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EXPERIMENTAL ARTICLES

Role of Putrescine in the Regulation of the Expression of the Oxidative Stress Defense Genes of *Escherichia coli*

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Abstract—The role of putrescine in the adaptive response of *Escherichia coli* grown aerobically in synthetic M9 medium with glucose to the H_2O_2-induced oxidative stress was studied. Under oxidative stress, the expression of the single-copy reporter gene fusions *oxyR'::lacZ* and *katG'::lacZ* was found to undergo biphasic changes, which were most pronounced in glucose-starved *E. coli* cells. The concentration-dependent activating effect of putrescine on the expression of the OxyR regulon genes was maximum when the *oxyR* gene was inhibited by high concentrations of hydrogen peroxide.

Key words: polyamines, oxidative stress, DNA topology, gene expression.

Polyamines, ubiquitous biogenic polycations of both prokaryotic and eukaryotic cells, are involved in many biosynthetic processes [1, 2], although their functions at a molecular level are as yet poorly understood. Some recent data indicate that polyamines may protect eukaryotic DNA from the action of reactive oxygen species and radiolysis products [3, 4]. However, the role of polyamines is not presumably limited to their functioning as free radical scavengers [5]. For instance, there is evidence that polyamines may activate the promoters of some eukaryotic genes, such as *c-myc*, acting as a preoncogen in malignant cellular processes [6]. The regulatory role of polyamines in the expression of microbial genes, the oxidative stress response genes in particular, remains to be studied.

There are two major regulons responsible for the protection of *E. coli* against oxidative stress, the OxyR and SoxRS regulons. The transcriptional regulators of these regulons, OxyR and SoxRS, induce the expression of the antioxidant defense genes in response to the action of hydrogen peroxide and superoxide anion, respectively. Of 30–40 proteins that are induced in the H_2O_2-treated *E. coli* cells, eight proteins are encoded by the OxyR regulon genes [7]. In such cells, the expression of the *oxyR* gene is believed not to be activated, since OxyR possesses the properties of an autorepressor [8]. However, some data indicate that the expression of *oxyR* in *E. coli* cells depends on their growth phase and the cellular level of cAMP [9, 10]. Therefore, the regulation of the *oxyR* gene expression may play an important biological role, which suggests the involvement of other cellular regulators, first of all polyamines, in the regulation of this gene.

Putrescine is the major polyamine in *E. coli* cells, whose concentration undergoes considerable changes under varying growth conditions [11]. This prompted us to investigate the role of this particular polyamine in the adaptive response of *E. coli* cells to hydrogen peroxide-induced oxidative stress.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The bacterial strains used in this study (see table) were a generous gift from B. Demple and B. Gonzalez-Flecha, the Department of Molecular and Cellular Toxicology of the Harvard School of Public Health (Boston, Massachusetts, the United States). In order to determine the OxyR regulon gene expression levels through the activity of β-galactosidase, the chromosomal DNAs of the *E. coli* strains MC4110 and RK4936 were marked, using bacteriophage λ, by the reporter gene fusions *oxyR'::lacZ* and *katG'::lacZ* [9, 12]. The strains were maintained on LB agar slants.

Experiments were carried out with the exponential-phase *E. coli* cultures grown aerobically in synthetic M9 medium with glucose. Material for inoculation was prepared as follows:. Cells, washed off of LB agar slants, were cultivated at 37°C in LB broth with 50 μg/ml streptomycin for 6 h, transferred to 500-ml flasks with 200 ml of M9 medium supplemented with the same amount of streptomycin and 100 μg/ml L-methionine, and cultivated for the next 16 h at 37°C on a shaker (100 rpm). This culture was used to inoculate four to six 250-ml flasks with 100 ml of M9 medium with the same additions as above. These flasks

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Bacterial strains used in this study

E. coli strain	Genotype
BGF930	Derivative of RK4936 with the gene fusion λ[Φ(*oxyR*'::*lacZ*)]
BGF931	Derivative of RK4936 with the gene fusion λ[Φ(*katG*'::*lacZ*)]
BGF940	Derivative of MC4100 with the gene fusion λ[Φ(*oxyR*'::*lacZ*)]
RK4936	AraD139(*argF-lac*)205*flb*B5301 *non-9 gyrA219 relA1* *rpsll50 metE*70 *btuB*::*Tn10*
MC4100	Δ(*lac*)*U169 rpsL*

were incubated under the same conditions as the 500-ml flask.

