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Changes in the Activity of Antioxidant Systems of *Escherichia coli* under Phosphate Starvation

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Abstract—Changes in the activity of antioxidant systems in *Escherichia coli* during phosphate starvation have been studied. It is shown that starvation was accompanied by a decrease in the intensity of respiration, an increase in the rate of superoxide production, and a decrease in the level of ATP. Simultaneously, there was a decrease in H_2O_2 in the medium and a significant increase in the expression of the *katG* and *katE* genes which encode the HPI and HPII catalases, respectively. At the same time, there was no drop in the membrane potential, which may indicate the retention of normal membrane activity in starving cells. It has been shown for the first time that the transition of *E. coli* to phosphate starvation is accompanied by significant changes in the status of glutathione. The most important of these are associated with a decrease in the level of reduced glutathione in the medium (GSH_{out}) and with a simultaneous increase in its content in the cytoplasm (GSH_{in}), as well as a shift in the GSH_{in} to oxidized glutathione form ($GSSG_{in}$) ratio towards reductive values, and $GSH_{out}/GSSG_{out}$ towards oxidative values. Among the mutants used in the work, the *gor trxB* double mutant, which is deficient in the synthesis of glutathione reductase and thioredoxin reductase, showed the most pronounced distinctive features. Compared to the parental strain, this mutant showed a multiple higher expression of *katG*::*lacZ*, the highest level of oxidized intra- and extracellular glutathione, and, accordingly, the lowest GSH/GSSG ratio in both compartments. In general, the data we obtained indicate that during phosphate starvation the interaction of the glutathione redox-system and regulons that control protection against reactive oxygen species creates conditions that allow maintaining the concentration of ROS below the toxic level. As a result, phosphate-starved *E. coli* cells can maintain high viability for a long period of time, which allows them to quickly resume growth after the addition of phosphate.

Keywords: antioxidant systems, *Escherichia coli*, phosphate starvation

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In their natural habitat, bacteria face various stresses, including those caused by the depletion of the main substrates. Most stresses are accompanied by inhibition of growth, so cells have universal mechanisms to deal with the negative consequences of a sharp slowdown in metabolic processes. These consequences may include increased production of reactive oxygen species (ROS) such as the superoxide anion ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2) and the hydroxyl radical ($HO^{\bullet}$), which can damage DNA, proteins, and cell membranes. To avoid the damaging effects of possible oxidative stress, cells tend to maintain ROS at a safe level. Bacteria have a set of enzymes that scavenge ROS and restore damaged macromolecules, as well as sensors and signaling pathways that perceive changes in the redox state and activate the mechanisms of its homeostasis [1].

Escherichia coli contains three superoxide dismutases (SODs): cytoplasmic Mn-SODs (*sodA*) and Fe-SOD (*sodB*) and periplasmic Cu-Zn-SOD (*sodC*). The main enzymes that remove H_2O_2 in vivo, include the alkyl hydroperoxide reductase Ahp (*ahpCF*) and catalases HPI (*katG*) and HPII (*katE*), whose gene expression is under the control of the transcriptional regulators OxyR and RpoS [2].

In addition, low-molecular-weight thiols are involved in maintaining intracellular redox homeostasis. The cytosol of *E. coli* contains two main thiol-based redox systems, the functions of which overlap significantly: the glutathione-glutaredoxin system and the thioredoxin system [3, 4]. Glutathione is the main thiol redox buffer. In the cytoplasm, glutathione is predominantly in a reduced state and its intracellular concentration is approximately 5 mM. Oxidized gluta-

Abbreviations. ROS, reactive oxygen species; GSH (L-γ-glutamyl-L-cysteinyl-glycine), reduced form of glutathione; GSSG, oxidized form of glutathione; in/out, in the cytoplasm/in the culture medium; SOD, superoxide dismutase.

thione (GSSG) is reduced by glutathione reductase (GOR), which is encoded by the *gor* gene and is a part of the OxyR regulon [3]. During exponential growth under aerobic conditions, glutathione can accumulate in the medium at the micromolar level and undergo continuous transmembrane circulation [5].

The thioredoxin system of *E. coli* consists of two thioredoxins (Trx1 and Trx2) encoded by *trxA* and *trxC*, and thioredoxin reductase (TrxR), encoded by *trxB* [3, 4, 6]. *E. coli* also contains three glutaredoxins (Grx1, Grx2, and Grx3) encoded by *grxA, grxB,* and *grxC* respectively, and two glutaredoxin-like proteins Grx4 (*grxD*) and NrdH (*nrdH*). Oxidized glutaredoxins, except for Grx4 and NrdH, are nonenzymatically reduced by glutathione [4, 6].

One of the stressful situations that can potentially be accompanied by disruption of redox homeostasis and ROS production is phosphate starvation (P_i). Phosphate is required by the cells of all living organisms for structural and energy purposes. Under natural conditions, the content of phosphate can vary in a significant range, up to its complete absence. When there is no phosphate in the medium, *E. coli* activates an adaptive response that includes the synthesis of several dozen proteins, some of which are included in the Pho regulon, which is a global regulatory network that interacts with the biosynthesis of polyphosphates, RpoS, and ppGpp [7, 8]. Proteomic analysis showed that the overall response of *E. coli* to phosphate starvation can include up to 400 genes, which make up almost 10% of the genome of this bacterium [9].

Previous evidence has been presented that *E. coli* growing on MOPS medium with glucose and six amino acids, maintain an active metabolism for about 3 days of phosphate starvation. Phosphate-starved cells utilized not only glucose, but also amino acids present in the nutrient medium, producing metabolites such as putrescine and spermidine, which are involved in the protection of nucleic acids from oxidative damage [10–12]. Based on experiments with mutants, it was suggested that the RpoS and LexA regulons, as well as the H-NS protein, are important for maintaining the viability of bacteria [10]. It is known that the expression of *katG* and *katE* increases with entry of *E. coli* into the stationary phase [2]. Considering that phosphate starvation causes bacterial growth arrest and transition to the stationary phase, it would be logical to expect the induction of KatG and KatE in such cells. However, VanBogelen et al. [9] did not find the induction of these catalases in response to starvation of *E. coli* for phosphate. Moreau et al. [11] found that a deficiency in the synthesis of alkyl hydroperoxide reductase (Ahp) and catalase HPI (KatG) significantly reduced the survival of phosphate-starved cells; however, gene inactivation of *oxyR* and *rpoS* had only a small effect on survival. The authors judged the production of ROS indirectly by measuring the concentrations of compounds that react with thiobarbituric acid. This method is often criticized for its lack of specificity.

In experiments with other bacteria, *Sinorhizobium meliloti*, it was shown that phosphate starvation stimulated H_2O_2-inducible catalase (*katA*) gene expression in cells. It was found that for gene transcription of *katA* a PhoB regulator was needed, and initiation occurred on a promoter other than the OxyR-dependent one that activates transcription of *katA* in response to adding H_2O_2 [13]. The authors observed a similar effect of starving for phosphate in *Agrobacterium tumefaciens* and *Pseudomonas aeruginosa*. Based on the fact that *phoB*-mutants were more sensitive to H_2O_2 than mutants *katA*, Yuan et al. assumed that in addition to *katA,* phosphate starvation induces other PhoB-regulated genes that play an important role in protecting cells from H_2O_2 [13].

