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EXPERIMENTAL ARTICLES

The Status and the Role of Glutathione under Disturbed Ionic Balance and pH Homeostasis in *Escherichia coli*

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Abstract—The study of glutathione status in aerobically grown *Escherichia coli* cultures showed that the total intracellular glutathione (GSH_{in} + $GSSG_{in}$) level falls by 63% in response to a rapid downshift in the extracellular pH from 6.5 to 5.5. The incubation of *E. coli* cells in the presence of 50 mM acetate or 10 μg/ml gramicidin S decreased the total intracellular glutathione level by 50 and 25%, respectively. The fall in the total intracellular glutathione level was accompanied by a significant decrease in the (GSH_{in} : $GSSG_{in}$) ratio. The most profound effect on the extracellular glutathione level was exerted by gramicidin S, which augmented the total glutathione level by 1.8 times and the (GSH_{out} : $GSSG_{out}$) ratio by 2.1 times. The gramicidin S treatment and acetate stress inhibited the growth of mutant *E. coli* cells defective in glutathione synthesis 5 and 2 times more severely than the growth of the parent cells. The pH downshift and the exposure of *E. coli* cells to gramicidin S and 50 mM acetate enhanced the expression of the *sodA* gene coding for superoxide dismutase SodA.

Key words: *Escherichia coli*, glutathione, ionic balance.

The circulation of ions, including protons, through the bacterial plasma membrane plays an important part in energy transduction, the maintenance of turgor pressure, the intracellular pH homeostasis, and the regulation of cell activity [1, 2]. There is evidence that the disturbance of pH homeostasis and the intracellular ionic balance is accompanied by phenomena typical of oxidative stress. For instance, the incubation of *Escherichia coli* cells in the presence of membrane-penetratable acetic acid enhances the expression of a large number of genes, including the antioxidant defense genes *katG*, *katE*, and *grxB* coding for hydroperoxidases HPI and HPII and glutaredoxin 2, respectively [3–5]. Furthermore, the pH downshift and bacterial growth at low pH are accompanied by an enhanced expression of the *ahpC* gene coding for alkylhydroperoxide reductase [5]. The major emphasis in these works was made on the study of the role of the enzymes (and their genes) that are involved in the degradation of oxidants, whereas the glutathione system of antioxidant defense remained virtually unstudied.

This work was undertaken to study changes in the status of glutathione and its role in the response of growing *E. coli* cells to stresses disturbing the intracellular ionic balance and pH homeostasis.

MATERIALS AND METHODS

The *Escherichia coli* strains used in this work are listed in Table 1. The strains were grown in a minimal M9 medium [6] supplemented with 0.4% glucose, 0.2% casamino acids, and 10 μg/ml thiamine. In experiments with gramicidin S, valinomycin, and nigericin, the content of K^+ ions in the medium was reduced to 1 mM. *E. coli* cells were collected from an overnight culture by centrifugation, resuspended in 100 ml of fresh growth medium, and incubated at 37°C in 250-ml flasks on a shaker (150 rpm). Bacterial growth was monitored by measuring culture turbidity at 670 nm. Acetate and antibiotics were added and pH was shifted when the pH of the cultivation medium declined to 6.5 in the process of bacterial growth. At this time, the culture density was about 0.4 g dry cell wt/l. The pH of the medium was rapidly shifted from 6.5 to 5.5 by adding the necessary amount of an HCl solution within 1–2 min.

The reduced (GSH) and oxidized (GSSG) forms of glutathione were assayed spectrophotometrically [7] as described earlier [8]. The activity of glutathione oxidoreductase (GOR) was determined at 30°C by measuring the oxidation rate of NADPH at 340 nm with GSSG as the enzyme substrate [9]. The intracellular content of K^+ ions was determined by the flame photometry of the cell samples obtained by the rapid filtration of cell suspensions through a membrane filter [10].

Protein was quantified by the method of Lowry *et al.* with bovine serum albumin as the standard. The expression of genes was evaluated by measuring β-galactosidase activity in strains carrying the promoter fusions of these genes with the structural gene of β-galactosidase [6].

