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EXPERIMENTAL ARTICLES

The Role of Thiol Redox Systems in the Resistance of *Escherichia coli* in the Stationary Phase

G. V. Smirnova[a], N. G. Muzyka[a], and O. N. Oktyabr'skii[a, b, 1]

[a] *Institute of Ecology and Genetics of Microorganisms, Ural Branch, Russian Academy of Sciences, ul. Goleva 13, Perm, 614081 Russia*

[b] *Perm State Technical University, Komsomol'skii pr. 29, Perm, 614990 Russia*

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Abstract—The effect of glutathione (*gshA*), thioredoxin (*trxA*), and thioredoxin reductase (*trxB*) mutations on the adaptation of *Escherichia coli* to prolonged glucose starvation was investigated. The *trxB* mutation had the worst consequences for the stationary-phase cells. These bacteria exhibited decreased survival, increased sensitivity to oxidants, and decreased expression of the *katE* and *sulA* genes. As the stationary phase proceeded, the physiological resistance to antibiotics increased in all the strains tested; however, the thiol redox system mutants were far less able to develop antibiotic resistance than the parent strain cells. During the stationary phase, a considerable shift was observed in the redox status of intra- and extracellular glutathione toward the oxidative values. These results indicate that the thiol redox systems play an important role in the adaptation of *Escherichia coli* to prolonged starvation and antibiotic resistance.

Keywords: starvation stress, glutathione, thioredoxin, gene expression, antibiotic resistance.

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In the life cycles of many bacteria, including *Escherichia coli*, periods of active growth are followed by periods of quiescence due to the absence of nutrient substrates and unfavorable environmental conditions. Transition to the stationary phase requires fundamental rearrangements in gene expression and bacterial metabolism. One of their consequences is increased resistance to many stress effects [1]. The main regulator of this general stress response is the RNA polymerase sigma subunit (the sigma factor σ^s) controlling the expression of more than 50 genes, including those involved in protection from oxidative stress. Antibiotic resistance of the stationary-phase *E. coli* cells was also shown to be associated with the activity of σ^s [2].

Increasing attention is presently being paid to the study of the redox regulation of cellular activity. Reactive oxygen species (ROSs), on the one hand, and glutathione (GSH), thioredoxins (Trx), and other thiol-containing molecules, on the other hand, play an important role in this process. The known functions of these thiols include their involvement in such processes as ROS neutralization, the regulation of antioxidant gene expression, and repair of the oxidative damage [3, 4]. The molecular mechanisms involved in the redox regulation of eukaryotic cells have been studied at length. The role of redox regulation involving thiols is much less studied in bacteria, especially as far as the processes related to the adaptation to stress are concerned.

Over the last decade, data have been accumulated indicating that, under aerobic conditions, oxidative stress is involved in the mechanism of bactericidal action of diverse classes of antibiotics [5–8]. This is another area of research where very little is known about the role of redox regulation with the involvement of the thiol compounds.

The goal of the present work was to study the role of thiol redox systems in the adaptation of *E. coli* to long-term starvation, as well as their resistance to oxidants and antibiotics during the stationary growth phase.

MATERIALS AND METHODS

The *E. coli* strains used in the work are listed in Table 1. The bacteria were grown in the minimal medium M9 [9] supplemented with 4 g/l of glucose. After centrifugation the cells from the overnight culture were resuspended in 100 ml of fresh M9 medium ($OD_{600} = 0.1$) with 0.4 g/l glucose and grown at 37°C in 250-ml flasks on shakers at 150 rpm. Glucose was exhausted at $OD_{600} = 0.5–0.6$, which corresponded to the midexponential growth phase, and afterward the bacteria were subjected to 3-day starvation. The growth was monitored by changes in the optical density of the culture at 600 nm. The number of viable cells was determined by counting the colonies grown on petri dishes with LB agar. The bacterial sensitivity