In general, the previously obtained data leave open the question of ROS production and the role of oxidative stress in phosphate-starving bacteria.

We have recently shown that during amino-acid starvation of *E. coli* growing under aerobic conditions glutathione plays an important role in response to stress [14]. Bacteria maintain a low level of intracellular free cysteine due to its high reducing activity towards Fe^{3+} ions, which contributes to the Fenton reaction [15, 16]. Unlike cysteine, GSH is a weak iron reducer in the highest oxidation state [15], so the incorporation of cysteine into glutathione serves as an effective mechanism for maintaining cysteine homeostasis with an increase in its intracellular concentration, which is observed when protein synthesis is stopped [14]. Reducing the excess of cysteine in cells is also achieved by its export to the medium and desulfurization with the formation of H_2S [14, 16, 17]. We have previously shown that amino acid starvation stimulates an increase in the intra- and extracellular pool of glutathione, as well as a change in the ratio of reduced/oxidized glutathione in the cytoplasm ($GSH_{in}/GSSG_{in}$) [14]. It is known that the redox status of glutathione and other thiol redox systems are closely interrelated both with each other and with the expression of enzymes that neutralize ROS [3].

Based on the data, it can be concluded that understanding the dynamics of changes in the redox status of glutathione will expand our knowledge of the mechanisms of bacterial response to stress induced by starvation. In this work, we studied changes in the activity of antioxidant systems during phosphate starvation of *E. coli*. We evaluated such indicators as the redox status of glutathione, the production of H_2O_2, and expression of antioxidant enzymes.

EXPERIMENTAL

Materials and reagents. We used reagents from Sigma Chemical Co.: 3-[*N*-morpholine]propanesulfonic acid (MOPS), 2-nitrophenyl-β-D-galactopyra-

Table 1. Strains of *Escherichia coli* and plasmids used in the work

Name	Genotype	Source
BW25113	Δ(*araD-araB*)*567*, Δ*lacZ4787*(::rrnB-3), λ^-,*rph-1*, Δ(*rhaD-rhaB*)*568*, *hsdR514*	CGSC[a]
JW2663	like BW25113 but *gshA*	CGSC
JW3467	like BW25113 but *gor*	CGSC
JW5856	like BW25113 but *trxA*	CGSC
JW0871	like BW25113 but *trxB*	CGSC
DM4000	*hisG4 argE3 thr-1*$^-$ *ara-14 xyl-5 mtl-1 tsx-33 ilv TS rpsL31 sulA*::*Mud1(bla lac)cam*	VMR[b]
NM3655	*gshA trxA*	IEGM[c]
NM3761	*gor trxB*	IEGM
pKT1033	*katG*::*lacZ*	TK[d]
pRS katE16	*katE*::*lacZ*	LPC[e]
pColV-K30	*iucC*::*lacZ*	IJA[f]

[a] *E. coli* Genetic Stock Center.
[b] Material courtesy of Prof. Volkert MR (University of Massachusetts Medical School, Worcester, United States).
[c] Museum of the Laboratory of Physiology and Genetics of Microorganisms (Institute of Ecology and Genetics of Microorganisms, Perm, Russia).
[d] Material courtesy of Prof. Tao K (Laboratory of Radiation Biology, Kyoto University, Kyoto, Japan).
[e] Material courtesy of Prof. Loewen PC (Department of Microbiology, University of Manitoba, Winnipeg, Canada).
[f] Material courtesy of Prof. Imlay JA (Department of Microbiology, University of Illinois, Urbana, Illinois, United States).

noside, deoxycholate, mercaptoethanol, horseradish peroxidase (HRP), Amplex Red, $DiBAC_4(3)$, ferricytochrome *C*, superoxide dismutase, 5,5'-dithiobis(2-nitrobenzoic acid, glutathione reductase; as well as agar (Fluka, United States), LB medium (Amresco, United States), dimethyl sulfoxide (DMSO) (Scharlau, Spain), and ethylenediaminetetraacetic acid (EDTA) (BioFrox, Germany); the remaining reagents were of analytical grade from Russian manufacturers.

Bacterial strains and growth conditions. The *Escherichia coli* BW25113 parent strain (wt) and single mutants: *gshA, gor, trxA* and *trxB*, were from the Keio collection [18] (Table 1). Double mutants: *gshA trxA* and *gor trxB*, were constructed by transduction with P1 phage using appropriate Keio strains. The parent strain and mutants listed above, carrying *katG*::*lacZ* [19], *katE*::*lacZ* [20], s*ulA*(*sfiA*)::*lacZ* [21] and *iucC*::*lacZ* [22] transcriptional gene fusions were created by methods of transformation and transduction.

Cells were grown in MOPS medium supplemented with 8.5 mM glucose and 2 mM KH_2PO_4 (or 2 mM NaH_2PO_4 when using a medium with a low content of K^+) [23]. Overnight cultures were centrifuged and diluted in 50 ml of fresh medium to an optical density of 0.1 at 600 nm (OD_{600}) and then grown to OD_{600} 0.6 at 37°C in 250 mL flasks, with shaking at 150 rpm. After centrifugation, cells were diluted to OD_{600} 0.25 in 100 ml of prewarmed fresh medium with phosphate (control) or without phosphate (P_i-starvation) and grown from 2 to 48 h as above. The specific growth rate (μ) was calculated using the equation $\mu = \Delta \ln OD_{600}/\Delta t$, where *t* is time (h).

Measurement of oxygen (dO_2) and potassium (K^+). Dissolved oxygen monitoring (dO_2) was performed in real time directly in flasks using a Clark InPro 6800 oxygen electrode (Mettler Toledo, Switzerland). Changes in the level of extracellular potassium were recorded using an ELIS-121K K^+-selective electrode (IT, Russia) directly in flasks in a medium with a low concentration of K^+ (0.2 mM), where *E. coli* cells were added prepared as above. Synchronous processing of all primary data from the sensors was carried out using the RS-232 and Modbus protocols of the Advantech OPC Server v3.0 software package (https://advantech-modbus-opc-server.soft-ware.informer.com/3.0/).

Determination of ATP concentration and the membrane potential. The ATP concentration was measured by the luciferin-luciferase method using the ATP Determination Kit (Molecular Probes, United States). For ATP extraction, 50 μL of the cell suspension was mixed with 450 μl of DMSO. The extraction was carried out for 5 min and the ATP concentration was determined according to the manufacturer's protocol.

Membrane potential changes were assessed using $\Delta\psi$-sensitive fluorescent dye $DiBAC_4(3)$ [24, 25]. Cell samples treated with protonophore carbonyl cyanide-*m*-chlorophenylhydrazone (20 μM) were used as a positive control. Fluorescent cells were counted using a Leica DM2000 fluorescence microscope (Leica, Germany) as described previously [25]. The total number of cells was counted in transmitted light. Approximately 1000 cells were counted for each sample. All experiments were carried out 3–6 times independently of each other.

