
EXPERIMENTAL
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The Role of Thiol Redox Systems in the Peroxide Stress Response of *Escherichia coli*

O. N. Oktyabrskii¹, N. G. Muzyka, V. Yu. Ushakov, and G. V. Smirnova

*Institute of Ecology and Genetics of Microorganisms, Ural Division, Russian Academy of Sciences,
ul. Goleva 13, Perm, 614081 Russia*

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Abstract—The effect of mutations in the genes encoding glutathione, glutaredoxin, thioredoxin, and thioredoxin reductase on the response of growing *Escherichia coli* to oxidative stress was studied. The *gshA* mutants defective in glutathione synthesis had the lowest resistance to high doses of H₂O₂, whereas the *trxB* mutants defective in thioredoxin reductase synthesis had the highest resistance to this oxidant, exceeding that of the parent strain. Among the studied mutants, the *trxB* cells demonstrated the highest basic levels of catalase activity and intracellular glutathione; they were able to rapidly reach the normal GSH level after oxidative stress. At the same time, these bacteria showed high frequency of induced mutations. The expression of the *katG* and *sulA* genes suggests that, having different sensitivity to high oxidant concentrations, the studied mutants differ primarily in their ability to induce the antioxidant genes of the OxyR and SOS regulons.

Keywords: oxidative stress, glutathione, thioredoxin reductase, gene expression.

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Reactive oxygen species (ROS) exert toxic and mutagenic effects on all cell forms by inducing oxidative damage of membrane lipids, proteins, and DNA. Both normal aerobic metabolism and exogenic chemical compounds can be sources of ROS, generating them directly or indirectly. Bacterial cells constitutively synthesize antioxidant enzymes and possess genetically controlled adaptive response mechanisms, due to which cells that have received low doses of oxidants become resistant to higher doses. In *Escherichia coli* cells, low doses of H₂O₂ induce the expression of a number of genes (some of these are controlled by the transcriptional factor OxyR) protecting the cells from peroxide-induced stress. The OxyR regulon includes the genes encoding catalase–hydroperoxidase HPI (*katG*), alkyl hydroperoxide reductase (*ahpCF*), as well as a number of other genes [1]. Oxidative damage of DNA induces also the activation of the SOS regulon which is controlled by two genes, *lexA* and *recA*, and plays a key role in DNA reparation and mutagenesis [2, 3].

In *E. coli* cells, a number of thiol redox systems are involved in the antioxidant system. A tripeptide, glutathione, as well as glutaredoxins (Grx1, Grx 2, and Grx C) and thioredoxins (Trx1, Trx 2), which belong to thiol disulfide oxidoreductases, are the key components of this system [4, 5].

Glutathione (GSH) is an important component of the antioxidant protective mechanisms in eukaryotic

cells. Although GSH plays a key role in the adaptation of *E. coli* cells to osmotic and cold stresses, as well as to some toxic agents, its role as an antioxidant is not well understood [6, 7].

Glutaredoxins induce the reduction of disulfides or mixed disulfides through the GSH pathway. The resultant glutathione disulfide (GSSG) is reduced by NADPH-dependent glutathione reductase (GOR). In *E. coli* cells, thioredoxin 1 may serve as an electron donor for ribonucleotide reductase, 3'-phosphoadenyl sulfate reductase (PAPS), and methionine sulfoxide reductase [4, 5]. The thioredoxin oxidized during the transfer of reducing equivalents to its substrates is reduced by NADPH-dependent thioredoxin reductase. There is a balance between the thioredoxin, glutaredoxin 1, and glutathione levels in *E. coli* cells. Many of their functions overlap and duplicate each other [4, 8]. It is noteworthy that, in *E. coli* cells, Grx1 is involved in the OxyR reduction. In turn, OxyR controls, directly or indirectly, the genes *gor*, *grxA*, and *trxC* encoding the syntheses of glutathione reductase, glutaredoxin, and thioredoxin 2, respectively. Hence, OxyR is involved both in the maintenance of the homeostasis of intracellular H₂O₂ and in the regulation of the thiol–disulfide status of the cell [1, 9].

This work is devoted to the study of the effect of thiol redox systems on the response of the growing *E. coli* cells to superoxide stress induced by hydrogen peroxide.