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

Table 1. *E. coli* bacterial strains used in the work

Strain	Genotype	Source
K12	wt	VKM
RI89	*ara D139* Δ(*araABC-leu*) *7679 galU galK* Δ*(lac)X74 rpsL thi phoR* Δ*ara-714 leu*$^+$	J. Beckwith
RI336	RI89 *gshA*::Tn *10* Kan^r	J. Beckwith
RI319	As RI89, but *trx*::Kan^r	J. Beckwith
RI363	As RI89, but *trxA*::Kan^r	J. Beckwith
RK 4936	pKT 1033 *katG*::*lacZ*	K. Tao
QC772	As GC 4468, but *sodA49* (*sodA*::*lacZ*)	D. Touati
AI 1340	As 1283 (JTG100), but *pRS 415 KatF5* (*katF*::*lacZ*)	A. Eisenstark
AI 1326	As JTG100, but *pRS KatE 16* (*katE*::*lacZ*)	A. Eisenstark
BM11	As RI89, but *katG*::*lacZ*	Laboratory Museum
BM21	As RI319 (*trxB*), but *katG*::*lacZ*	Laboratory Museum
BM31	As RI336 (*gshA*), but *katG*::*lacZ*	Laboratory Museum
BM41	As RI363 (*trxA*), but *katG*::*lacZ*	Laboratory Museum
NM131	As RI89, but *sulA*::*lacZ*	Laboratory Museum
NM141	As RI336 (*gshA*), but *sulA*::*lacZ*	Laboratory Museum
NM151	As RI363 (*trxA*), but *sulA*::*lacZ*	Laboratory Museum
NM161	As RI319 (*trxB*), but *sulA*::*lacZ*	Laboratory Museum
NM251	As RI89, but *sodA*::*lacZ*	Laboratory Museum
NM261	As RI319 (*trxB*), but *sodA*::*lacZ*	Laboratory Museum
NM271	As RI363 (*trxA*), but *sodA*::*lacZ*	Laboratory Museum
NM281	As RI336 (*gshA*), but *sodA*::*lacZ*	Laboratory Museum
NM321	As RI89, but *katF*::*lacZ*	Laboratory Museum
NM331	As RI336 (*gshA*), but *katF*::*lacZ*	Laboratory Museum
NM341	As RI363 (*trxA*), but *katF*::*lacZ*	Laboratory Museum
NM351	As RI319 (*trxB*), but *katF*::*lacZ*	Laboratory Museum
NM381	As RI89, but *katE*::*lacZ*	Laboratory Museum
NM391	As RI336 (*gshA*), but *katE*::*lacZ*	Laboratory Museum
NM401	As RI319 (*trxB*), but *katE*::*lacZ*	Laboratory Museum
NM411	As RI363 (*trxA*), but *katE*::*lacZ*	Laboratory Museum

to oxidants and antibiotics was assessed with the disk method by measuring the diameter of the growth inhibition zones around the disks with the test substances. The cells for lawn formation on the plates with LB agar where the disks were placed were sampled from the growing culture and after 1, 2, or 3 days of glucose starvation. In order to assess the bacterial sensitivity to antibiotics, their minimal inhibitory concentration (MIC) on plates with agar was also determined as the lowest antibiotic concentration, which prevents visible growth after 20 h of incubation at 37°C; the survival of cells in serial culture dilutions on plates with the LB medium containing different antibiotic concentrations was measured as well.

Gene expression was determined by measuring the β-galactosidase activity in the strains carrying a fusion of the corresponding promoters with the β-galactosidase structural gene [9]. The reduced (GSH) and oxidized (GSSG) glutathione was determined spectrophotometrically in the sequence we described earlier [10]. Protein content was measured using Lowry's method.

Each result is shown as the mean of at least three independent experiments ± the standard deviation. Student's *t*-test was used for analysis. The $P < 0.05$ value was used as a minimum to determine the statistical significance. The following reagents provided by

Sigma Chemical Co. (St. Louis, MO, United States) were used in the work: agar, casamino acids, thiamine, deoxycholate, glutathione reductase, mercaptoethanol, DTNB, EDTA, NADPH, GSH, GSSG, NEM, ONFG, and antibiotics. All other reagents used in this work were of analytical grade.

RESULTS

To study the role of thiol redox systems in the survival of *E. coli* at their transition to the stationary growth phase, we used strains carrying mutations in the genes encoding the components of these systems: *gshA* (γ-glutamylcysteine synthetase, the first enzyme of glutathione synthesis), *trxA* (thioredoxin I), and *trxB* (thioredoxin reductase). In the growing cultures, prior to cessation of growth, only the *trxA* mutants differed significantly in survival from the cells of the other strains. The survival rate of these bacteria was 26% higher than in the parent type cells and 51% higher than in the *trxB* mutants (Fig. 1). Two hours after the cessation of growth, the survival of all strains significantly increased and remained at an elevated level during the subsequent 48 h of observation. After 72 h of the stationary phase, bacterial survival was close to the values characteristic of the growing cultures. In general the thioredoxin reductase mutation was the most critical for bacterial survival in both phases, while the thioredoxin A synthesis mutation was the least critical.