Measurement of extracellular superoxide and H_2O_2. Extracellular superoxide was determined by using it ability to restore ferricytochrome *c* [26]. To determine H_2O_2, the cells were grown as described above. At certain intervals, aliquots of the culture with a volume of 2 mL were taken, passed through membrane filters, and the concentration of H_2O_2 in filtrates was determined using the Amplex Red–Horseradish peroxidase system [27] using a Shimadzu RF-1501 spectrofluorimeter (Shimadzu, Japan) (λ_{ex} 563 nm and λ_{em} 587 nm). The H_2O_2 concentration in samples was calculated from the calibration curve.

Measuring glutathione and gene expression. Extracellular and intracellular glutathione was determined spectrophotometrically by the cyclic method with 5,5'-dithiobis(2-nitrobenzoic acid) and glutathione reductase [28], modified as described previously [5]. To determine extracellular glutathione, samples of cell suspensions were passed through membrane filters (0.45 μm). One part of the filtrate was analyzed for total glutathione and the other for GSSG content. To determine intracellular glutathione, 10 ml of culture was centrifuged at 8000 *g* within 5 min, the precipitate was resuspended in 5 ml of a cold aqueous solution of 20 mM EDTA and disrupted by ultrasound at 0°C. Protein precipitation and determination of GSH and GSSG were performed as described previously [5]. Calibration curves were built from known concentrations of GSH and GSSG, which were processed as samples of cell suspensions. The glutathione concentration was expressed as the ratio of the value obtained from the calibration curves to the OD_{600} at the time of sampling.

Changes in the expression of the analyzed genes were assessed by determining the activity of β-galactosidase [29] of *E. coli* strains carrying the corresponding gene fusions.

Determination of colony forming units (CFU). To calculate CFU, serial dilutions were prepared (from 10^{-1} to 10^{-8}) of each sample in 0.9% sterile NaCl. One milliliter of diluted culture (usually 10^{-6} and 10^{-7} to obtain single colonies) was mixed with 3 ml of molten soft LB-agar (0.8%) at 42°C and poured onto Petri plates with solid LB-agar (1.5%). The number of colonies on the plates was counted after 24 h of incubation at 37°C.

Statistical analysis. Each result is presented as an average value (at least three independent experiments) ± standard error of the mean. For analysis we used the Student's *t*-test. Values of $p < 0.05$ were considered statistically significant. The results were analyzed using the Statistica 8.0.360 program (Statsoft Inc. 2007).

RESULTS

The effect of phosphate starvation on the growth and energy parameters of E. coli

In the absence of phosphate limitation, the maximum specific growth rate (μ_{max}) of the exponentially growing *E. coli* parent strain (wt) and *gshA*, *gor*, *trxA*, *trxB*, *gshA trxA* and *gor trxB* mutants was 0.69 ± 0.02, 0.71 ± 0.01, 0.65 ± 0.01, 0.54 ± 0.01, 0.63 ± 0.01, 0.63 ± 0.01, and 0.57 ± 0.01 h^{-1} respectively (Fig. 1a). When the density reached ~1 p.u. at a wavelength of 600 nm, the growth rate gradually decreased in all strains due to a drop in oxygen concentration and accumulation of acidic metabolic products.

After the bacteria were transferred from the growing culture to the medium without phosphate, the growth rate rapidly decreased, reaching a value of about 0.25 h^{-1} in 20–40 min, after which in all strains (including the parent) a phase of a slower decrease in the growth rate was observed (Fig. 1b). The growth observed in the first phase appeared to be associated with consumption of trace amounts of P_i that entered the medium along with centrifuged cells. After 24 h, growth stopped in all strains. The addition of phosphate resulted in regrowth at the rate seen in the growing culture (*data not provided*). Growth retardation in cultures growing under conditions of phosphate starvation was also recorded by a decrease in CFU when compared with a culture consuming phosphate (Figs. 1c, 1d). During the first 2 h of incubation of the parent strain ~~o~~in a medium with phosphate, the number of CFU increased by 4.5 times, while in the starving culture this indicator increased by 2.9, and in the mutants, by an average of 2.1 times. The smallest increase in CFU (by 1.5 times) was observed for the *trxA* mutant, which is defective in the synthesis of thioredoxin-1.

The parent strain and *gshA*, *gor*, *trxA*, *trxB*, *gshA trxA*, and *gor trxB* mutants, exponentially growing on a medium with phosphate contained 3.07 ± 0.08, 2.85 ± 0.06, 3.13 ± 0.1, 2.34 ± 0.11, 2.80 ± 0.1, 2.84 ± 0.06, and 2.3 ± 0.06 μM ATP/OD_{600} respectively (zero time in Fig. 2a). When phosphate was depleted, synchronously with a decrease in the growth rate, the ATP level decreased in all strains by an average of 2.3 times after 1 h and by 5.3 times after 24 h (Fig. 2a).

In all strains, including the parental one, during growth in the presence of phosphate, the proportion of cells stained with the fluorescent dye $DiBAC_4(3)$, varied from 2.0 to 3.2% and changed little during growth (zero time in Fig. 2b). Tteatment with the protonophore carbonyl treatment with carbonyl cyanide-*m*-chlorophenylhydrazone (20 μM CCCP) increased the number of cells permeable to $DiBAC_4(3)$, up to 54% within 20 min (*not shown*). During 48 h of phosphate starvation, no increase in the number of stained cells was observed in all strains, which indicates that starving cells retained their membrane potential.