¹ Corresponding author; e-mail: oktyabr@iegm.ru

Table 1. The *E. coli* strains used in this study

Strain	Relevant genotype	Source or reference
DM4000	<i>hisG4 argE3 thr-1⁻ ara-14 xyl-5 mtl-1 rpsL31 tsx-33 ilv TS sulA::Mud1(bla lac) cam (sulA::lacZ)</i>	M. Volkert*
RI89	MC1000 <i>phoR Δara-714 leu⁺</i>	J. Beckwith**
RI336	RI89 <i>gshA::Tn10 Kan^r</i>	J. Beckwith**
RI363	RI89 <i>ΔtrxA::Kan^r</i>	J. Beckwith**
RI319	RI89 <i>trxB::Kan^r</i>	J. Beckwith**
WP812	DHB4 <i>grxA::Kan^r</i>	J. Beckwith**
BM11	RI89 <i>katG::lacZ</i>	Present work
BM31	RI336 <i>katG::lacZ</i>	Present work
BM41	RI363 <i>katG::lacZ</i>	Present work
BM21	RI319 <i>katG::lacZ</i>	Present work
NM131	RI89 <i>sulA::lacZ</i>	Present work
NM141	RI336 <i>sulA::lacZ</i>	Present work
NM151	RI363 <i>sulA::lacZ</i>	Present work
NM161	RI319 <i>sulA::lacZ</i>	Present work
NM171	WP812 <i>sulA::lacZ</i>	Present work
Plasmid pRK4936/pKT1033 with <i>katG::lacZ</i>		K. Tao et al.***

Notes: * University of Massachusetts, United States.

** Harvard University, United States.

*** Tokyo University, Japan.

MATERIALS AND METHODS

The *Escherichia coli* strains used in this work are listed in Table 1. Strains NM131, NM141, NM151, NM161, and NM171 were obtained by a nonspecific transduction with the bacteriophage P1 of the *sulA::lacZ* fusion from strain *E. coli* DM4000 to strains RI89, RI319, RI336, and RI363 [10]. Strains BM11, BM21, BM31, and BM-41 were obtained by transferring the plasmid pRK4936/pKT1033 [11] with the *katG::lacZ* fusion to strains RI89, RI319, RI336, and RI363, respectively.

The strains were grown overnight in minimal M9 medium [12] supplemented with 0.4% glucose, 0.2% casamino acids, and 10 µg/ml thiamine. The cells were precipitated by centrifugation, resuspended in fresh medium (100 ml), and cultivated in 250-ml flasks on a shaker (150 rpm) at 37°C. The growth of the cells was estimated from the optical density of the culture at 670 nm. Hydrogen peroxide (H₂O₂) was added to the medium when the culture density was 0.4 g dry wt cells/l. The numbers of viable cells were determined by counting colonies on petri dishes with agarized medium after plating the dilutions of the culture obtained from the samples treated or untreated with H₂O₂.

The reduced (GSH) and oxidized (GSSG) forms of glutathione were determined spectrophotometrically [13], as described earlier [14]. Protein concentrations were determined by the method of Lowry et al. The expression of the *sulA* and *katG* genes was studied by measuring the β-galactosidase activity of the strains harboring the promoter fusions of these genes with the structural gene of β-galactosidase [12].

The catalase activity was measured separately for each catalase (HPI and HPII) [15]. A catalase activity unit (U) is defined as 1.0 µmol H₂O₂ degraded in 1 min per 1.0 mg of total protein. The intracellular concentration of H₂O₂ was determined using scopoletin on a Shimadzu RF-1501 spectrofluorimeter (Japan) with excitation and emission wavelengths of 350 and 460 nm, respectively, and expressed in µmols per one liter of the medium (µM) [16]. The frequency of mutations was determined according to the numbers of colonies grown on petri dishes with LB agar in the presence of rifampicin [16].

All the experiments were performed at least in triplicate. The results presented in this paper are the mean values ± the standard deviation. The statistical significance of the data was evaluated by using Student's *t*-test for significance level *P* < 0.05.

Agar, casamino acids, thiamine, deoxycholate, glutathione reductase, mercaptoethanol, DTNB, EDTA, NADPH, GSH, GSSG, NEM, ONPG, and antibiotics from Sigma Chemical Co., St. Louis, MO, United States were used. All other reagents used were of analytical grade.

