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Involvement of *soxRS* Regulon in Response of *Escherichia coli* to Oxidative Stress Induced by Hydrogen Peroxide

H. Semchyshyn, T. Bagnyukova, and V. Lushchak*

Department of Biochemistry, Vassyl Stefanyk Precarpathian National University, 76025 Ivano-Frankivsk, Ukraine; fax: +38 (03422) 31-574; E-mail: lushchak@pu.if.ua

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Abstract—The effect of hydrogen peroxide on the activity of *soxRS* and *oxyR* regulon enzymes in different strains of *Escherichia coli* has been studied. Treatment of bacteria with 20 μM H_2O_2 caused an increase in catalase and peroxidase activities (*oxyR* regulon) in all strains investigated. It is shown for the first time that oxidative stress induced by hydrogen peroxide causes in some *E. coli* strains a small increase in activity of superoxide dismutase and glucose-6-phosphate dehydrogenase (*soxRS* regulon). This effect is cancelled by chloramphenicol, an inhibitor of protein synthesis in prokaryotes. The increase in *soxRS* regulon enzyme activities was not found in the strain lacking the *soxR* gene. These results provide evidence for the involvement of the *soxRS* regulon in the adaptive response of *E. coli* to oxidative stress induced by hydrogen peroxide.

Key words: *Escherichia coli*, hydrogen peroxide, *soxRS* regulon, superoxide dismutase, glucose-6-phosphate dehydrogenase

Oxidative stress response in *E. coli* is accompanied by the activation at least of two regulons, *soxRS* and *oxyR* [1-4]. The first includes about 15 genes and is under two-stage control of the regulatory protein SoxR, which is a sensor for superoxide anion and expression regulator of SoxS protein, which in turn is a transcriptional activator of some structural genes [2, 3, 5, 6]. The *soxRS* regulon is also responsible for the resistance of *E. coli* to antibiotics, organic compounds, nitric oxide radicals, and heavy metals [7-9]. This regulon includes genes such as *sodA* (Mn-superoxide dismutase), *zwf* (glucose-6-phosphate dehydrogenase), *fpr* (NADPH:ferredoxin oxidoreductase), *acnA* (aconitase A), *nfsA* (nitrate reductase A), *fumC* (fumarase C), *nfo* (endonuclease IV), and *micF* (regulatory RNA) [2, 3, 10].

In response to hydrogen peroxide exposure, *E. coli* expresses about 30 genes, which include nine genes of the *oxyR* regulon. The *katG* (hydroperoxidase HPI), *gorA* (glutathione reductase), *ahpC* (NADPH-dependent alkyl hydroperoxide reductase), *oxyS* (regulatory RNA), and *grxA* (glutaredoxin) genes are among them [2-4, 10]. For a long time it was widely believed, that the *soxRS* regulon is not sensitive to hydrogen peroxide [1-3, 6, 10, 11]. However, in our previous studies it was found that some enzyme activities of the *soxRS* regulon increased as a result of H_2O_2-induced stress [12-14]. There are now several reports on weak activation of some genes of this regulon by hydrogen peroxide [15, 16].

The aim of the present work was to expand our understanding of *E. coli* response to hydrogen peroxide exposure, particularly to study possible mechanisms of the H_2O_2 effect on superoxide dismutase (SOD) and glucose-6-phosphate dehydrogenase (G6PDH) activities.

MATERIALS AND METHODS

Bacteria and chemicals. The strains used in this study were as follows: *E. coli* KS400 (wild type K12, *met B*) kindly provided by Dr. I. Andreeva (Institute of Epidemiology and Microbiology, Moscow, Russia); AB1157 (wild type K12, F^- *thr-1 leuB6 proA2 his-4 thi-1 argE2 lacY1 galK2 rpsL supE44 ara-14 xyl-15 mtl-1 tsx-33*) kindly provided by Dr. I. Andreeva, Dr. B. Demple (Harvard School of Public Health, Boston, USA), and Dr. J. Imlay (University of Illinois, Urbana, USA); MP180 (wild type K12, HfrH *thi-1*), CHS7 (*lacY rpsL thi-1*), and B23 (wild type B) kindly provided by Dr. P. Loewen (University of Manitoba, Winnipeg, Canada); MC4100 (*araD139 relA1 thi rpsL150 flbB5301 Δ(lacU139) deoC7 ptsF25*) and GS071 (MC4100 *ΔsoxRS-*

Abbreviations: G6PDH) glucose-6-phosphate dehydrogenase; GR) glutathione reductase; SOD) superoxide dismutase; TEMED) *N*,*N*,*N*′,*N*′-tetramethylethylenediamine.