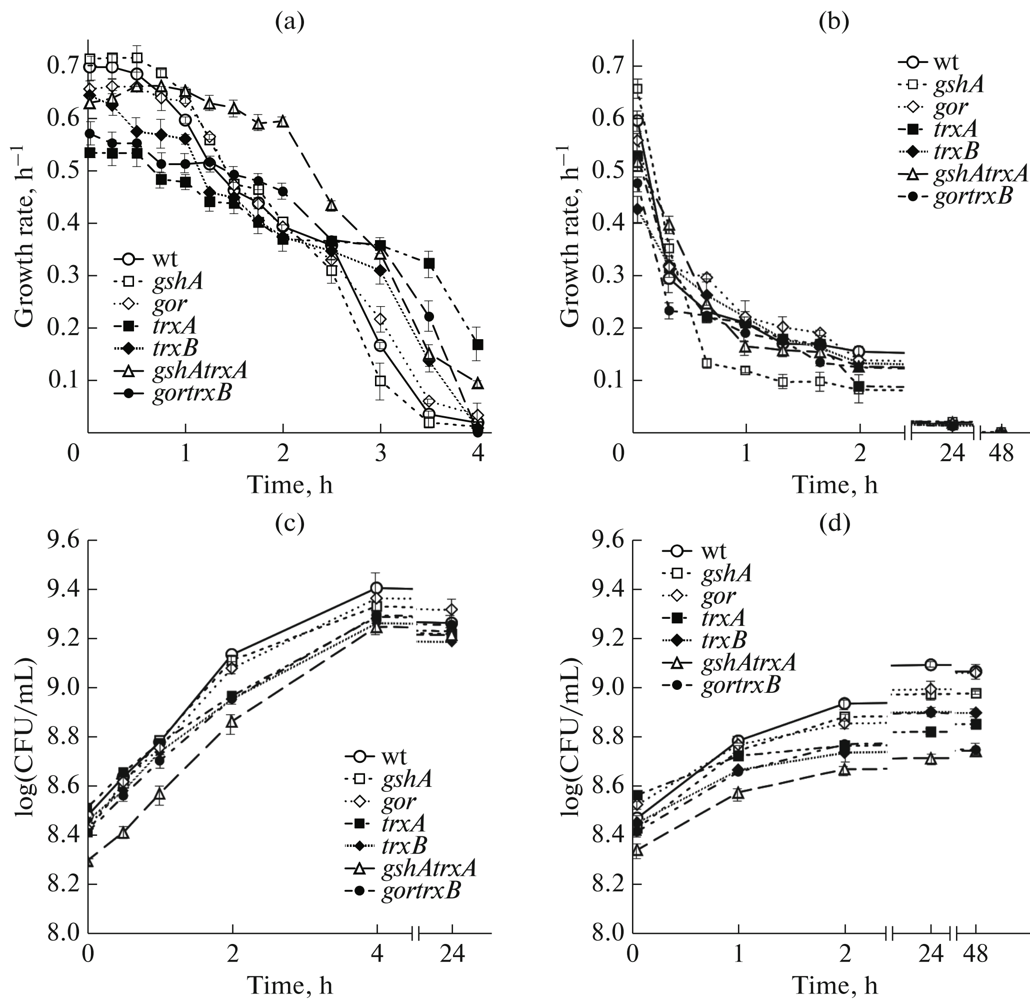


Fig. 1. Changes in the growth rate and number of CFUs in growing and phosphate-starving *E. coli*. The specific growth rate in a medium with phosphate (a) and without phosphate (b); CFU in a medium with phosphate (c) and without phosphate (d).

A decrease in the growth rate at the initial phase of incubation in a culture medium without phosphate was accompanied by a drop in the oxygen level (Fig. 2c). We hypothesize that this may be due to aerobic glucose metabolism. The falling dO_2 rate for the parent strain at this phase was 4.3% OD_{600}^{-1} min^{-1} compared to 6.3% OD_{600}^{-1} min^{-1} for the same period after the transfer of bacteria to the medium with phosphate. In all strains, including the parental one, after 10–20 min from the start of incubation, phosphate depletion led to a sharp increase in dO_2 and the level of K^+ in the medium, which indicates a decrease in oxygen consumption and the release of part of the K^+ from cells (Fig. 2c). Then, the slow consumption of oxygen and K^+ resumed, corresponding to the growth of bacteria at a low rate. The rate of decrease of dO_2 at this phase (40–60 min after transfer to medium without phosphate) was 0.27% OD_{600}^{-1} min^{-1}, which was almost 5 times lower than the corresponding rate in a medium with phosphate (1.28% OD_{600}^{-1} min^{-1}).

The Effect of Phosphate Starvation on Oxidant Production and Antioxidant Gene Expression in E. coli

The growth of bacteria in the presence of phosphate was accompanied by the accumulation of H_2O_2 in the environment. In a starving culture, the concentration of extracellular hydrogen peroxide was kept at a low level; with the addition of phosphate, the resumption of growth was accompanied by a rapid increase in H_2O_2 in the medium (Fig. 2g). In the presence of phosphate, for all mutants (except *gor*) the extracellular concentration of H_2O_2 was lower than that of the parent strain. The double mutant *gshA trxA* had the lowest H_2O_2 level and was 35% of the parental level. For the *gor* mutant*,* which is deficient in glutathione reductase, the extracellular concentration of H_2O_2 was 12% higher than that of the parental strain (Fig. 2e).

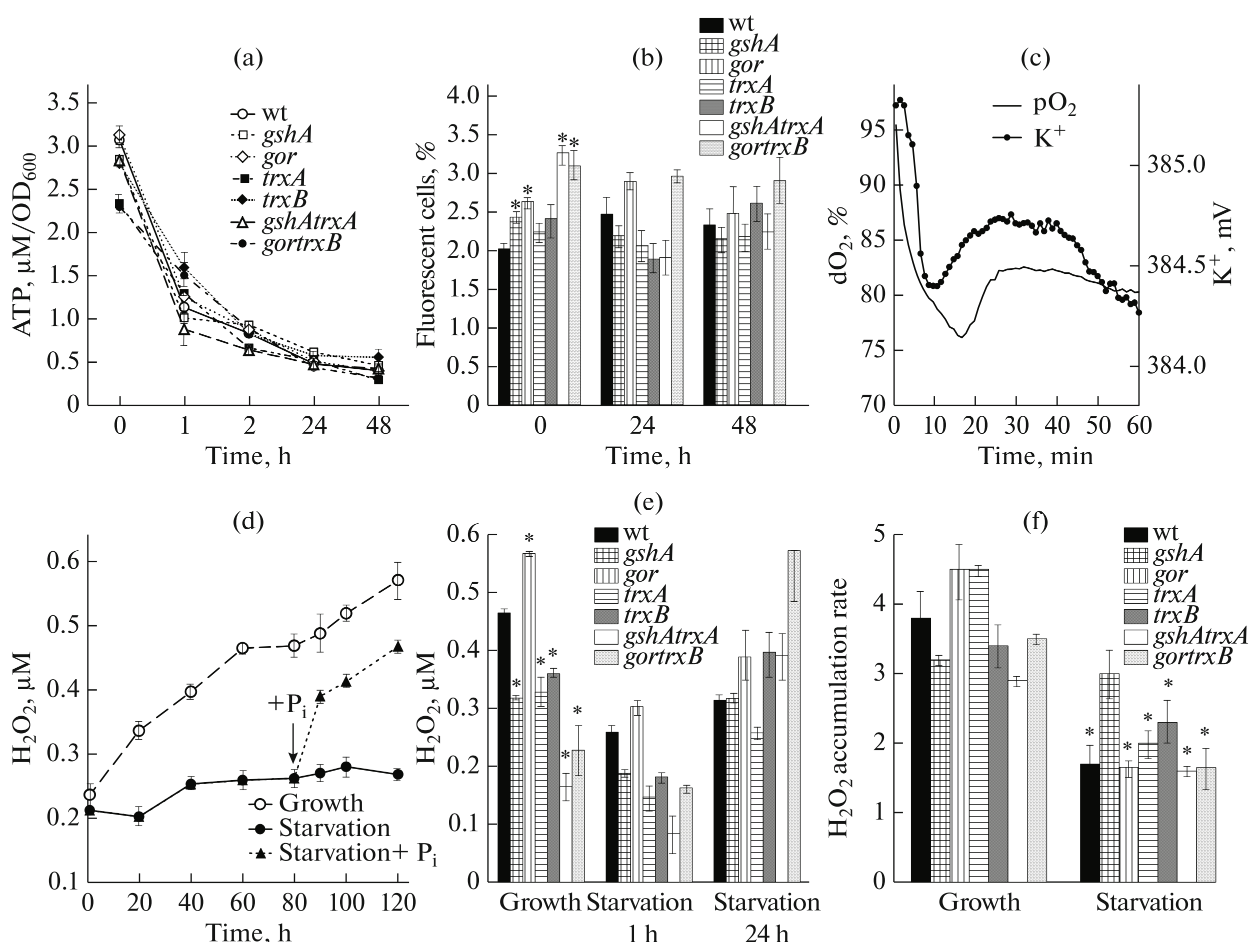


Fig. 2. Changes in physiological parameters of *E. coli* during phosphate starvation. (a) ATP level; (b) is the percentage of cells that have lost their membrane potential (stained with the fluorescent dye $DiBAC_4(3)$); (c) is the content of dissolved oxygen (dO_2) and extracellular potassium (K^+) in the culture of *E. coli* BW25113 (wt), 0.5 mV corresponds to 8 μM K^+; (d) the extracellular concentration of H_2O_2 in *E. coli* BW25113(wt); (e) the accumulation of H_2O_2 in the culture medium of mutants for thiol redox systems; (f) the rate of accumulation of H_2O_2 in the medium of the studied strains for 2 h of growth or starvation (nmol OD_{600}^{-1} min^{-1}). $*R < 0.05$ between the values of the parameters obtained for the mutants relative to the parental strain (b, e) or between values in growing and starving cultures for each strain (f).