In the growing culture, the thioredoxin reductase-deficient strain (*trxB*) exhibited increased resistance to peroxide and menadione (a generator of superoxide radicals). The strain devoid of glutathione (*gshA*) had the highest sensitivity to these oxidants. In the course of starvation, the resistance of the *trxB* mutants to oxidants decreased and altered insignificantly in the rest of the strains.

The resistance of bacteria to antibiotics is known to increase significantly during the stationary phase. In this work, we tested the effect of mutations in the thiol redox systems on the resistance of starving bacteria to three antibiotics with different mechanisms of action. During the stationary phase, the resistance of all the tested strains to rifampicin, ciprofloxacin, and ampicillin increased significantly. The testing showed that, under experimental conditions, this resistance was the consequence of physiological adaptation, rather than the result of mutant selection. It is noteworthy that the

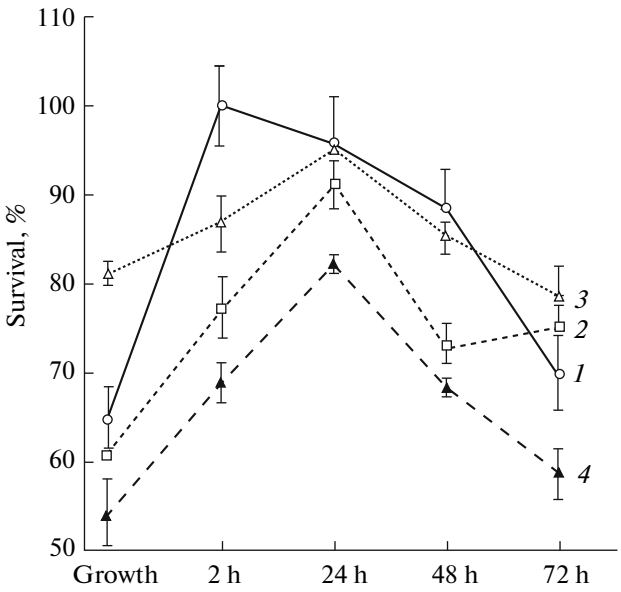

Fig. 1. Effect of mutations in the thiol redox systems on survival of *E. coli* during long-term starvation: RI89 (parent strain, wt) (*1*), RI336 (*gshA*) (*2*), RI363 (*trxA*) (*3*), and RI319 (*trxB*) (*4*). The number of viable parent strain cells 2 h after the cessation of growth was accepted as 100%.

mutants in thiol redox systems were far less capable of developing antibiotic resistance than the parent strain. For example, after 3 days of starvation, the survival of bacteria on the plates with LB agar containing 100 μg/ml rifampicin increased (compared to the level in the growing culture) 754-fold in the wild type cells and 18-, 13-, and 16-fold in the *gshA*, *trxA* and *trxB* mutants, respectively (Table 2). In the test with the MIC determination, resistance to ciprofloxacin in the parent type cells increased 64-fold, whereas in the *trxB* and *gshA* mutants it increased 4- and 32-fold, respectively. Under the same conditions, resistance to ampicillin in the parent type cells increased eightfold; in the *trxB* and *gshA* mutants, it increased only twofold. These data suggest that the differences in survival between *E. coli* mutants exposed simultaneously to antibiotic treatment and starvation stress increase many times more than in the case of cells subjected only to starvation stress. It is notable that the influence of mutations on bacterial resistance to antibiotics dif-

Table 2. Effect of the thiol redox systems mutations on resistance to rifampicin in *E. coli* during long-term starvation

Time after cessation of growth, h	Number of colonies per 10^9 cells			
	RI89 (wt)	RI336 (*gshA*)	RI363 (*trxA*)	RI319 (*trxB*)
0	7.2 ± 0.7	17.4 ± 9.9	13.3 ± 1.5	3.1 ± 0.2
24	18.8 ± 0.8	16.1 ± 7.9	32.2 ± 2.2	6.2 ± 0.2
48	3905 ± 81	190 ± 18.5	260 ± 11	19.3 ± 4.6
72	5431 ± 121	317 ± 24	173 ± 42	51.5 ± 3.8