* To whom correspondence should be addressed.

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*zjc2205 zjc2204::*Tn*10kan*) kindly provided by Dr. G. Storz (National Institutes of Health, Bethesda, USA).

Glucose-6-phosphate was supplied by Sigma (USA); *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (TEMED), quercetin, NADPH, NADP, and oxidized glutathione were purchased from Reanal (Hungary). All inorganic chemicals were obtained from Reakhim (Russia). Medium for bacterial cultivation was from Nutrient Media (Makhachkala, Russia).

Growth conditions and cell extracts. Bacteria were grown in a nutrient broth for cultivation of microorganisms containing 10.05 g/liter sprat tryptic hydrolyzate and 4.95 g/liter NaCl. For experimentation, bacteria were cultivated at 37°C over 17-18 h without aeration. The resulting bacterial cultures were diluted 1 : 100 v/v with fresh culture medium heated to 37°C and grown at the same temperature under aeration over 4-5 h (middle exponential phase) to reach an optical density of approximately 0.45 at 600 nm. Then hydrogen peroxide was added to aliquots of culture, followed by incubation of cultures at 37°C.

Cells were harvested by centrifugation at 3000*g* for 10 min and washed twice with medium containing 50 mM potassium phosphate buffer (pH 7.0) and 0.5 mM EDTA. Then the cells were resuspended in the same medium and disrupted by sonication for 6-8 min at 4°C using a System UZDN-2T disintegrator equipped with a cup horn and operated at 22 kHz. Cell debris was removed by centrifugation at 4000*g* for 10 min at 4°C, and cell-free extracts were kept on ice for immediate use.

Enzyme activity measurements. Dismutation of hydrogen peroxide by catalase was measured spectrophotometrically at 240 nm with an SF-46 spectrophotometer (LOMO, Russia) using molar extinction coefficient for hydrogen peroxide of 39.4 $M^{-1}\cdot cm^{-1}$ [17]. Catalase activity was assayed in 2 ml of medium containing (final concentrations) 50 mM potassium phosphate buffer (pH 7.0), 0.5 mM EDTA, 10 mM hydrogen peroxide, and 20 µl of cell suspension. Blanks were run in the absence of hydrogen peroxide. One unit of catalase activity was defined as the amount of supernatant protein that utilizes 1 µmol of hydrogen peroxide per min.

Peroxidase activity was evaluated at 436 nm by the method of Claiborn and Fridovich [18]. Reaction medium contained 50 mM potassium phosphate buffer (pH 7.0), 0.5 mM EDTA, 0.4 mM *o*-dianisidine, 10 mM H_2O_2, 0.3 mM NaN_3, and 20 µl of cell extract in a final volume of 2 ml. One unit of peroxidase activity was defined as the extract protein amount producing a change of 0.01 optical density unit at 436 nm per min.

Superoxide dismutase (SOD) activity was assayed at 406 nm as the inhibition rate of quercetin oxidation by superoxide anion [19] in reaction medium containing 30 mM Tris-HCl buffer (pH 9.0), 0.3 mM EDTA, 0.8 mM TEMED, 14 µM quercetin, and 3-30 µl of cell extract in a final volume of 2 ml. One unit of SOD activity was defined as the amount of supernatant protein that inhibited the maximal rate of quercetin oxidation by 50%. The parameter was calculated with the computer program Kinetics [20].

Glutathione reductase (GR) activity was measured by following the consumption of NADPH at 340 nm in reaction medium containing 50 mM potassium phosphate buffer (pH 7.5), 0.5 mM EDTA, 1 mM oxidized glutathione, 0.25 mM NADPH, and 20 µl of cell extract in a final volume of 1.5 ml [21]. Two blanks were run, without GSSG or extract.