The biomass was estimated by measuring the optical density of cultures at 600 nm (OD_{600}). If necessary, the cultures were diluted with a physiological saline solution. Optical density was measured with an SF-46 spectrophotometer (LOMO, Russia).

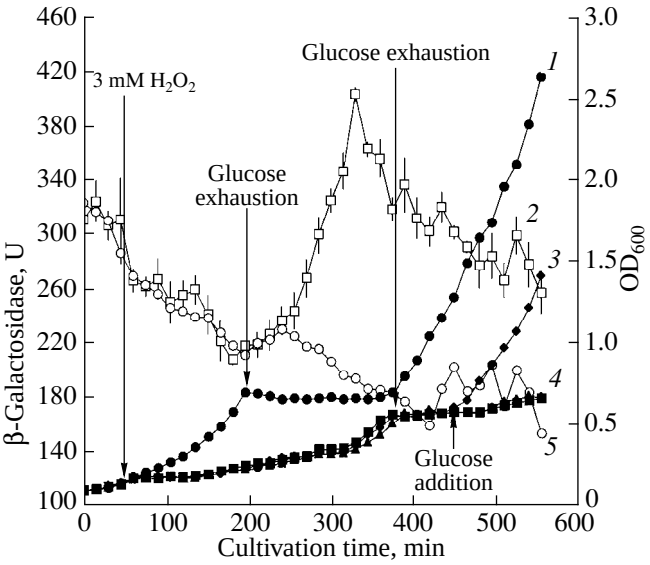

Fig. 1. Effect of glucose deficiency on the *oxyR* expression (estimated through β-galactosidase activity) in the *E. coli* BGF930 cultures subjected to oxidative stress: *1*, growth (OD_{600}) of the control (unstressed) culture; *2*, the β-galactosidase activity of H_2O_2-stressed cells; *3*, growth of the H_2O_2-stressed culture after the addition of glucose; *4*, growth of the glucose-starved H_2O_2-stressed culture; and *5*, the β-galactosidase activity of unstressed cells. Cells were grown in M9 medium with 0.02% glucose on a shaker. Oxidative stress was induced by adding hydrogen peroxide to 50-min-old cultures to a final concentration of 3 mM. The arrows indicate the times of the hydrogen peroxide and glucose additions and glucose exhaustion in the growth media.

β-Galactosidase was assayed by the Miller method using *E. coli* cells, which were preliminarily permeabilized through the treatment with sodium dodecyl sulfate and chloroform [13].

Statistics. Experimental results were statistically processed using the Statistica version 5.0 software program for Windows 95 (StatSoft, Inc.). The results presented are the means of measurements performed at least in triplicate. The error bars in the figures represent standard errors.

RESULTS AND DISCUSSION

The degree of expression of the *oxyR* gene in the exponential-phase cultures of *E. coli* BGF930 with the single-copy *oxyR*'::*lacZ* fusion was evaluated by the activity of β-galactosidase. During the growth of this strain in synthetic M9 medium with glucose without hydrogen peroxide, the degree of expression of the *oxyR* gene gradually decreased (Fig. 1). At the same time, according to the data of Gonzalez-Flecha and Demple [9], the expression of the *E. coli oxyR* gene in LB broth is biphasic. The absence of the stage of an increased expression of *oxyR* during the growth of *E. coli* in synthetic medium with glucose can presumably be explained by the low rate of endogenous formation of H_2O_2 in aerobically grown *E. coli* cells [14]. The inhibitory effect of glucose on the *oxyR* expression was also observed in the LB broth-grown *E. coli* culture [9].