RESULTS

Out of all the studied strains grown on media without H₂O₂, bacteria carrying the mutant genes *trxB* and *grxA* were characterized by the highest growth rates; the growth rate of the *gshA* mutants was lowest (data not presented). The addition of the oxidant inhibited bacterial growth in a dose-dependent manner; the cells of *E. coli* RI336 (*gshA*), defective in glutathione synthesis, were the least resistant to H₂O₂. This effect was most pronounced at H₂O₂ concentration of 5 mM, when the growth rate of the mutant dropped to almost zero (Fig. 1). Under the same conditions, strains RI319 (*trxB*) and WP812 (*grxA*), defective in thioredoxin reductase and glutaredoxin 1 synthesis, were more resistant to H₂O₂ than the parent strain RI89. The responses of the parent cells and the cells of strain RI363 (*trxA* mutants), defective in thioredoxin synthesis, to the oxidant-induced stress were similar. A correlation between the effect of H₂O₂ on bacterial growth and on cell viability was observed. When treated with 5.0 mM H₂O₂, the *trxB* mutants showed the highest resistance in both respects, whereas the *gshA* mutants demonstrated the least resistance.

An increase in the intracellular concentration of H₂O₂ may cause an increase in the numbers of induced

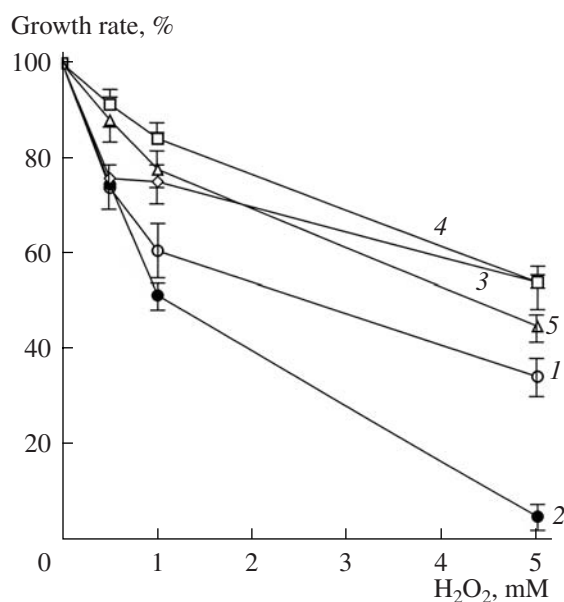


Fig. 1. Effect of H_2O_2 on the growth of *E. coli* cells defective in the genes controlling various redox systems: (1) RI89 (parent strain); (2) RI336 (*gshA*); (3) WP812 (*grxA*); (4) RI319 (*trxB*); (5) RI363 (ΔtrxA). The specific growth rate (μ) of the strains grown in the absence of H_2O_2 is taken as 100%.

mutations [2]. In our case, the strains grown in the absence of H_2O_2 showed no difference in the mutation frequency. The treatment of cells with 5 mM H_2O_2 caused a statistically significant increase in the mutation frequency only in the mutants defective in thioredoxin reductase synthesis (Fig. 2). It is remarkable that, after the exposure of the studied strains to another mutagenic factor, UV radiation (254 nm), the *trxB* mutants showed the highest rates of mutations as well (data not presented).

In aerobically growing *E. coli* strains, at high concentration of the exogenic H_2O_2 , cells detoxify hydrogen peroxide with the aid of the HPI catalase encoded by the *katG* gene, whereas the HPII catalase encoded by the *katE* gene plays an important role in H_2O_2 detoxication during the stationary phase [1, 17]. It is quite possible that the difference in the activity of the HPI catalase may cause the difference in the responses of the studied strains to peroxide-induced stress. To confirm this assumption, a number of strains were constructed, each of them simultaneously carrying a mutation in the genes controlling one of the redox systems and the *katG::lacZ* fusion (Table 1). Bacteria grown under aerobic conditions were exposed to 1 mM H_2O_2 ; the activity of β -galactosidase was measured during one hour after the exposure. As expected, the treatment of cells with this oxidant resulted in an increase in the *katG::lacZ* expression in all the studied strains. The *katG::lacZ* induction indices (the ratio between the β -galactosidase activity levels in the cells treated with