The accumulation rate H_2O_2 in the medium, calculated as nmol OD_{600}^{-1} min^{-1}, was minimal in *gshA* and *gshA trxA* mutants. In other cases, there was no significant difference from the parent (Fig. 2f). At 1 h after the start of fasting the level of H_2O_2 in all strains was on average 44% lower than in the growing culture. The degree of reduction was generally proportional to the content of H_2O_2 before starvation, with the exception of the *gor trxB* double mutant, whose H_2O_2 level decreased by 29% from the original. The accumulation rate of H_2O_2 in the starving culture was also lower than in the growing one in all strains, except *gshA* (Fig. 2f). Further observation revealed that after 24 h of fasting, the extracellular level of H_2O_2 in *gshA* and *trxB* mutants was restored to values close to those in the growing culture, and in the parental strain and *gor* and *trxA* mutants it slightly increased, but did not reach the values in the growing culture. We note the sharp increase (more than 2 times) in the production of H_2O_2 in double mutants (Fig. 2e).

In contrast to H_2O_2, the production of superoxide during phosphate starvation not only did not decrease, but even increased by 2–3 times in comparison with the culture that was not limited in phosphate (Fig. 3a). This effect was equally observed both in the parental strain and in the *gshA* and *gshA trxA* mutants. An increase in superoxide production corresponded to a slowdown in respiration during starvation (Fig. 2c). The addition of phosphate led to a decrease in superoxide formation to the level observed in the culture with phosphate (Fig. 3a).

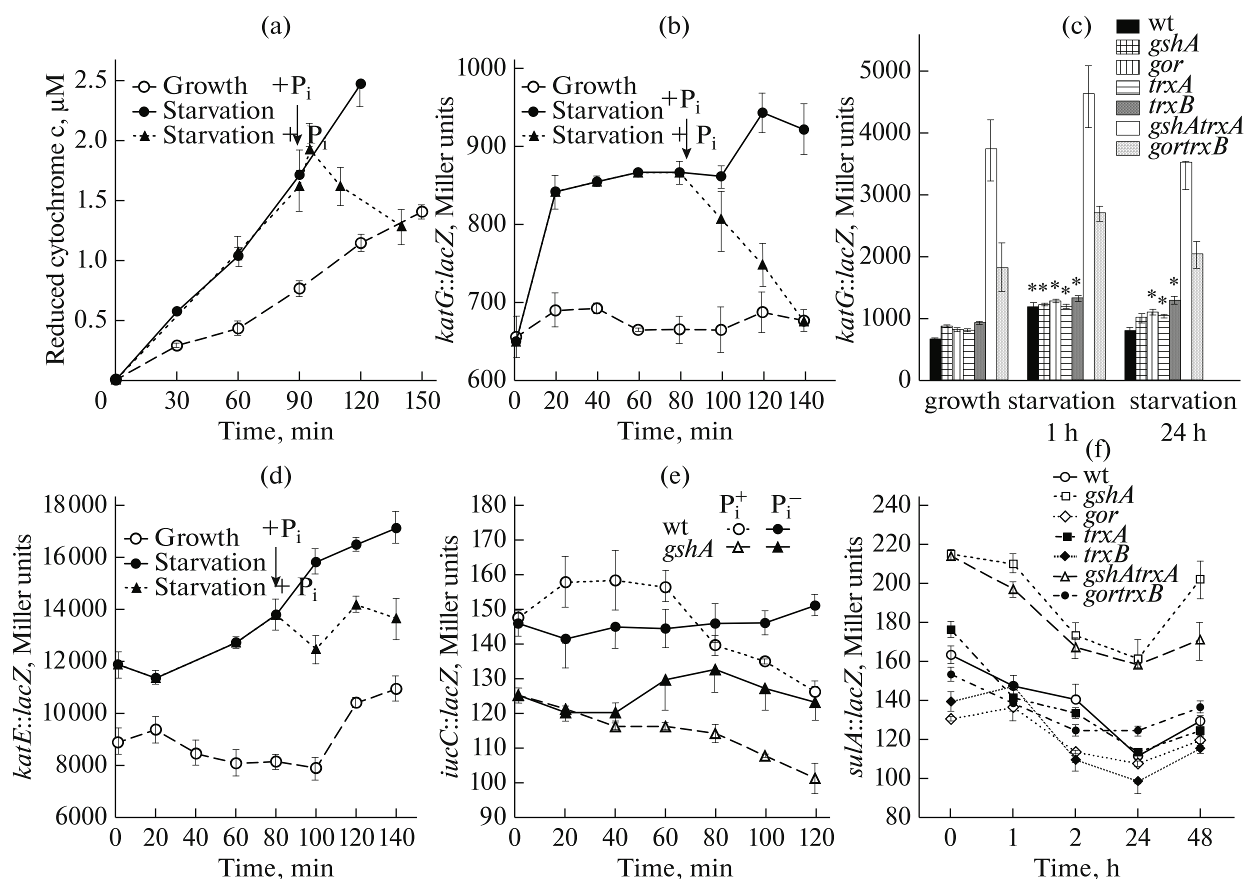
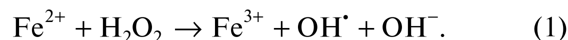


Fig. 3. Superoxide production and antioxidant gene expression in growing and phosphate-starved *E. coli*. (a) Production of superoxide in strain BW25113 (wt); (b) expression of *katG*::*lacZ* in strain BW25113 (wt); (c) expression of *katG*::*lacZ* in mutants for thiol redox systems (**R* < 0.01 between the values of parameters in growing and starving cultures for each strain); (d) expression of *katE*::*lacZ* in strain BW25113 (wt); (e) expression of *iucC*::*lacZ* in strains BW25113 (wt) and JW2663 *(gshA)*; (f) expression of *sulA*::*lacZ* in the studied mutants during phosphate starvation.

The decreased extracellular H_2O_2 levels observed during phosphate starvation may be the result of both a decrease in peroxide production and an increase in the activity of hydrogen peroxide degrading enzymes such as catalases G and E. The gene expression of *katG*, which encodes catalase G, is regulated by the transcription factor OxyR, which is activated by H_2O_2, and the regulator of the general stress response RpoS during the transition to the stationary phase [30]. *katE*, which encodes catalase E, is expressed under the control of RpoS, whose level ~~of~~ is inversely proportional to the specific growth rate [31].