The addition of hydrogen peroxide to the growth medium led to an increase in the *oxyR* gene expression, which peaked 30–40 min before the glucose exhaustion in the medium and growth cessation (Fig. 1). This peak in the degree of the *oxyR* expression approximately corresponds to the maximum level of cAMP in glucose-limited batch *E. coli* cultures [15], which confirms the inhibitory effect of glucose on the expression of the *E. coli oxyR* gene revealed by Christman *et al.* [9].

During the growth of *E. coli* in M9 medium with glucose, as in the case of its growth in LB broth without glucose [9], the expression of the *oxyR* gene was biphasic, with nearly equal durations (~ 2 h) of its induction phases and suppression (Fig. 1). The maximum *oxyR* expression preceded the rise in the H_2O_2-suppressed growth rate of *E. coli*, suggesting that bacterial cells reached a state of adaptation to H_2O_2. This suggestion is confirmed by the increase in the growth rate of the glucose-starved culture in response to the addition of glucose (Fig. 1). The peak in β-galactosidase activity was followed by its gradual decrease, irrespective of whether glucose was added again or not.

These data may be interpreted as an indication that cAMP is a regulator of the *oxyR* gene in *E. coli* [9]. Catabolic glucose repression is obviously due to the inability of the CAP protein to promote the binding of the RNA polymerase to the *oxyR* gene promoter in the absence of cAMP. Polyamines, putrescine in particular, may influence protein–nucleic acid interactions by

