at optimum levels. This primarily concerns the dissolved oxygen content and pH, which significantly influence the polyamine pool in the cell and the medium [19, 20]. The culture grown under these is characterized by high values of cell density and a specific growth rate; therefore, increased hydrogen peroxide concentrations (10 mM) could be used in these studies.

When grown in the cultivator under conditions of high cell density, the transformed strain *E. coli* BGF930 (pBR322) was characterized by a higher baseline level of *oxyR* expression than the flask-grown culture (Fig. 5). Against this background, the addition of hydrogen peroxide to a concentration of 10 mM resulted in an immediate 1.5-fold increase in the β-galactosidase activity; upon the second addition of hydrogen peroxide, the increase occurred again. In contrast, in cultures grown on shakers with low cell densities, the response to the addition of H_2O_2 proceeded after a considerable lag (Figs. 2, 3). Although the reasons for these phenomena remain unclear, culture growth in a cultivator with intense aeration and, accordingly, an increased respiration rate and enhanced formation of reactive oxygen species presumably provides for a high baseline level of *oxyR* expression and the preadapted state of the cells that promotes rapid response to the stress.

The peak values of *oxyR* activity in response to oxidative stress occurred simultaneously with a 1.5- to 2.5-fold increase in the putrescine pool in the cell (subsequently, putrescine was released into the medium). The increase in the putrescine pool is consistent with the above data on the stimulatory effect of putrescine on the level of *oxyR* expression (Fig. 3). The increase in the total putrescine pool in the cells and in the medium during *E. coli* cultivation under oxidative stress points to a significant activation of putrescine synthesis. We demonstrated this earlier for the wild strain *E. coli* K-12 (VKM) under analogous conditions [7].

Our research on the DNA topology under oxidative stress in *E. coli* BGF930 (pBR322) cultures with relatively high cell densities (Fig. 5) revealed a clear-cut biphasic pattern of changes in the DNA supercoiling degree. The relaxation period was followed by an

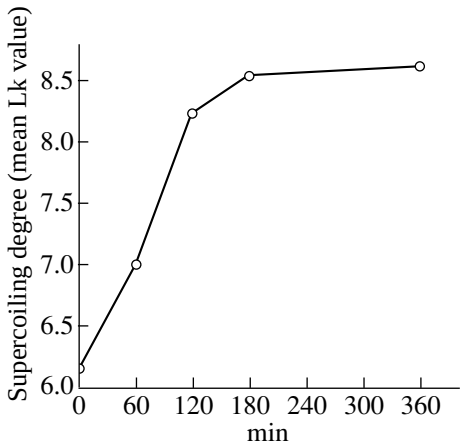

Fig. 6. Effect of putrescine on the DNA topology in *E. coli* BGF930 (pBR322). Cells were grown on a shaker (100 rpm) overnight at 37°C, transferred to fresh M9 medium containing 20 mM putrescine, and cultivated on a shaker under the same conditions. The supercoiling degree was determined in samples taken from the culture.

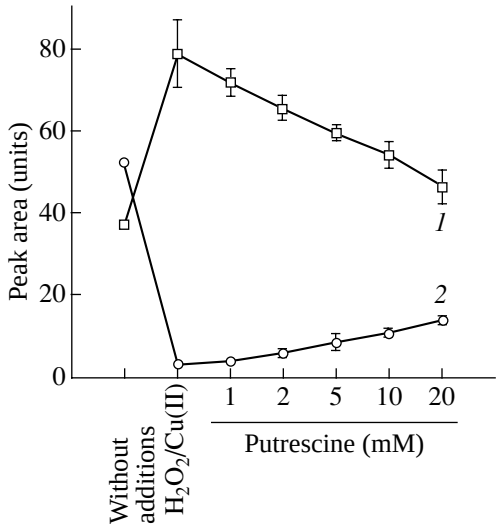

Fig. 7. Protective effect of putrescine on the DNA of plasmid pBR322 damaged by the products ($OH^{\bullet}$) of Fenton reaction: *1*, relaxed plasmid fraction; *2*, supercoiled plasmid fraction.

increase in the supercoiling degree, which exceeded the initial level 2 h after the increase in the putrescine pool in the cells and in the medium.

Our data on the effect of exogenous putrescine on the DNA topology revealed a comparable temporal pattern of the manifestation of the putrescine topological activity (Fig. 6). Hence, the rapid change in the level of *oxyR* expression in response to oxidative stress in relatively dense *E. coli* cultures is apparently unrelated to the increase in the DNA supercoiling degree. However, putrescine obviously exerts a direct stimulatory influence on *oxyR* activity under these circumstances.

Based on the data obtained, the regulatory function of putrescine under stress is likely to result from the interaction of two factors: (1) the direct effect of putrescine on the promoter activity of the stress-related *oxyR* gene and (2) the indirect effect mediated by changes in the DNA topology. The latter factor is apparently involved in the preadaptive strategy based on enhancement of the promoter activity of genes via the increase in the degree of negative DNA supercoiling [8].

Our studies on the contents of other polyamines characteristic of *E. coli* (spermidine, spermine, and cadaverine) failed to reveal any appreciable increase in their amounts under oxidative stress (data not shown). Hence, in this bacterial species, putrescine is of greater importance in terms of cell protection against oxidative stress than other polyamines.

In addition to the direct effect of putrescine on the expression level of *oxyR* regulon genes and its indirect effect mediated by DNA topology, one cannot rule out the existence of other possible putrescine-involving protective mechanisms. In light of earlier data on the protective properties of another natural-polyamine spermine in relation to ROS-induced DNA damage [3], studies concerning analogous properties of putrescine seemed to be of considerable interest.

The destructive effect of H_2O_2 on DNA involves its conversion to $OH^{\bullet}$ in the presence of metals with variable valencies (the Fenton reaction) [1]. We used a mixture of 1.5 mM H_2O_2 and 0.5 mM $CuCl_2$ (Cu(II)). At these substrate concentrations, the Fenton reaction resulted in the formation of $OH^{\bullet}$ amounts sufficient to secure almost the complete conversion of the supercoiled fraction of plasmid pBR322 into the relaxed form as a result of rupturing one or both DNA strands (Fig. 7). The addition of putrescine (within the physiological concentration range) to the plasmid prior to the onset of the Fenton reaction produced a significant protective effect manifested in an increase in the amount of the supercoiled plasmid fraction and a concomitant decrease in the amount of the relaxed fraction (Fig. 7). The protective effect was directly proportional to the polyamine concentration.

Hence, the data on the putrescine role in the protection of *E. coli* against oxidative stress demonstrate that putrescine is involved in at least three mechanisms providing for the adaptation of this bacterium to the destructive effect of hydrogen peroxide: (1) the regulation of the expression level of the *oxyR* regulon; (2) the regulation of the promoter activity of genes by altering the DNA topology; and (3) the protection of the structural integrity of DNA.

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