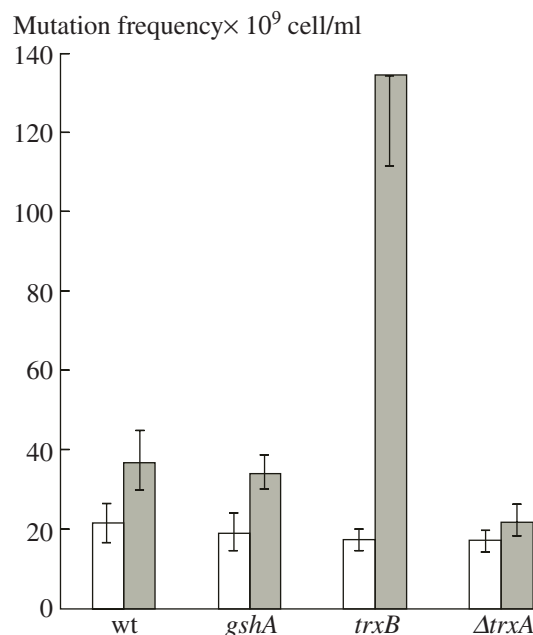


Fig. 2. The mutagenic effect of H_2O_2 on the *E. coli* cells defective in the genes controlling the redox systems: mutation frequency in the cultures treated with 5 mM H_2O_2 for 1 h (grey bars); mutation frequency in untreated cells (white bars). Strains NM131 (parent strain, wt), NM141 (*gshA*), NM161 (*trxB*), and NM151 (ΔtrxA) were used.

H_2O_2 and in untreated cells) were distributed as follows: the highest induction was detected in the *trxB* mutants (4.5), whereas the lowest one was observed in the *gshA* mutants (2.8); in the parent cells and the cells of the *trxA* mutants, these indices were 3.4 and 3.7, respectively.

The basic levels of the catalase HPI activity, as well as of the intracellular H_2O_2 concentration, in the cells grown in the absence of the oxidant were measured (Table 2). The catalase activity in the *trxB* mutants was the highest; in the *gshA* mutants it was the lowest. The distribution pattern of the total catalase activity (HPI + HPII) was much the same. Among the studied strains, the *grxA* mutants had the lowest level of intracellular H_2O_2 . No statistically significant changes in H_2O_2 concentrations were detected in the other strains in comparison to the parent strain. It is noteworthy that this

Table 2. Activity of the catalase HPI (U) and the intracellular H_2O_2 concentration (μM) in the *E. coli* strains defective in the genes controlling the redox systems

Strain	HPI	H_2O_2
NM131 (wt)	5.01 ± 0.65	0.054 ± 0.005
NM141 (<i>gshA</i>)	4.65 ± 0.42	0.041 ± 0.002
NM151 (<i>trxA</i>)	7.95 ± 1.07	0.059 ± 0.01
NM161 (<i>trxB</i>)	19.5 ± 2.75	0.051 ± 0.006
NM171 (<i>grxA</i>)	6.13 ± 0.84	0.039 ± 0.001

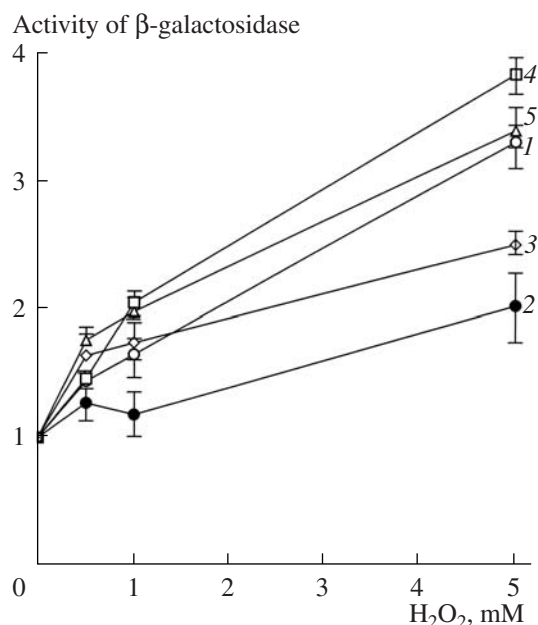


Fig. 3. The effect of H_2O_2 on the expression of the *sulA::lacZ* fusion in the *E. coli* cells defective in the genes controlling the redox systems. The β -galactosidase activity is expressed in specific units. One unit corresponds to the β -galactosidase activity before the addition of H_2O_2 . (1) NM131 (parent strain); (2) NM141 (*gshA*); (3) NM171 (*grxA*); (4) NM161 (*trxB*); and (5) NM151 ($\Delta trxA$).

phenomenon was observed even in the cells of the *trxB* mutant, whose catalase activity was high.