Glucose-6-phosphate dehydrogenase (G6PDH) activity was measured by monitoring NADP reduction in reaction medium containing 50 mM potassium phosphate buffer (pH 7.5), 0.5 mM EDTA, 5.0 mM $MgSO_4$, 0.2 mM NADP, 1 mM glucose-6-phosphate, and 40 µl of cell extract in a final volume of 2 ml [21]. One unit of GR or G6PDH activity is defined as the amount of supernatant protein that utilizes or produces 1 µmol of NADPH per min, respectively. All reactions were started by addition of cell extract and activities were measured at 25°C and expressed per mg protein in supernatant.

Protein concentration and statistics. Protein content was determined by the Coomassie brilliant blue G-250 dye-binding method [22] using bovine serum albumin as the standard. Experimental data are expressed as means ± SEM and statistical testing used Student's *t*-test.

RESULTS AND DISCUSSION

In previous works, it has been shown that oxidative stress induced by hydrogen peroxide at the concentrations lower than sublethal (up to 100 µM) increased the levels of oxidatively modified proteins and lipids in *E. coli* strain KS400 [4, 12-14]. Incubation of bacteria with 20 µM H_2O_2 for 20 min caused the maximal effect for lipid oxidation. These conditions were used to investigate the influence of oxidative stress on activities of some antioxidant enzymes of the *soxRS* and *oxyR* regulons in *E. coli* KS400. The results are presented in Table 1. As a result of H_2O_2-induced stress, the activities of catalase, peroxidase, and GR increased 1.9-, 2.7-, and 1.5-fold, respectively, which coincide with literature data on the increase in *oxyR* regulon enzyme activities [1-3, 10]. Exogenous hydrogen peroxide did not change the activity of SOD, a member of the *soxRS* regulon, which is supposed to be not activated by H_2O_2 [1, 6, 10, 11]. Unexpectedly, a small but significant increase (by 1.4-fold) was found in the activity of G6PDH, another member of the *soxRS* regulon.

It is well known that different strains may have different sensitivities to oxidants [23, 24]. Since we found earlier that bacteria of *E. coli* strain AB1157, unlike KS400 strain, do not grow under oxygenation [12], further we investigated effects of hydrogen peroxide on the activities

Table 1. Activities of antioxidant and associated enzymes in *E. coli* KS400 under oxidative stress induced by 20 μM H_2O_2 for 20 min

Enzyme	Control	Stress
SOD, U/mg protein	21.8 ± 4.0	18.7 ± 1.3
Catalase, μmol/min per mg protein	23.5 ± 12.6	45.2 ± 14.4*
Peroxidase, ΔOD_{436}/min per mg protein	1.77 ± 0.16	4.93 ± 0.66**
GR, nmol/min per mg protein	42.6 ± 5.1	65.4 ± 8.5*
G6PDH, nmol/min per mg protein	30.6 ± 2.9	43.3 ± 5.8*

Note: Data are mean ± SEM (n = 3-7).
* $p < 0.05$.
** $p < 0.01$.

of antioxidant and associated enzymes in AB1157 strain. This strain is widely used for biochemical and microbiological investigations in many laboratories. Therefore, we studied *E. coli* AB1157 cultures from different sources. The results of hydrogen peroxide effects on the activities of *soxRS* and *oxyR* regulon enzymes in AB1157 strain are shown in Table 2. The activities of catalase and peroxidase increased in this strain from two sources, by 2.6- and 1.8-fold (from Dr. Andreeva) and 3.1- and 4.0-fold (from Dr. Demple). The GR activity (*oxyR* regulon) rose 1.5-fold in the first case and was unchanged in the second case. The activities of SOD and G6PDH increased in both cases, by 1.5- and 1.6-fold, respectively. Hence, in AB1157 strain oxidative stress induced by hydrogen peroxide enhanced slightly the activities of both *soxRS* regulon enzymes tested.