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

Strain	Phenotype	Source
AB1157	*thr-1 leuB6 thi-1 arg-E3 his-4 proA2 tsx-33*	
	supE44 lacY1 galK2 ara-14 xyl-5 mt-1 rpsL	*E. coli* Genetic Stock Center
JTG10	AB1157 *gshA* (*gshA20*::*Tn10 km*$^{-}$)	B. Demple
NM23	As AB1157, but with plasmid pKT1033 (*katG*::*lacZ*)	Laboratory collection
NM31	As AB1157, but with plasmid pRS*katE16* (*katE*::*lacZ*)	Laboratory collection
NM51	As AB1157, but with plasmid pRS415*KatF5* (*katF*::*lacZ*)	Laboratory collection
NM122	As AB1157, but with plasmid pGOR-12 (*gor*::*lacZ*)	Laboratory collection
QC772	GC4468 *sodA49* (*sodA*::*lacZ*)	D. Touati
TN521	Δ(*lac*)*U169 rpsL* Δ*soxRS soxR*$^{+}$ *soxS*::*lacZ*	B. Demple
Plasmids		
pRK4936/pKT1033 with *katG*::*lacZ*		K. Tao
pRSkatE16 with *katE*::*lacZ*		A. Eisenstark
pRS415KatF with *katF*::*lacZ*		A. Eisenstark
pGOR-12 with *gor*::*lacZ*		A. Eisenstark

The data presented in the paper are the means of at least three independent measurements ± the standard error of the mean. The data obtained were processed using Student's *t*-test statistics at significance level $P < 0.05$.

Gramicidin S, valinomycin, nigericin, casamino acids, thiamine, and reagents used for the assay of GSH, GSSG, β-galactosidase, and glutathione oxidoreductase were purchased from Sigma (United States). All other reagents used in the work were of analytical grade.

RESULTS AND DISCUSSION

Immediately after the addition of 50 mM acetate (hereinafter also called acetate stress) to a growing *E. coli* AB1157 culture when its pH was 6.5, the specific growth rate μ of the culture fell from 0.67 ± 0.04 h^{-1} to zero and then gradually increased to 0.29 ± 0.06 h^{-1} (Fig. 1). The mutant *E. coli* JTG10 (*gshA*) cells defective in glutathione synthesis were about twice as sensitive to high acetate concentrations (100 and 150 mM) as the parent AB1157 cells (data not presented). The rapid shift in pH from 6.5 to 5.5 resulted in a drop of the specific growth rate of strain AB1157 to 0.12 ± 0.08 h^{-1}, followed by a gradual increase in μ to 0.32 ± 0.06 h^{-1} (Fig. 1). The effect of pH downshift on the parent AB1157 and the mutant JTG10 strains was the same.

It is known that gramicidin S augments the potassium permeability of both the cytoplasmic and the outer membrane of *E. coli* cells [11], thus inducing cell lysis and ultimately death. In our experiments, gramicidin S at a concentration of 10 μg/ml did not induce cell lysis but decreased the cell survival rate threefold. At a concentration of 1 μg/ml, this antibiotic insignificantly influenced cell survival.

After the addition of gramicidin S to the growth medium of *E. coli* AB1157 at a concentration of 10 μg/ml, the specific growth rate of this strain gradually decreased from 0.53 ± 0.029 to 0.26 ± 0.012 h^{-1} (Fig. 1). The effect of the tenfold lower concentration of gramicidin S (1 μg/ml) on the growth of this strain was almost the same (data not presented). The *gshA* mutation considerably enhanced the sensitivity of *E. coli* cells to gramicidin S, as is evident from the fact that 45 min after the addition of 10 μg/ml gramicidin S, the specific growth rate of the mutant cells was five times lower than that of the parent cells (data not presented).

The addition of 50 mM acetate to the growth medium of *E. coli* AB1157 resulted in a twofold decline in the intracellular level of the total glutathione (from 18.9 ± 0.57 to 9.53 ± 1.2 μmol/g dry cell wt). The pH downshift decreased this level to the greatest degree, from 18.9 ± 0.57 to 6.9 ± 0.8 μmol/g dry cell wt, while the effect of gramicidin S was the least pronounced (the total glutathione level fell from 17.7 ± 1.3 to 13.3 ± 0.7 μmol/g dry cell wt) (Fig. 2). In all of these cases, the decline in the total glutathione level was largely due to a decrease in the intracellular concentration of reduced glutathione (GSH_{in}), whereas the intracellular concentration of oxidized glutathione ($GSSG_{in}$) remained at a constant level. This resulted in a considerable decline in the (GSH_{in} : $GSSG_{in}$) ratio (Table 2). Noteworthy is the fact that the incubation of *E. coli* AB1157 cells in the presence of 1 μg/ml gramicidin S not only failed to diminish the intracellular GSH content but even slightly increased it (by 28%).