To study the role of thiol redox systems in the induction of SOS responses to peroxide-induced stress, we used a number of strains, each of them carrying a mutation in the genes controlling one of the redox systems and the *sulA::lacZ* fusion. In *E. coli* cells, the *sulA* (*sfIA*) gene was incorporated into the SOS regulon controlled by the genes *recA* and *lexA*. As in the case of the *katG::lacZ* fusion, during the exposure to H_2O_2 , the highest *sulA::lacZ* expression was detected in the *trxB* mutants, whereas the lowest expression was observed in the *gshA* mutants (Fig. 3). This index was lower (by 50%) in the mutants defective in glutathione synthesis in comparison to parent cells.

Due to the abovementioned results, it was of interest to elucidate the effect of glutathione added to the medium on the *sulA::lacZ* expression. The growth of *gshA* mutants in the presence of exogenic glutathione resulted in an increase in the base level of expression by 12% (Fig. 4, line 4), as well as in an increase in the *sulA::lacZ* expression by 50% (as compared to the cells grown in the presence of the oxidant, but without GSH) in response to hydrogen peroxide (Fig. 4, line 5). The addition of the iron chelator 2,2'-dipyridyl to the culture with H_2O_2 and GSH eliminated the H_2O_2 -induced expression of *sulA::lacZ* almost completely (Fig. 4,

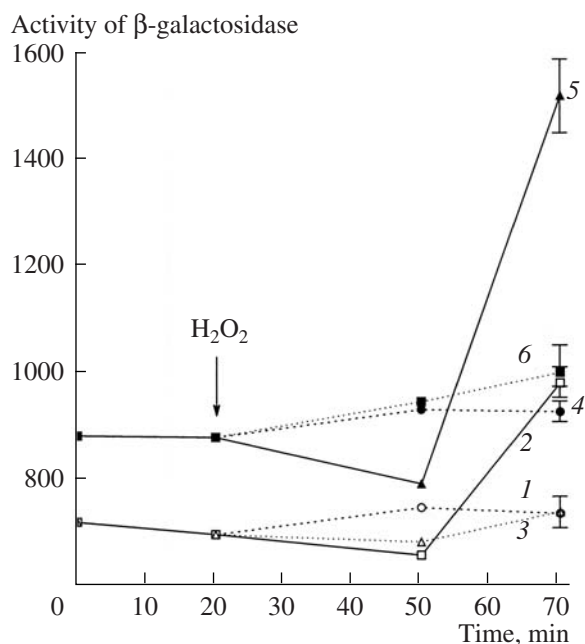


Fig. 4. Expression of *sulA::lacZ* fusion in strain NM141 (*gshA*) after various types of treatment: (1) control, the cells were grown on an aerated M9 medium without any additions; (2) M9 + 1 mM H_2O_2 ; (3) M9 + 0.1 mM 2,2'-dipyridyl; (4) M9 + 0.1 mM GSH; (5) M9 + 0.1 mM GSH + 1 mM H_2O_2 ; (6) M9 + 0.1 mM GSH + 1 mM H_2O_2 + 0.1 mM 2,2'-dipyridyl. The time of H_2O_2 addition is indicated by the arrow; other compounds were added at the early stage of cultivation (zero time).

line 6). It should be noted that the presence of exogenic glutathione in the medium did not affect bacterial growth in the presence of the oxidant.

Similar to other cell types, glutathione concentration in *E. coli* cytoplasm is the highest among all thiol redox systems. Due to this fact, as well as to the low concentration of its oxidized (GSSG) form, glutathione is the main intracellular redox buffer. It was of interest to elucidate how the glutathione level changes in the *E. coli* cells that have mutations in the genes controlling the redox systems.