When we obtained these data, a few reports had appeared that iRNA levels of some *soxRS* regulon genes were elevated by exogenous hydrogen peroxide [15, 16]. The genes *sodA* (Mn-SOD), *soxS* (a regulator of *soxRS* regulon expression), *micF* (regulatory RNA), and others were among them. However, the noted effect was registered at the hydrogen peroxide concentrations of 100 μM and higher. On the other hand, only 10 μM H_2O_2 was enough to increase the expression of *oxyR* regulon genes more than 10-fold [15].

To elucidate a possible mechanism of this rise in SOD and G6PDH activities under the treatment of bacteria with 20 μM H_2O_2, we used an inhibitor of protein synthesis in prokaryotes, chloramphenicol. In this case, a suspension of bacterial strain AB1157 from Dr. Imlay (USA) was used. Activities of all enzymes tested except peroxidase were the same in cells treated with chloramphenicol (30 μg/ml) and then with hydrogen peroxide compared to control bacteria (without H_2O_2 and chloramphenicol). At the same time, the peroxidase activity dropped 2-fold after treatment with the protein synthesis inhibitor and H_2O_2. Hence, protein synthesis *de novo* is needed to increase activities of all tested enzymes including SOD and G6PDH.

Further we were interested is regulatory protein SoxR involved in the response of *E. coli* to oxidative stress induced by H_2O_2. We used wild-type strain MC4100 and its isogenic derivative GS071 (*ΔsoxR*). In MC4100 strain, like AB1157, increased activities of enzymes of both antioxidant regulons, *soxRS* and *oxyR* (Table 3), were found. After treatment of bacteria with H_2O_2, the activities of catalase and peroxidase increased 3.1- and 4.8-fold, respectively, while the SOD and G6PDH activities were increased 1.3-fold. The GR activity did not change under these conditions. At the same time, in mutant strain GS071 (*ΔsoxR*) the catalase and peroxidase activi-

Table 2. Activities of antioxidant and associated enzymes in *E. coli* AB1157 from different sources under H_2O_2-induced oxidative stress (other information as in Table 1)

Enzyme	Control	Stress
AB1157 (from Dr. Andreeva)		
SOD, U/mg protein	11.7 ± 0.9	17.1 ± 2.5*
Catalase, μmol/min per mg protein	15.5 ± 2.7	39.9 ± 9.5**
Peroxidase, ΔOD_{436}/min per mg protein	2.29 ± 0.33	4.17 ± 0.86*
GR, nmol/min per mg protein	28.1 ± 1.7	42.8 ± 6.5*
G6PDH, nmol/min per mg protein	9.94 ± 0.56	16.0 ± 2.3**
AB1157 (from Dr. Demple)		
SOD, U/mg protein	28.0 ± 4.7	40.6 ± 3.2*
Catalase, μmol/min per mg protein	12.4 ± 1.6	38.7 ± 6.1***
Peroxidase, ΔOD_{436}/min per mg protein	0.244 ± 0.026	0.984 ± 0.149***
GR, nmol/min per mg protein	53.4 ± 6.8	57.8 ± 6.5
G6PDH, nmol/min per mg protein	4.04 ± 0.075	6.63 ± 0.96*

Note: Data are mean ± SEM (n = 4-7).
* $p < 0.05$.
** $p < 0.025$.
*** $p < 0.005$.

ties elevated by 3.4- and 4.0-fold, respectively, whereas the activities of SOD, G6PDH, and GR were near control values (Table 3). It should be noted that in wild strain MC4100 as in the case with AB1157 strain the increase in both regulon enzyme activities stimulated by hydrogen peroxide was blocked by chloramphenicol. It can be concluded that the *soxRS* regulon as well as the *oxyR* regulon take part in adaptive response of *E. coli* to oxidative stress induced by hydrogen peroxide.

It has been shown previously that stress development in *E. coli* assessed by alteration of oxidized protein and lipid levels as well as by catalase activity depended on the manner of oxidative stress induction, e.g. on hydrogen peroxide concentrations and exposure time [4, 12, 14]. Therefore, we investigated the influence of different H_2O_2 concentrations and different times of exposures on activities of *soxRS* regulon enzymes. The effects of different hydrogen peroxide concentrations on the SOD and G6PDH activities in MC4100 strain bacteria are shown in Fig. 1. We used low, not higher than sublethal, hydrogen peroxide concentrations, which are usually used to study experimental oxidative stress in bacteria [24].