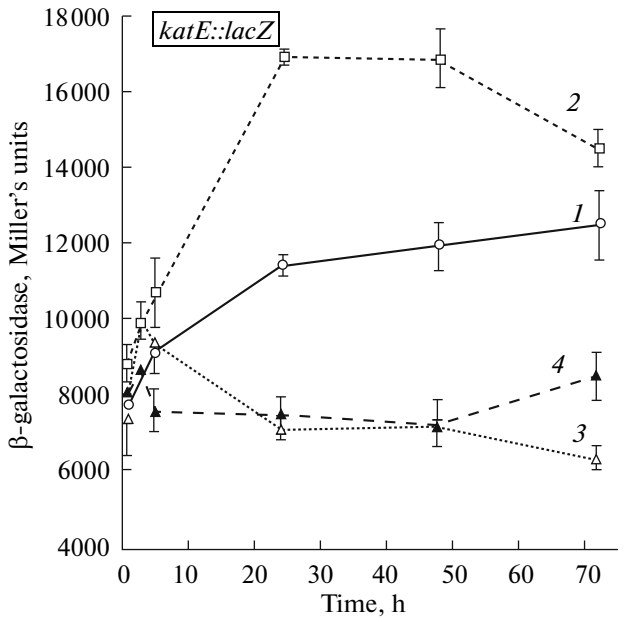

Fig. 2. Expression of the *katE::lacZ* fusion during long-term starvation of *E. coli*. NM381 (parent strain, wt) (*1*), NM391 (*gshA*) (*2*), NM411 (*trxA*) (*3*), and NM401 (*trxB*) (*4*).

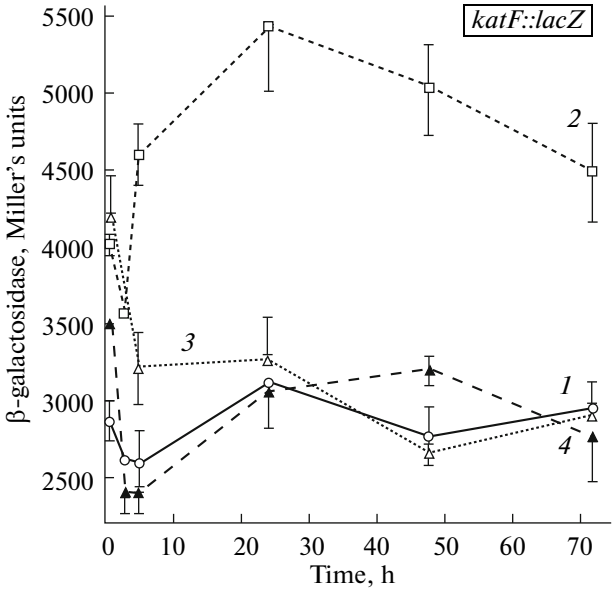

Fig. 3. Expression of the *katF::lacZ* fusion during long-term starvation of *E. coli*: NM321 (parent strain, wt) (*1*), NM331 (*gshA*) (*2*), NM341 (*trxA*) (*3*), and NM351 (*trxB*) (*4*).

fered substantially in growing and stationary cultures (Table 2).

In order to study the role of thiol redox systems in gene expression, the *E. coli* strains we constructed earlier and carrying simultaneously the *gshA*, *trxA*, and *trxB* mutations and the fusion of the *katF*, *katG*, *katE*, *sodA*, and *sulA* promoters with the β-galactosidase structural gene were used. The gene *katF* (*rpoS*) encodes the global σ^s regulator controlling the expression of stress genes, including those induced by starvation and transition of *E. coli* cells to the stationary growth phase [1]. The gene *katE* is a member of the RpoS regulon and encodes catalase–hydroperoxidase HPII, the activity of which increases significantly during the stationary phase [1]. The gene *katG* encodes catalase–hydroperoxidase I (HPI) and is controlled by both the global regulator OxyR regulating the adaptive response to peroxide stress and the sigma factor σ^s [11, 12]. The gene *sodA* encodes Mn-superoxide dismutase (Mn-SodA) and is a member of the SoxRS regulon, which is activated by superoxide [13]. The gene *sulA* (*sfiA*) is a member of the SOS regulon, which plays an important part in DNA repair [14]. Thus, the use of these strains makes it possible to reveal the influence of thiol redox systems on the expression of the genes that are components of the four stress regulons.