Among untreated bacteria, the *trxB* mutants were characterized by the highest level of intracellular GSH, and the *trxA* mutants by the lowest level (Table 2, Fig. 5a). Under these conditions, the level of intracellular oxidized glutathione ($GSSG_{in}$) was very low in all the studied strains. The $GSH_{in}/GSSG_{in}$ ratio, a parameter characterizing the redox state of glutathione, was lowest in the *trxA* mutants (48); in the *trxB* mutants, it was maximal (187). After 1 h of the oxidant treatment, the GSH_{in} concentration in the parent strain and in the mutants *grxA* and *trxA* decreased markedly, whereas in the *trxB* mutants it remained high (Fig. 5a). In untreated cells, the highest level of extracellular GSH was observed in the *grxA* mutants; the lowest one, in the *trxB* mutants (Fig. 5b). In 20 min after the addition of H_2O_2 , the GSH_{out} concentration decreased in all strains;

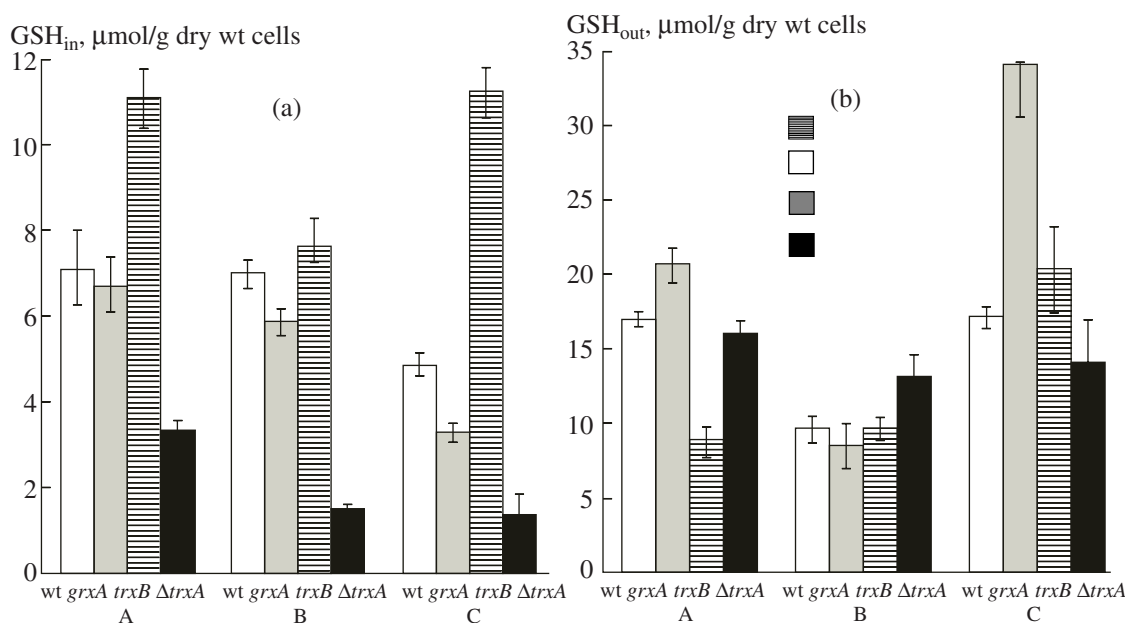


Fig. 5. The effect of 5 mM H_2O_2 on the concentrations of (a) intracellular and (b) extracellular glutathione in *E. coli* cells defective in genes controlling the redox systems: (A) control, untreated cells; (B) after 20 min of the treatment with H_2O_2 ; (C) after 60 min of the treatment with H_2O_2 . Strains NM131 (parent strain, wt), NM171 (*grxA*), NM161 (*trxB*), and NM151 ($\Delta trxA$) we used.

however, after 60 min, it increased up to the control level (in the parent cells and the *trxA* mutants) or even higher (in the mutants *grxA* and *trxB*).

DISCUSSION

It has been previously demonstrated that, in *E. coli* cells, the glutathione and thioredoxin redox systems are particularly important for genetic regulation of the antioxidant protective mechanisms [18, 19], although they do not contribute much to the direct ROS detoxication. We have found that the *E. coli* cells that have mutations in the genes controlling the redox systems were considerably different in their responses to oxidative stress induced by the exogenic hydrogen peroxide (especially at high concentrations of this oxidant). For example, the lack of GSH resulted in a sharp increase in the cell sensitivity to H_2O_2 ; conversely, the lack of thioredoxin reductase resulted in an increase in the rate of bacterial growth upon the treatment with H_2O_2 , so that the growth rate and viability of the *trxB* mutants were higher than those of the parent cells. Also, it has been demonstrated that the sensitivity of both strains to hydrogen peroxide correlated to some degree with the level of the *katG* expression. The catalase activity of the *trxB* mutants was high as well. The data on the behavior of the *trxB* mutants agree well with the previously published results obtained by Takemoto et al., who, using *E. coli* strains different from those used in this study, showed that the viability, *katG* expression, and catalase activity in the growing *trxB* mutants treated with hydrogen peroxide (2–6 mM) were higher than in the parent strain [20].