Hydrogen peroxide at a concentration of 10 μM did not significantly increase SOD activity (Fig. 1a). At higher concentrations, the enzyme activity was 145% (20 μM), 112% (50 μM), and 139% (100 μM) compared to control. The G6PDH activity slightly increased at all concentrations used. So, its activities were 138, 137, 131, and 143% at 10, 20, 50, and 100 μM H_2O_2, respectively.

Figure 2 shows the effects of oxidative stress induced by 20 μM hydrogen peroxide over different exposures on the SOD and G6PDH activities in MC4100 strain bacteria. In the chosen time intervals, incubation time with hydrogen peroxide had only a small influence on these enzyme activities. For example, 10 min stress did not affect the SOD activity, whereas treatment within 20-60 min caused an increase of 1.3-fold in the enzyme activity. The G6PDH activity was 151% after 30 min stress and 146% after 60 min when compared with control.

In the abovementioned work [15], an increase of 2.1- and 4.7-fold was quantified for iRNA stationary level of the *sodA* gene (Mn-SOD) in response to treatment with 500 and 1000 μM hydrogen peroxide, respectively. At H_2O_2 concentrations lower than 100 μM, the effect was found only for *oxyR* regulon genes. Similar results were obtained by others [16]. Dependence of the effect on regulatory genes *soxR* and *soxS* was shown in both cases. Interestingly, no change in transcription level of the *zwf* gene (G6PDH) was found in these studies. In our instance, G6PDH activity rose with simultaneous increase in SOD activity in response to hydrogen peroxide treatment (Figs. 1b and 2b). In contrast to SOD, 10 μM H_2O_2 was enough to observe the effect for G6FDH. In other words, if the results obtained by others are taken into consideration, the increase in G6PDH activity in our case seems not to depend on its gene transcription, and other mechanisms are involved in the effect detected. However, it conflicts with our results obtained with GS017 strain (*ΔsoxRS*), which did not demonstrate any variation of the two enzyme activities of the *soxRS* regulon in response to hydrogen peroxide, unlike MC4100 wild-type (Table 3).

Table 3. Activities of antioxidant and associated enzymes in *E. coli* MC4100 and GS071 under H_2O_2-induced oxidative stress (other information as in Table 1)

Enzyme	Control	Stress
MC4100		
SOD, U/mg protein	25.5 ± 2.1	34.1 ± 1.6**
Catalase, μmol/min per mg protein	5.48 ± 0.66	16.9 ± 1.2**
Peroxidase, ΔOD_{436}/min per mg protein	0.117 ± 0.020	0.557 ± 0.096***
GR, nmol/min per mg protein	67.3 ± 3.8	71.5 ± 2.2
G6PDH, nmol/min per mg protein	7.11 ± 0.45	8.91 ± 0.75*
GS071		
SOD, U/mg protein	22.6 ± 4.7	24.2 ± 6.1
Catalase, μmol/min per mg protein	4.22 ± 0.24	14.4 ± 1.28***
Peroxidase, ΔOD_{436}/min per mg protein	0.126 ± 0.016	0.508 ± 0.121**
GR, nmol/min per mg protein	67.7 ± 3.1	75.7 ± 5.1
G6PDH, nmol/min per mg protein	8.58 ± 1.25	7.77 ± 1.19

Note: Data are mean ± SEM (n = 4-7).
* $p < 0.05$.
** $p < 0.01$.
*** $p < 0.001$.

We also tested *E. coli* wild-type strains MP180, B23, and CSH7. For simpler analysis, all results are summarized in Table 4. It shows that oxidative stress induced by treatment of the bacteria with 20 μM H_2O_2 for 20 min caused different effects on GR, SOD, and G6PDH activities in various strains. Only catalase and peroxidase activities demonstrate a strong increase in all strains. It seems this might be due to specific role of the last two enzymes in cell defense against hydrogen peroxide. At the same time, it is possible that the H_2O_2 effect on GR, SOD, and G6PDH activity, which just "assist" the main enzymes, depends on the sensitivity of different strains to oxidants.