When growing on phosphate, the expression of *katG*::*lacZ* was maintained at a constant level, but rapidly increased with the onset of starvation (Fig. 3b). The addition of phosphate reduced expression of *katG* to the level observed in growing culture. In a growing culture, expression of *katG*::*lacZ* in single mutants was higher than in the parental strain by an average of 25%, and in double mutants *gshA trxA* and *gor trxB* by 5.6 and 2.7 times, respectively (Fig. 3c). Fasting for 1 hour led to an increase expression of *katG*::*lacZ* in all strains, including the parent strain by 76%, and in single mutants by an average of 47%. Double mutants retained a high level of expression of *katG*::*lacZ*. It is noteworthy that in double mutants grown with phosphate and starved for 1 h, low levels of H_2O_2 corresponded to the highest expression of *katG*::*lacZ*. Although 24 h after the onset of fasting, the expression level of *katG*::*lacZ* in all strains slightly decreased compared to the values observed after 1 h of starvation, it still remained higher than in bacteria growing on a medium with phosphate (Fig. 3c). A fasting-induced increase in the expression of *katE*::*lacZ* (Fig. 3d) occurred. In a phosphate-containing culture of the parental *E. coli* strain the expression of this fusion increased by 17% in 2 h (from 8852 ± 851 to 10 377 ± 72 Miller units), while in the starving culture it increased by 86% (up to 16486 ± 220).

The major cause of DNA damage and death *E. coli* under peroxide stress was the formation of highly toxic hydroxyl radicals in the Fenton reaction [32]:



$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^{\bullet} + OH^{-}. \quad (1)$$

The free iron pool in *E. coli* was maintained at a low level and was strictly controlled by the Fur transcriptional regulator [33]. One of the members of the Fur regulon is *iucC*, which encodes a protein involved in the synthesis of siderophore aerobactin. A decrease in the level of intracellular free iron stimulates the expression of *iucC*. Using *iucC*::*lacZ* [22] makes it possible to trace the degree of induction of the Fur regulon and obtain an indirect estimate of the state of the free iron pool. As can be seen from Fig. 3e, in the parent *E. coli* strain and the *gshA* mutant, after 2 hours of fasting, the expression of *iucC*::*lacZ* rose by 21% compared to cells growing on phosphate ($p < 0.05$). This may indicate a slight decrease in the content of free iron in the cytoplasm.

In response to DNA damage *E. coli* induce the SOS regulon that controls the genes involved in DNA repair [34]. To study the expression of SOS genes, we used a transcriptional fusion of *sulA*, a mediator of filamentation in the SOS response, with *lacZ* [21]. In cultures of *E. coli* growing on a medium with phosphate, the highest level of expression of *sulA*::*lacZ* occurred in the *gshA* and *gshA trxA* strains (215 ± 4 Miller units), which is 32% higher than in the parental strain (163 ± 4) (Fig. 3f). The lowest expression was in the *gor* strain (130 ± 1). All strains (including the parent) showed a statistically significant decrease in *sulA*::*lacZ* expression during 24 h of phosphate starvation, by an average of 26%, which may indicate a decrease in DNA damage. After 48 h of fasting, in all the studied strains, the expression level of *sulA*::*lacZ* slightly increased compared to the 24-h point (by a maximum of 25% in the mutant *gshA*), but did not reach the values typical for a growing culture.

The Effect of Phosphate Starvation on the Level and Redox Status of Glutathione in E. coli Cells

We have previously shown that the levels of intracellular (GSH_{in}) and extracellular (GSH_{out}) of glutathione, as well as the redox status of glutathione, change significantly under various stresses [3, 5, 14, 35, 36]. It was of interest to test the effect of phosphate starvation on these parameters.

In cells growing on phosphate *E. coli* had the highest concentration of GSH_{in} found in the *gor* mutant (11.1 ± 0.07 μM/OD_{600}), the smallest occurred in the *trxA* mutant (3.3 ± 0.6). The *gor trxB*, *trxB* mutants and the parent strain contained 4.7 ± 0.4, 7.2 ± 0.2, and 7.7 ± 0.3 μM/OD_{600}, respectively. After 1 h of cultivation in a medium without phosphate, all strains showed a statistically significant ($p < 0.05$) increase in GSH_{in}. The smallest increase (by 1.6 times) occurred in the parent strain, the largest occurred in *gor trxB* (4.4 times) (Fig. 4a). Elevated GSH_{in} persisted in all strains after 24 h of starvation. By 48 h of starvation, all strains showed a tendency to decrease in intracellular GSH, although to a different extent. In the parent strain, as well as in *trxA* and *trxB* mutants the GSH_{in} level still remained higher than in cultures growing on phosphate, and in *gor* and *gor trxB* it approached this value (Fig. 4a).

It is known that *E. coli* accumulate glutathione in micromolar concentrations in the medium [37]. The level of extracellular glutathione depends on the cultivation conditions and can change significantly in stressful situations [3]. We have shown that during growth on a medium with phosphate, the level of GSH_{out} was maintained in the range from 1.2 ± 0.15 (*trxA*) to 3.0 ± 0.03 (*gor*) μM/OD_{600} and correlated with the values of GSH_{in} ($r = 0.95$). Phosphate starvation in all strains was accompanied by a decrease in GSH_{out} levels, which was faster during the first hour (Fig. 4d). By this time and further, until the end of cultivation, the differences in GSH_{out} between strains sharply decreased. After 48 h of fasting, the GSH_{out} approached trace amounts (Fig. 4d). As follows from the comparison of the data presented in Figs. 4a and 4d, a significant contribution to the increase in the GSH_{in} pool in the process of starvation can come from the medium.

The level of intracellular oxidized glutathione ($GSSG_{in}$) in the medium with phosphate in the parental strain was 0.16 ± 0.02 μM/OD_{600} (Fig. 4b). $GSSG_{in}$ concentrations close to this value were found in g*or*, *trxA,* and *trxB* strains: 0.20 ± 0.06, 0.19 ± 0.04, and 0.19 ± 0.01, respectively. Mutants lacking both *gor trxB* reductases were markedly different from other strains, with a level of $GSSG_{in}$ that was twice as high as that of the parent strain ($p < 0.05$). There was no statistically significant change in the concentration of $GSSG_{in}$ during starvation in all strains except *gor trxB*. In this strain, 1 hour after the onset of starvation, the level of $GSSG_{in}$ increased by 2.3 times ($p < 0.05$), and then, during further cultivation, it decreased to a level close to the initial value (Fig. 4b).

In strains growing in the presence of phosphate, the concentration of oxidized extracellular glutathione ($GSSG_{out}$) was on average 3 times lower than GSH_{out} (Fig. 4c). In the parent strain and *trxA* and *trxB* mutants a low level of $GSSG_{out}$ was also preserved during starvation. The exception was the *gor trxB* mutant, whose concentration of $GSSG_{out}$ in cells growing on phosphate was only 1.5 times lower than GSH_{out}, while during starvation it increased by 3.6 times, reaching the level of GSH_{in}, which was observed in the growing culture and after 48 hours of starvation. For the *gor* mutant the fasting level of $GSSG_{out}$ almost doubled (Fig. 4e).