As was expected, transition of the parent type cells to the stationary phase induced the expression of *katE* (Fig. 2). However, the presence of the *trxA* and *trxB* mutations inhibited *katE* expression and the expression of this gene in the *gshA* mutants was 1.5 times higher than in the parent type cells. The *gshA* mutation also led to increased *katF* expression (Fig. 3).

It was previously shown that *katG* expression in the growing *E. coli* cells differed significantly in the thiol redox system mutants [15, 16]. During the stationary phase, *katG* expression in all the strains tested, except for *gshA*, gradually decreased to values at which there were no significant differences between the strains. In the *gshA* mutants, the decline in *katA* expression was preceded by its 25% increase. The *sodA* expression in the *trxA* and *trxB* mutants measured prior to the cessation of growth was twice as high as in the parent strain cells. Immediately after the cessation of growth, the *sodA* expression dropped 1.5-, 1.4-, 2.6-, and 2.5-fold in the parent type cells—*gshA*, *trxA*, and *trxB*, respectively.

Prior to the stationary phase, the highest level of the *sulA* gene expression was observed in the *trxA* mutants; the lowest level, in the *trxB* mutants. No significant changes in *sulA* expression during the stationary phase were noted; after 3 days of starvation, the levels of expression for different strains varied between 5 and 35% (Fig. 4).

Monitoring the changes in the glutathione status during the stationary phase showed that the intracellular reduced glutathione concentration (GSH_{in}) increased over the first 24 h of starvation and then declined, falling on the third day to half of the level recorded in the growing culture (Table 3). The intracellular oxidized glutathione concentration ($GSSG_{in}$) increased throughout the observation period, exceeding the baseline level by a factor of 4 on the third day.

Due to the increased GSH_{in} level, the bacteria retained a high $GSH/GSSG_{in}$ ratio over the first 24 h of starvation; after that, the glutathione redox status shifted dramatically towards the oxidized values, which could be seen from a tenfold decrease in the $GSH/GSSG_{in}$ ratio. Decreasing initially over the first hour of starvation, extracellular glutathione (GSH_{out}) remained at a level of about 1 μmol/1 g dry cells during the whole subsequent period, whereas oxidized glutathione ($GSSG_{out}$) continued to increase; in the final analysis, the $GSH/GSSG_{out}$ ratio decreased almost fourfold.

DISCUSSION

The present work demonstrated that normal functioning of thiol redox systems is of great importance for the adaptation of *E. coli* to long-term starvation. The *trxB* mutation had the most critical consequences for the stationary-phase cells. Thioredoxin reductase-deficient bacteria had a decreased survival rate, were less resistant to the action of oxidants and antibiotics, and had a low level of expression of the *katE* and *sulA* genes present in the two most important stress regulons. Earlier, it was reported that *E. coli trxB* exhibit the properties that single them out noticeably from the general series of mutants in thiol redox systems in respect to peroxide stress and irradiation with long-range UV light [15–17]. Another important observation is associated with an increased level of *katF* and *katE* expression in the mutants deficient in glutathione synthesis, which gives evidence of an important role of this tripeptide in the regulation of the σ^s-controlled regulon.

What are the possible pathways of influence of thiol redox systems on the adaptation of *E. coli* to starvation stress? Thioredoxin is a small protein containing the redox-active Cys-X1-X2-Cys site capable of reversible oxidation and reduction. The oxidized form of thioredoxin is reduced by thioredoxin reductase. Despite the fact that the intracellular thioredoxin concentration is very low, its reductive activity in relation to the transcription factors is approximately 1000 times higher than that of GSH, which makes thioredoxin more specific for redox-regulated signal reactions. Among the pathways through which thioredoxin may be involved in redox regulation, its participation should be noted in reduction of the disulfide bonds in the regulatory proteins or formation of conjugates with these proteins. Proteomic analysis revealed 80 thioredoxin-associated proteins in *E. coli*, many of which are directly or indirectly involved in the regulation of the cellular functions [18]. It was shown that in single *E. coli* mutants in the redox systems, the rate of formation of disulfide bonds in the cytoplasm is distributed in the following way (in relative units, with 1.0 being the value for the wild type): *trxB* – 9.7; *trxA* – 1.4; *gshA* – 1.4; *grxA* – 0.9 [19]. The high disulfide level in the *trxB* mutant might explain the specific properties of this mutant.