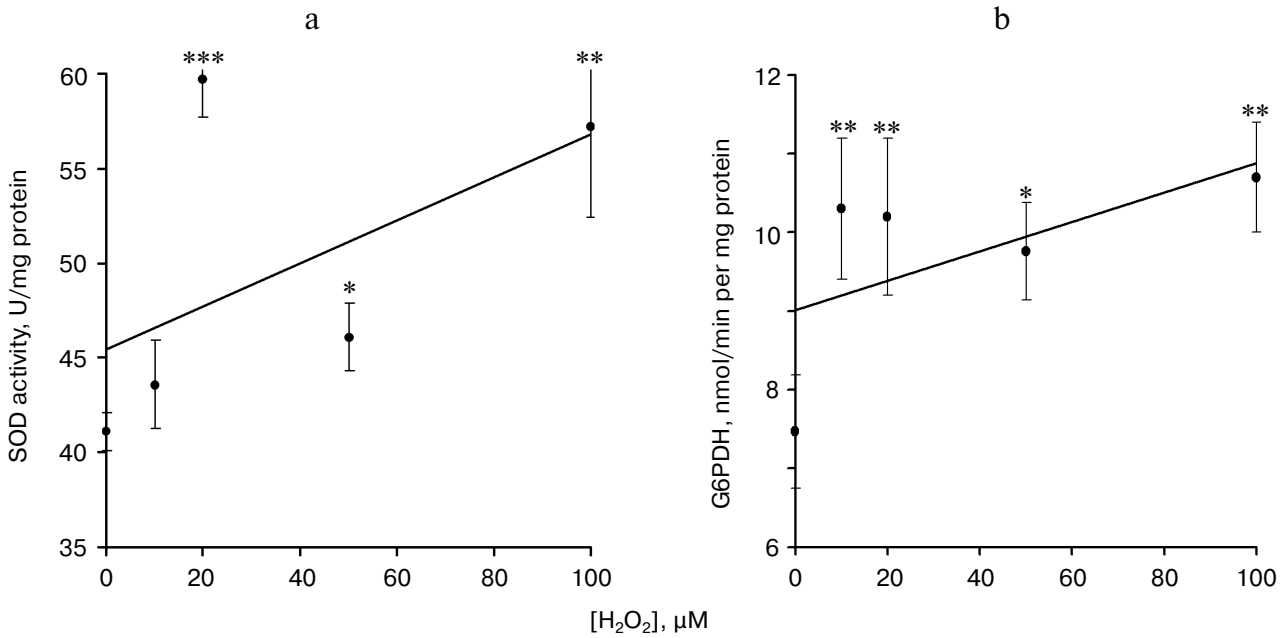

Fig. 1. SOD (a) and G6PDH (b) activities in *E. coli* MC4100 under oxidative stress induced by different concentrations of hydrogen peroxide for 20 min. Data are mean ± SEM (n = 4-5). * Significantly different from control (without H_2O_2) values with $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