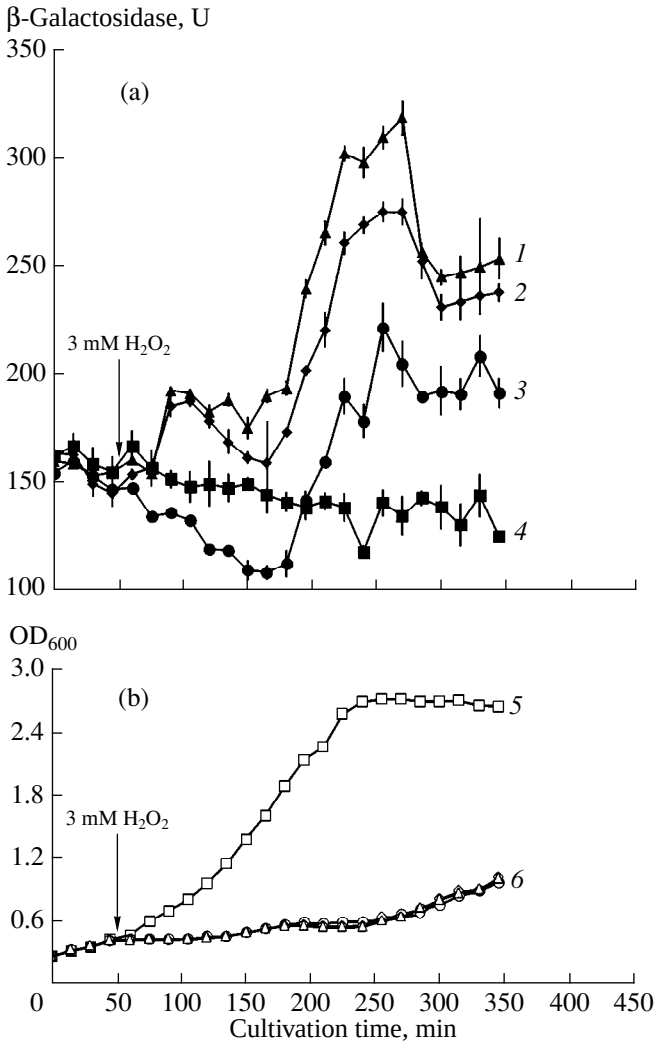

Fig. 2. Effect of putrescine on (a) the *oxyR* expression (estimated through β-galactosidase activity) and (b) growth (OD_{600}) of the *E. coli* BGF940 cultures subjected to oxidative stress: (*1*, *2*, and *3*) H_2O_2-stressed cells grown in the presence of 10, 5, and 0 mM putrescine, respectively; (*4* and *5*) the control (unstressed cells grown without putrescine); and (*6*) growth of H_2O_2-stressed cells in the presence of different putrescine concentrations. Cells were grown in M9 medium with 0.3% glucose on a shaker. Oxidative stress was induced by adding hydrogen peroxide to 50-min-old cultures to a final concentration of 3 mM.

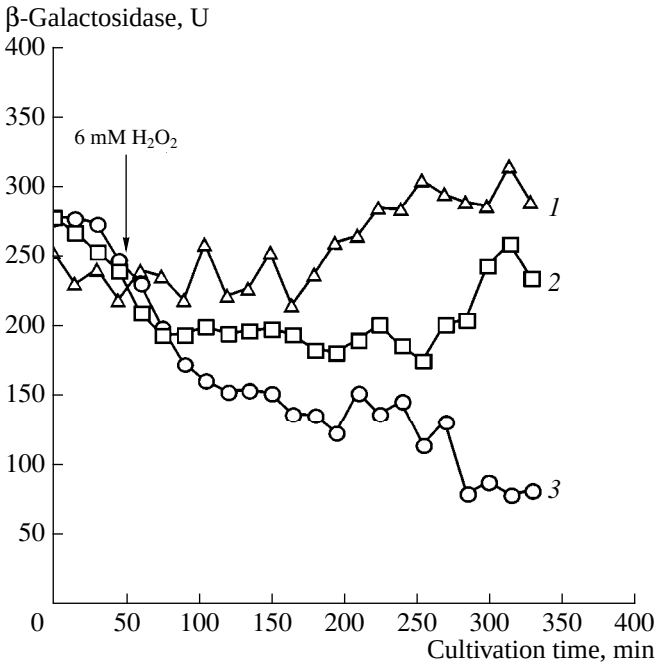

Fig. 3. Effect of culture density on the *oxyR* expression (estimated through β-galactosidase activity) in *E. coli* BGF930 subjected to oxidative stress: *1*, *2*, and *3* correspond to culture densities (OD_{600}) of 1.0, 0.2, and 0.1, respectively. Oxidative stress was induced by adding hydrogen peroxide to 50-min-old cultures to a final concentration of 6 mM.

releasing the *lac* gene repressor from the DNA–repressor complex [16]. This property of polyamines is due to the presence of positive charges in their molecules, which are located at distances, providing their optimal interactions with polyanionic DNA strands [17]. Taking into account our earlier data that polyamines are involved in the adaptation of *E. coli* to various stresses [18, 19], we attempted to evaluate the effect of putrescine on the degree of expression of the *oxyR* gene during the adaptation of this bacterium to oxidative stress.

These investigations were carried out with the *E. coli* BGF940 marked by the single-copy *oxyR*'::*lacZ* fusion. As in the case with strain BGF930, the growth of strain BGF940 in synthetic M9 medium with glucose in the absence of hydrogen peroxide was not accompanied by noticeable changes in the *oxyR* expression level (Fig. 2). In these experiments, to provide for unlimited growth throughout the cultivation period, the concentration of glucose in the medium (0.3%) was considerably higher than in the glucose starvation experiments (0.02%). For this reason, the initial β-galactosidase activity and the amplitudes of its biphasic H_2O_2-induced changes were lower than in glucose-starved cells. When putrescine was added to the medium at physiological concentrations, it exerted a concentration-dependent stimulating effect on the expression of the *oxyR* gene, shortening the lag phase in the β-galactosidase activity rise and increasing its maximum level (Fig. 2).