The redox status of glutathione reflects the GSH/GSSG ratio. Due to the fact that the initial levels of $GSSG_{in}$ all strains changed in a narrow range, differences in GSH_{in}/$GSSG_{in}$ correlated with GSH_{in}, which varied over a wider range (Fig. 4c). In our

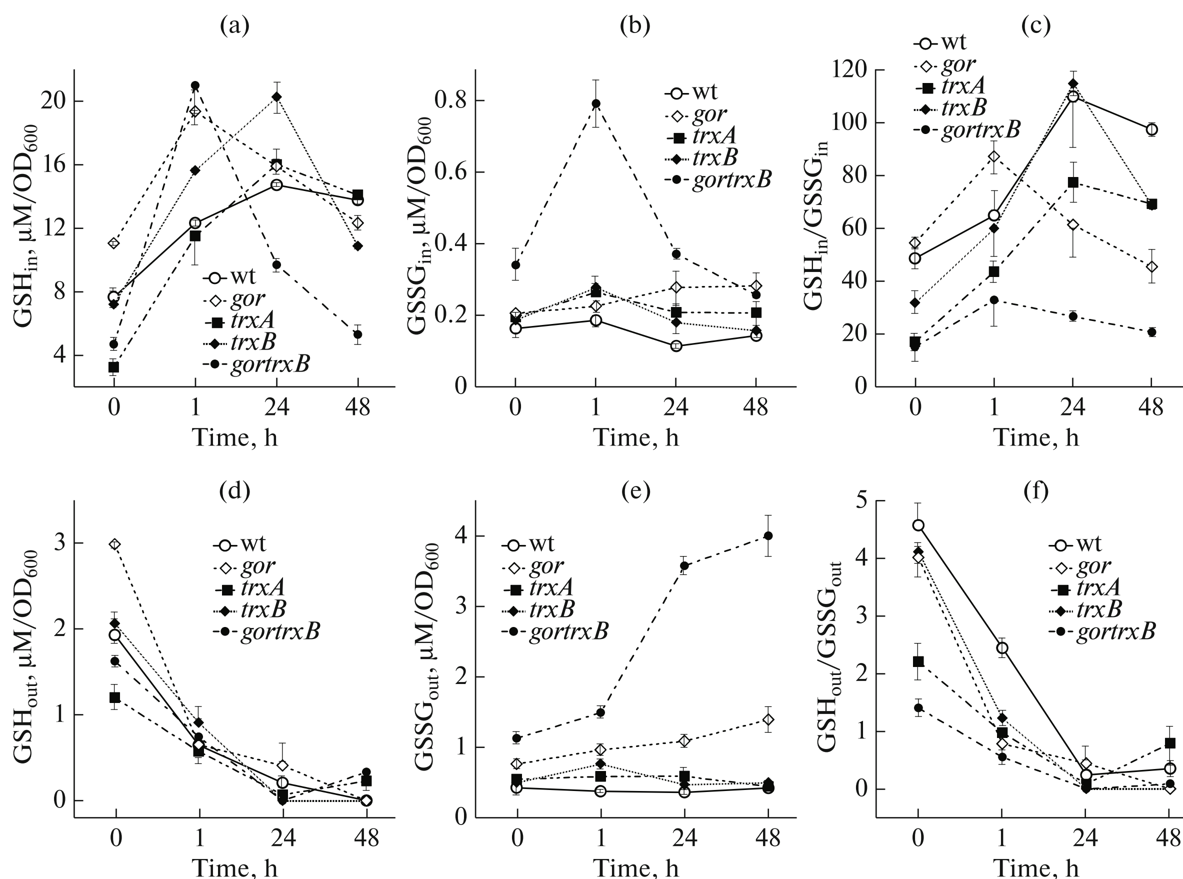


Fig. 4. The changing glutathione status in phosphate-starved *E. coli*. (a) Intracellular reduced glutathione (GSH_{in}); (b) intracellular oxidized glutathione ($GSSG_{in}$); (c) $GSH_{in}/GSSG_{in}$; (d) extracellular reduced glutathione (GSH_{out}); (e) extracellular oxidized glutathione ($GSSG_{out}$), (f) $GSH_{out}/GSSG_{out}$.

experiments in a medium with phosphate, the $GSH_{in}/GSSG_{in}$ for the parent strain was 48.6 ± 3.9. The absence of both reductases in the *gor trxB* strain sharply reduced this value by more than three times. During the phosphate starvation, there was a significant increase in $GSH_{in}/GSSG_{in}$ towards reductive values that reached a maximum for *gor* after 1 hour (87 ± 6), and for *trxA*, *trxB,* and the parent strain (77 ± 13, 114 ± 6 and 110 ± 19, respectively) at 24 h after the start of cultivation (Fig. 4c). The smallest increase was observed in *gor trxB* (32 ± 10), which is associated with the lowest content of GSH_{in} among other strains and the largest in $GSSG_{in}$. In all strains growing on phosphate, $GSH_{out}/GSSG_{out}$ changed from 1.4 ± 0.16 for *gor trxB* to 4.6 ± 0.3 in the parent strain, averaging about 3.3, which is 10 times less than $GSH_{in}/GSSG_{in}$. In contrast to $GSH_{in}/GSSG_{in}$, $GSH_{out}/GSSG_{out}$ in all strains significantly decreased towards oxidative values during starvation (Fig. 4f).

It should be noted that the $GSH_{in}/GSSG_{in}$ values obtained in this work were significantly lower than those observed earlier when growing *E. coli* BW25113 on M9 medium, where this ratio varied from 330 to 500 under normal growth conditions [14]. In our work, in the same bacteria growing on the MOPS medium, the ratio of $GSH_{in}/GSSG_{in}$ was about 5 times lower, which is associated with an increase in the level of $GSSG_{in}$. This increase in $GSSG_{in}$ may indicate a higher intensity of oxidative processes in the MOPS medium, where the concentration of Fe^{2+} ions was 5 times more than in M9.

DISCUSSION

An increase in ROS production, activation of components of antioxidant systems, and oxidative damage to biomolecules, together with a decrease in survival or growth rate, are usually considered as indicators of oxidative stress in the response of bacteria to stresses that are not directly related to the action of ROS. As a result of studying the dynamics of changes in the oxygen content in the medium, we showed that after the depletion of phosphate in the presence of glucose, the bacteria retained their respiratory activity, although at a lower level than those growing with phosphate.

Under aerobic conditions, *E. coli* produce superoxide and H_2O_2 when molecular oxygen randomly receives electrons from reduced flavoprotein cofactors. Other, including extracellular, sources of ROS are also possible [38]. Under the conditions we chose, the transition to phosphate starvation was accompanied by a decrease in the accumulation of H_2O_2 in the medium of all studied strains. This could be a consequence of a decrease in the intensity of respiration, as evidenced by the increase in oxygen content. However, an increase in the rate of superoxide production and gene expression of *katG* and *katE* during the transition to starvation indicates an increase in ROS production. Expression of both genes is controlled by RpoS, which responds to a decrease in growth rate and transition to the stationary phase [30]. Expression of *katG* may also be controlled by the transcriptional regulator OxyR, which responds to an increase in intracellular H_2O_2 and apart from *katG* activates transcription of other genes (*ahpFC, gorA,* etc.) involved in the protection of *E. coli* from peroxide stress [39]. Thus, the decrease in the accumulation of H_2O_2 in the medium observed during phosphate starvation may be due to the induction of antioxidant enzymes.