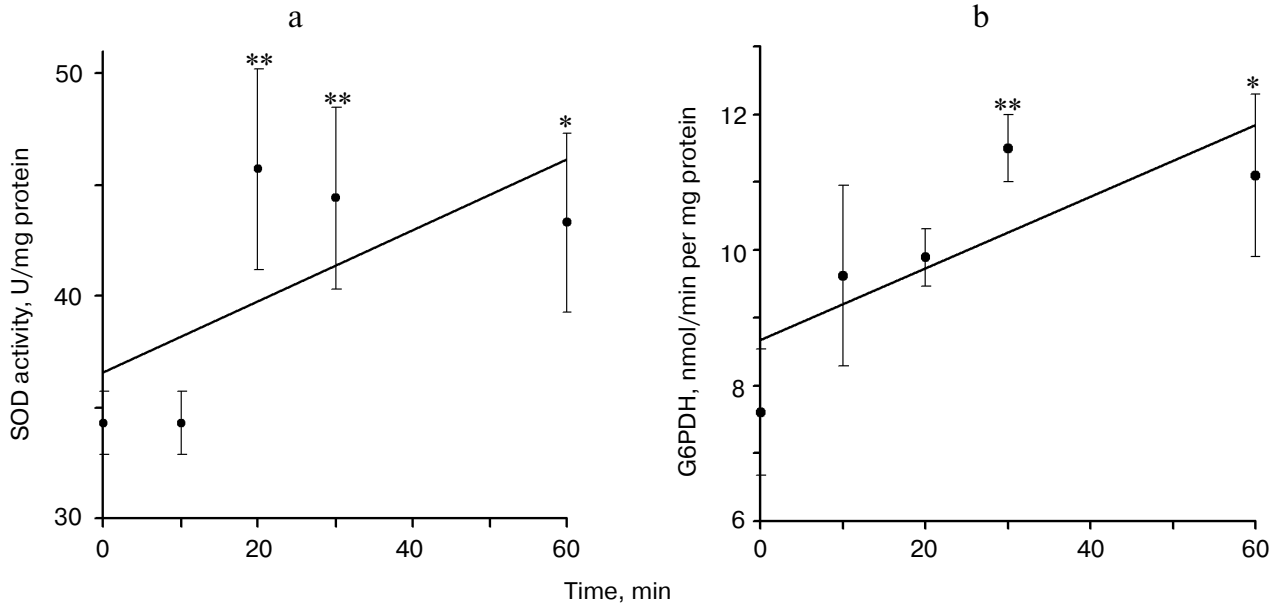

Fig. 2. SOD (a) and G6PDH (b) activities in *E. coli* MC4100 under oxidative stress induced by 20 μM H_2O_2 for different time periods. Data are mean ± SEM (n = 3-5). * Significantly different from control (without H_2O_2) values with $p < 0.05$ and ** $p < 0.025$.

Although much is known about *E. coli* defense against oxidative stress, many other possible mechanisms are intensively discussed and evidently many of them are still unknown. For example, an overlap between activations of some bacterial regulons has been known recently [25, 26]. This means that bacteria may use different defensive mechanisms against the same oxidants. In *Salmonella typhimurium* and *E. coli*, hydrogen peroxide induces enzymes that are directly involved in cell defense against H_2O_2 and at the same time heat shock proteins

Table 4. Changes in *oxyR* regulon and *soxRS* regulon enzyme activities in different strains of *E. coli* under H_2O_2-induced oxidative stress

E. coli strain	Changes in enzyme activty				
	oxyR regulon			*soxRS* regulon	
	catalase	peroxidase	GR	SOD	G6PDH
KS400	+	+	+	—	+
AB1157 (from Dr. Andreeva)	+	+	+	+	+
AB1157 (from Dr. Demple)	+	+	—	+	+
AB1157 (from Dr. Imlay)	+	+	+	+	+
MC4100	+	+	—	+	+
MP180	+	+	—	—	—
B23	+	+	+	+	—
CSH7	+	+	—	+	—

Note: "+", activity increased; "–", activity did not increase.

and others [27, 28]. As for SOD and G6PDH, transcription of the respective genes in *E. coli* is under control of not only SoxR and SoxS proteins, but they are controlled also by regulatory proteins of other regulons, for example, by MarA, Rob, and SoxQ, the main function of which is the regulation of *E. coli* adaptive response to antibiotics [7, 29, 30]. All these facts cannot explain the mechanisms of overlapping between distinct pathways for the regulation of genes, and the reasons why different regulators for the same genes exist are still not understood.

Hence, for the first time we demonstrated that hydrogen peroxide causes an increase in activities of *soxRS* regulon enzymes, SOD and G6PDH. Although H_2O_2 effects can be observed on iRNA levels, the final step in cell adaptive response is the activities of the respective enzymes. It is well known that not only transcription, translation, and posttranslational modification, but other mechanisms may be involved in the enzyme activity regulation. That is why in this case our method, which is based on enzyme activity measurements, is better to study bacterial adaptive response to oxidative stress.

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