Further experiments showed that the response of the *oxyR* gene to oxidative stress was largely determined by the proportion between the concentrations of hydrogen peroxide and the biomass (Fig. 3): the same H_2O_2 concentrations led to a substantial increase in the β-galactosidase activity of dense *E. coli* cultures with $OD_{600} = 1$, to a small increase in the β-galactosidase activity of dilute cultures with $OD_{600} = 0.2$, and exerted a slight inhibitory effect on the β-galactosidase activity of dilute cultures with $OD_{600} = 0.1$. Similar results (not

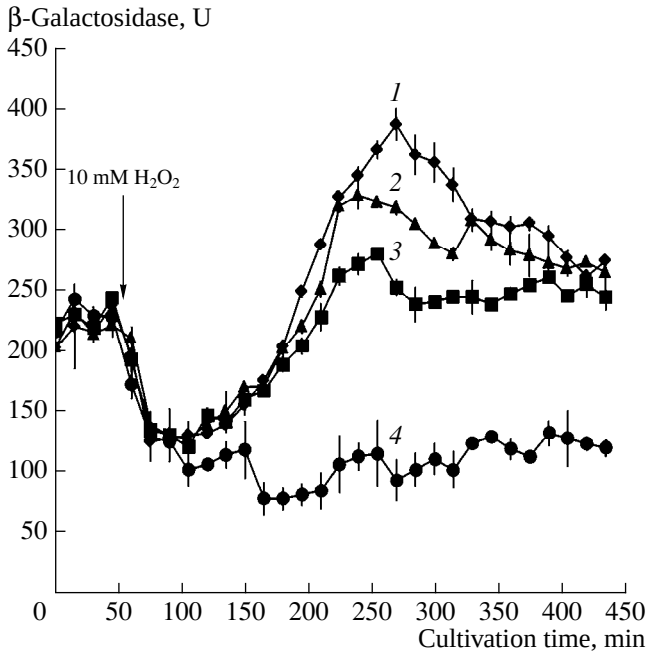

Fig. 4. The *oxyR* expression (estimated through β-galactosidase activity) in *E. coli* BGF930 cells subjected to severe oxidative stress and grown in the presence of different putrescine concentrations (mM): *1*, 5; *2*, 10; *3*, *2*; and *4*, 0. Oxidative stress was induced by adding hydrogen peroxide to 50-min-old cultures to a final concentration of 10 mM.

presented here) were obtained in the experiments in which cultures had the same density but the H_2O_2 concentrations were different. These data can be explained by the different physiological states of cells in cultures with different densities, which may have different cellular levels of putrescine.

The low level of expression of the *oxyR* gene in the presence of hydrogen peroxide may be due to the autoinhibition of this gene by the oxidized form of its own product OxyR [8]. This supposition is consistent with the finding of Bryans *et al.* that putrescine competes with the *lac* gene repressor for the binding sites in the operator regions of DNA [6].

To prove this supposition, we measured the degree of expression of the *oxyR* gene at high H_2O_2 concentrations as a function of the putrescine concentration (Fig. 4) and found that putrescine increased β-galactosidase activity by a factor of 2.5 at a concentration of 2 mM and by a factor of 4 at a concentration of 5 mM. At higher concentrations, the stimulatory effect of putrescine on β-galactosidase diminished. A comparison of these results with the effect of moderate concentrations of hydrogen peroxide (Fig. 2) showed that putrescine, as a regulator of the *oxyR* gene, is most efficient when oxidative stress is severe, which confirms the supposition that putrescine competes with oxidized OxyR, which serves as the *oxyR* gene autoinhibitor. These data suggest that polyamines may play an important role in the regulation of all OxyR regulon genes.