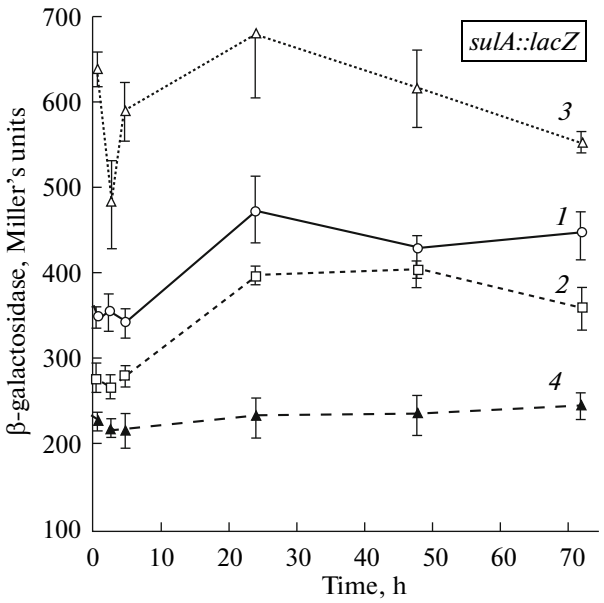

Fig. 4. Expression of the *sulA::lacZ* fusion during long-term starvation of *E. coli*: NM131 (parent strain, wt) (*1*), NM141 (*gshA*) (*2*), NM151 (*trxA*) (*3*), and NM161 (*trxB*) (*4*).

Table 3. Status of the intra- and extracellular glutathione in *E. coli* during long-term starvation

Time after cessation of growth, h	Intracellular glutathione, μmol/g dry biomass			Extracellular glutathione, μmol/g dry biomass		
	GSH	GSSG	GSH/GSSG	GSH	GSSG	GSH/GSSG
0	15 ± 1.6	0.13 ± 0.02	115	2.1 ± 0.16	0.18 ± 0.02	11.7
2	23.8 ± 2.3	0.15 ± 0.03	159	1.17 ± 0.16	0.35 ± 0.04	3.3
4	22.9 ± 2.1	0.22 ± 0.03	104	1.26 ± 0.16	0.29 ± 0.04	4.3
24	31.7 ± 2.5	0.20 ± 0.05	159	1.38 ± 0.17	0.28 ± 0.05	4.9
48	19.6 ± 2.1	0.37 ± 0.03	53	2.05 ± 0.18	0.53 ± 0.04	3.9
72	7.1 ± 1.7	0.5 ± 0.03	14	1.12 ± 0.15	0.39 ± 0.04	2.9

A large number of reactions proceeding in the cells are associated with the transfer of oxidative–reductive equivalents; therefore, the maintenance of a certain redox state in the cytoplasm is an important condition for normal cell activity. The glutathione redox activity, its high intracellular level, and the possibility of its maintenance in the reduced state all make GSH the most important intracellular redox buffer. The intracellular glutathione concentration is in excess of the level of NADPH and other intracellular redox systems by a factor of 500–1000, which is why changes in the glutathione redox state can directly reflect the changes in the redox status of the cells [20]. The glutathione and thioredoxin redox systems function simultaneously in the cells complementing and doubling each other [21, 22]. The present work showed that, when *E. coli* are exposed to prolonged starvation, the redox status of the intracellular glutathione sharply shifted toward oxidative values decreasing by an order of magnitude below the value recorded in the growing culture. Importantly, a simultaneous decrease in the GSH/GSSG ratio was observed on the outside of the cells. Such a change in the redox status of glutathione on both sides of the cytoplasmic membrane could contribute to the adaptation to prolonged starvation by modulating the activity not only of the biological molecules in the cytoplasm but also of those exposed to the environment.

The data obtained also indicate an important role of thiol redox systems in the resistance of bacteria to antibiotics during the stationary phase. At present, the evidence is being accumulated that, under aerobic conditions, the specific action of antibiotics on certain targets is accompanied by the development of oxidative stress, which may be regarded as a common mechanism of antibiotic-mediated cell death [7]. Our data, are, in part, consistent with this concept, because a considerable decrease in antibiotic resistance observed in the redox system-deficient strains correlated with their increased sensitivity to oxidants, which could have resulted partially from the low-level expression of the antioxidant gene *katE*.

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