The most pronounced changes in the level of extracellular glutathione were observed under the action of gramicidin S (Table 2). After 30 min of incubation in the presence of 10 μg/ml gramicidin S, the content of the total glutathione in the medium increased from

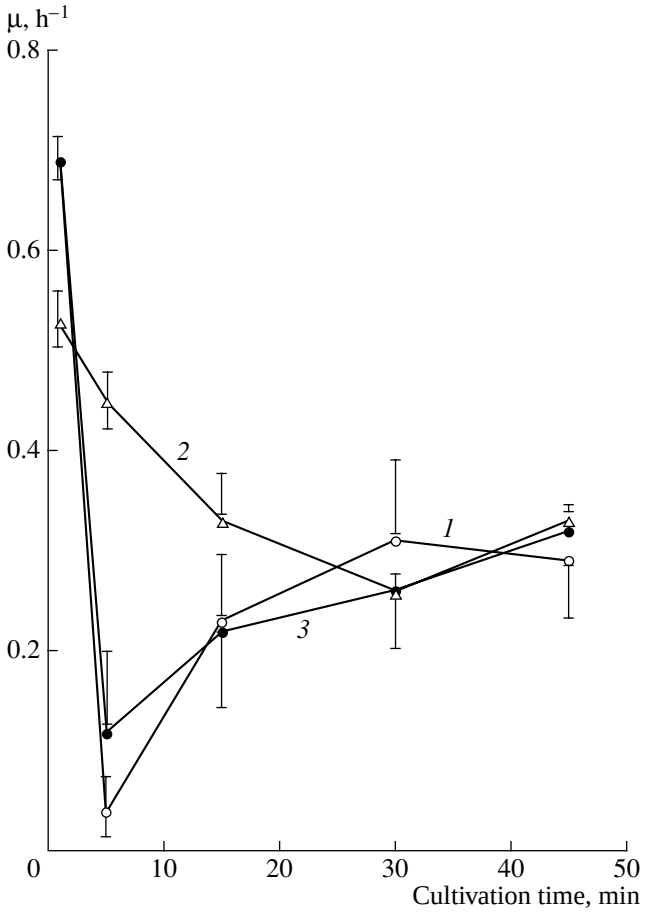

Fig. 1. The effect of (*1*) 50 mM acetate, (*2*) gramicidin S (10 μg/ml), and (*3*) pH downshift from 6.5 to 5.5 on the specific growth rate μ of aerobically grown *E. coli* AB1157 cells. Acetate and gramicidin S were added to the culture when its density reached 0.4 g dry cell wt/l. The pH of the medium was shifted by adding rapidly (within 1–2 min) a solution of HCl. Before the addition of gramicidin S, the culture was supplemented with 10 mM EDTA.