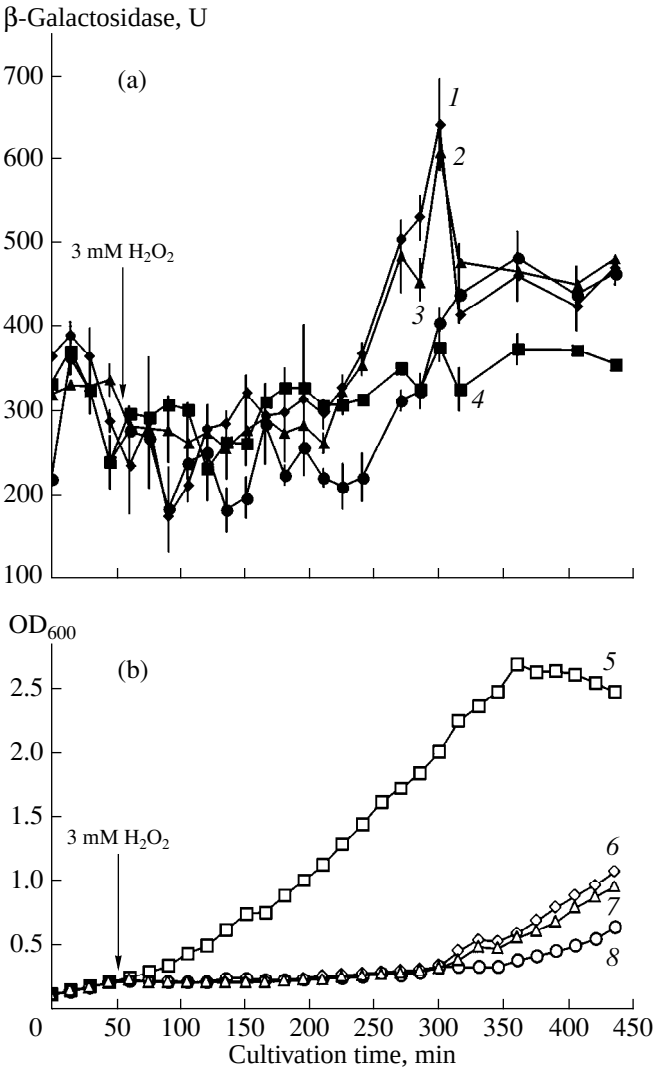

Fig. 5. Effect of putrescine on (a) the *katG* expression (estimated through β-galactosidase activity) and (b) growth (OD_{600}) of the *E. coli* BGF931 cultures subjected to oxidative stress: (*1*, *2*, and *3*) H_2O_2-stressed cells grown in the presence of 5, 10, and 0 mM putrescine, respectively; (*4* and *5*) control (unstressed cells grown without putrescine); and (*6*, *7*, and *8*) growth of H_2O_2-stressed cells in the presence of putrescine (5, 10, and 0 mM, respectively). Cells were grown in M9 medium with 0.3% glucose on a shaker. Oxidative stress was induced by adding hydrogen peroxide to 50-min-old cultures to a final concentration of 3 mM.

This suggestion was verified by measuring the degree of expression of the *katG* gene encoding the HPI catalase, the key enzyme in the oxidative stress defense of bacterial cells. In these experiments, we used the *E. coli* strain BGF931 harboring the single-copy *katG*'::*lacZ* gene fusion (Fig. 5).

As can be seen from Fig. 5, putrescine considerably accelerated and enhanced the expression of the *katG* gene, much as it enhanced the expression of the *oxyR* gene. The stimulatory effects of putrescine at 5 and 10 mM concentrations were almost the same,

indicating that the effects were saturated at these putrescine concentrations. The analysis of the growth curves showed that the activation of the *katG* and *oxyR* genes by putrescine may explain the beneficial effect of this compound on the H_2O_2-inhibited growth of *E. coli* (Fig. 5).

It should be noted that the stimulatory effect of putrescine on the *katG* gene expression can be mediated through the enhanced expression of the *oxyR* gene.

Thus, the regulation of the expression of the OxyR regulon genes, *oxyR* in particular, may be one of the mechanisms responsible for the protective effect of putrescine against the H_2O_2-induced oxidative stress in *E. coli*. On the other hand, the possibility that putrescine is also involved in the regulation of other oxidative stress defense regulons of *E. coli* cannot be excluded, which calls for further studies along this research line.

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