The significant changes in the redox status of glutathione discovered during phosphate starvation are of particular interest. The most important ones are associated with a decrease in the level of GSH in the medium and with an increase in its content in the cytoplasm, as well as with a shift in $GSH_{in}/GSSG_{in}$ towards reductive values, and $GSH_{out}/GSSG_{out}$ towards oxidative values.

Earlier, an increase in GSH_{in} was found in *E. coli* growing under aerobic conditions on M9 medium, under the action of ciprofloxacin [40, 41] and chloramphenicol [14], in amino-acid starvation [14], and in glucose depletion [35]. In addition, under the action of ciprofloxacin and chloramphenicol, an increase in GSH_{out} was simultaneously observed, whereas during amino acid starvation, changes in GSH_{out} were absent. The changes in the status of glutathione during phosphate starvation we revealed are similar to those that occurred during growth arrest of *E. coli* due to glucose depletion when there was an increase in GSH_{in} at the same time and a decrease in GSH_{out} [35]. Previously, it was shown that factors that reduce the energy status of cells inhibit the GSH transmembrane cycle and stimulate GSH are imported from the medium [5]. This may explain the similar changes in glutathione status inside and outside the cell when glucose and phosphate are depleted. In both cases, fasting was accompanied by a significant decrease in the level of ATP [35, and this work]. An increase in the ATP level was noted under the action of ciprofloxacin [41] and chloramphenicol [42].

In the works cited above, it was established that stress-induced changes in the status of glutathione are associated with its participation in cysteine homeostasis. Bacteria maintain a low level of intracellular free cysteine due to its high redox activity and ability to reduce intracellular iron, thereby supplying a substrate for the Fenton reaction [15, 16]. GSH is a weak iron reducer [15], thus the incorporation of cysteine into the glutathione molecule serves as one of the effective mechanisms for maintaining cysteine homeostasis with an increase in its intracellular concentration, which is observed when protein synthesis is stopped [14]. Reducing the excess of cysteine in cells is also achieved by its export to the medium and desulfurization with the formation of H_2S [14, 16, 17]. The increase in GSH_{in} levels we observed during phosphate starvation; it can also be a consequence of the intensification of glutathione synthesis when an excess of cysteine occurs due to inhibition of protein synthesis and other metabolic processes that consume cysteine.

It is known that redox systems of glutathione and thioredoxin largely functionally overlap and duplicate each other. As a result, the absence of components of one of the systems often does not manifest itself phenotypically, and only multiple mutations affecting both systems lead to significant metabolic disorders. In addition, the redox systems of glutathione and thioredoxin closely interact with the OxyR regulon, which is activated by H_2O_2. Thus, under peroxide stress, the loss of glutathione reductase or thioredoxin reductase can be compensated by an increase in the expression of *katG* (and GSH synthesis in the first case) [6].

The behavior of single mutants under phosphate starvation generally corresponds to what was observed earlier under peroxide stress. According to such an integral indicator as the growth rate, mutants (with the exception of *gshA*) differed insignificantly from the parental strain and from each other. The data on the *gor trxB* double mutant are of particular interest. By comparison with the parental and single mutants, this strain was characterized by many times higher expression of *katG*::*lacZ*, the highest levels of oxidized intra- and extracellular glutathione and, accordingly, the lowest GSH/GSSG ratio in both compartments. After 48 h of phosphate starvation, this strain (as well as at *gshA trxA*) had the lowest CFU. The double mutant *gshA trxA* had similar properties (excluding indicators related to glutathione status). The data on the behavior of the *gor trxB* mutant provide additional evidence for the presence of oxidative stress in phosphate starvation. Considering all the listed properties, the *gor trxB* double mutant can be considered as a convenient model for studying the response of bacteria to stresses not associated with the direct action of ROS.

The results we obtained are summarized in Fig. 5.

Based on the results we obtained, it can be said that during phosphate starvation in *E. coli* the rate of ROS production increases, but the induction of catalase

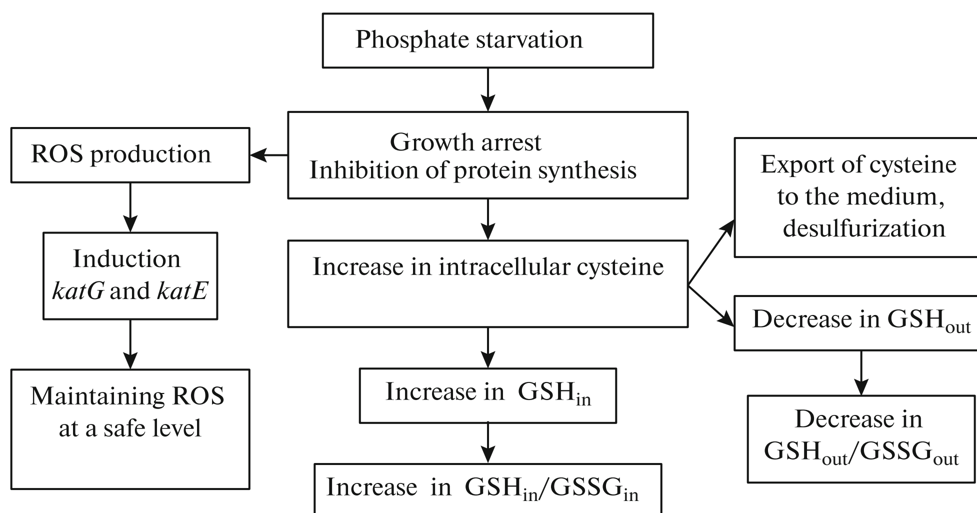


Fig. 5. The proposed response pattern of antioxidant systems of *E. coli* during phosphate starvation.

prevents the accumulation of H_2O_2 above the toxic level. Combined with low levels of free iron, this allows bacteria to maintain conditions that reduce the risk of DNA damage, as evidenced by the lack of upregulation of the *sulA* gene in starved cells, included in the SOS regulon. It is also noteworthy that a significant decrease in the ATP pool, which is observed as the cell growth rate decreases, is not accompanied by a large drop in the membrane potential and complete outflow of potassium, which may indicate the preservation of normal membrane activity in starving cells. Phosphate starvation causes a significant shift in the status of glutathione inside and outside cells, which, apparently, reflects the work of the mechanisms of intracellular cysteine homeostasis. The totality of the ongoing changes leads to the fact that under the described conditions, *E. coli* can maintain high viability for a long time, allowing it to quickly resume growth after the introduction of phosphate into the medium. Mutations in individual components of thiol redox systems do not lead to critical disorders causing cell death due to coordinated changes in the activities of other antioxidant systems.

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COMPLIANCE WITH ETHICAL STANDARDS

This article does not contain any studies involving human participants or animals as research subjects.

CONFLICT OF INTERESTS

The authors declare that they have no conflicts of interest.

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