Despite the fact that the *trxB* mutants have a high basic level of catalase activity (Table 2), as well as a high level of H_2O_2 -induced expression of the *katG* gene, they exhibited high numbers of H_2O_2 -induced mutations and higher expression of the *sulA::lacZ* fusion (as compared to that in all the studied strains). The *E. coli* gene *sulA* (*sfiA*) is involved in the SOS regulon, one of the functions of which is reparation of the DNA impaired by various agents, including hydrogen peroxide [3]. On the other hand, the enhanced sensitivity of the *gshA* mutants to hydrogen peroxide was accompanied by the low level of the H_2O_2 -induced expression of the *sulA::lacZ* fusion.

Hence, taking into account the parameters mentioned above, we should agree that the properties of the mutants *trxB* and *gshA* were opposing; their different sensitivities to high H_2O_2 concentrations were determined by their capacity for induction of the antioxidant genes involved in the OxyR and SOS regulons. Our measurements have also shown that the stationary levels of H_2O_2 in untreated cells are similar to those in the mutants studied. This indicates that, in spite of the decrease in a redox system activity, the cells maintain their intracellular H_2O_2 homeostasis by enhancing the catalase activity (like the *trxB* mutants) or by some other mechanism. It should be remembered that the rate of H_2O_2 production, rather than its stationary intracellular concentration, is the factor that governs the OxyR regulon activation and, accordingly, determines the increase in the *katG* expression [16].

Another property of the *trxB* mutants which distinguishes them from other studied strains is the high basic

Table 3. Intracellular glutathione ($\mu\text{mol/g}$ dry wt. cells.) in the *E. coli* strains defective in the genes controlling the redox systems

Strain	GSH	GSSG	GSH/GSSG
NM131 (wt)	7.01 ± 1	0.044 ± 0.001	159
NM151 (<i>trxA</i>)	3.23 ± 0.3	0.067 ± 0.001	48
NM161 (<i>trxB</i>)	11.04 ± 0.8	0.059 ± 0.006	187
NM171 (<i>grxA</i>)	6.59 ± 0.7	0.066 ± 0.003	144

level of intracellular glutathione, as well as their ability to rapidly reach a normal GSH level after oxidative stress. Interestingly, these bacteria had the lowest basic level of extracellular glutathione. One can assume that, under normal conditions, the decrease in the amount of glutathione excreted into the medium is one of the factors that cause an increase in the glutathione concentration in the cytoplasm of these bacteria. GSH excretion by bacteria is common knowledge; however, the functions of extracellular glutathione are poorly understood. It is assumed that detoxication of harmful agents at "remote sites" may be one of its functions. In this regard, one should note that, in all the studied strains, the level of extracellular glutathione either returned to the basic level after one hour of the treatment with H_2O_2 (parent calls and the *trxA* mutant) or even increased (*trxB* and *grxA*). On the whole, in the *trxB* mutants, the high level of intracellular glutathione and the ability to substantially increase the level of extracellular glutathione (in response to the treatment with H_2O_2) create a high reducing potential both in the cell cytoplasm and in the surrounding environment, which may additionally increase the resistance of these mutants to exogenic H_2O_2 .

In this work it was demonstrated that the mutants deficient in GSH synthesis had a low level of H_2O_2 -induced expression of *sulA::lacZ* fusion. The addition of glutathione to these cultures significantly enhanced the expression of *sulA::lacZ* fusion in the presence of the oxidant without affecting the growth rate. One of the possible explanations of this phenomenon is that the simultaneous effect of GSH and H_2O_2 inflicts more damage on DNA, resulting in the enhanced induction of the SOS response. It is a well-known fact that, in bacterial cells, as in other organisms, exogenic glutathione decomposes to glutamate and cysteinyl glycine upon entering the cell. The latter, unlike GSH, is able to reduce free or chelated Fe^{+3} , which, in turn, triggers a series of reactions resulting in the production of ROS in the presence of O_2 [6]. The assumption that such reactions were observed in our case was confirmed by the fact that the exogenic glutathione did not increase H_2O_2 -induced expression of *sulA::lacZ* fusion during iron chelation by 2,2'-dipyridyl.

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