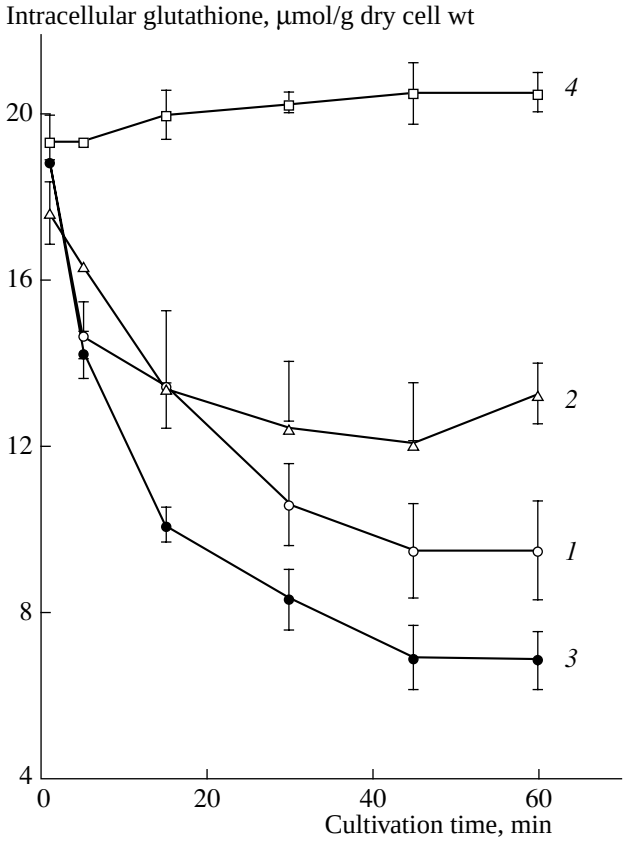

Fig. 2. The effect of (*1*) 50 mM acetate, (*2*) gramicidin S (10 μg/ml), and (*3*) pH downshift from 6.5 to 5.5 on the intracellular level of the total glutathione in aerobically grown *E. coli* AB1157 cells. Curve *4* refers to untreated (control) cells. The cells were exposed to acetate, gramicidin S, and acidification stresses at time $t = 0$. The other conditions are as described in the legend to Fig. 1.

8.6 to 15.6 μmol/g dry cell wt. A comparison of changes in the levels of intracellular and extracellular glutathione (Fig. 2, Table 2) indicated that the increase in the extracellular glutathione level was likely due to its transport from the cytoplasm into the medium. The incubation of *E. coli* AB1157 cells in the presence of 1 μg/ml gramicidin S gave rise to a 2.5-fold increase in the extracellular glutathione level. Thus, gramicidin S at the low concentration raised the glutathione content both inside and outside cells.

The pH downshift augmented the concentration of the total extracellular glutathione by 26%, whereas acetate stress did not induce significant changes in the level of extracellular glutathione (Table 2).

In *E. coli* AB1157, as in other organisms, GSSG is reduced by the NADPH-linked glutathione oxidoreductase (EC 1.6.4.2). All of the treatments under study caused a small increase (by 10–15%) in the activity of this enzyme, likely due to the enhanced expression of its gene *gor*.

To investigate possible changes in the membrane permeability, we measured intracellular potassium levels. In response to the addition of 50 mM acetate, the intracellular level of potassium in *E. coli* AB1157 cells initially increased from 0.59 ± 0.05 to 1.46 ± 0.26 mmol/g dry cell wt and then decreased to 0.91 ± 0.13 mmol/g dry cell wt (Fig. 3). The maximum in the intracellular potassium level coincided in time with the maximum growth rate of cells (Fig. 1). The mutant JTG10 cells incubated in the presence of 50 mM acetate accumulated two times less potassium than the parent cells. The maximum intracellular potassium level was reached already 5 min after the addition of acetate and persisted for about 40 min (data not presented). The effect of pH downshift on the concentration of intracellular potassium was statistically insignificant in both strains. Gramicidin S at concentrations of 1 and 10 μg/ml irreversibly decreased the intracellular potassium level by 40 and 30%, respectively. The rate of potassium excre-

Table 2. Glutathione status in the *E. coli* cells subjected to different stresses

Stress	GSSG	GSH	GSH : GSSG
Intracellular glutathione			
Control	0.06 ± 0.01	20.2 ± 0.15	337
50 mM acetate	0.077 ± 0.01	10.5 ± 1.0	136
pH downshift from, 6.5 to 5.5	0.072 ± 0.007	8.25 ± 0.7	115
Gramicidin S, 10 μg/ml	0.079 ± 0.005	12.3 ± 1.7	156
Extracellular glutathione			
Control	0.55 ± 0.04	8.03 ± 0.53	14.6
50 mM acetate	0.63 ± 0.03	8.08 ± 0.26	12.8
pH downshift from, 6.5 to 5.5	0.66 ± 0.07	9.98 ± 0.44	15.1
Gramicidin S, 10 μg/ml	0.49 ± 0.03	15.12 ± 0.94	30.9

Note: Samples for analysis were taken 30 min after the treatments. Before the addition of gramicidin S, the *E. coli* culture was supplemented with EDTA, which decreased the concentrations of intracellular GSH and extracellular GSSG by 1.7 mM/g dry cell wt and increased the concentration of extracellular GSH by 1.5 mM/g dry cell wt.

tion from the mutant cells was two times higher than that from the parent cells.

Experiments showed that 10 μM nigericin, 10 μM valinomycin, and 20 μM carbonyl cyanide *m*-chlorophenylhydrazone (CCCP, an efficient uncoupler of oxidative phosphorylation) inhibited the growth of *E. coli* AB1157 cells by 50, 64, and 100%, respectively. In this case, the extracellular content of glutathione increased by 27, 65, and 100%, respectively. Changes in the intracellular glutathione level were less pronounced. The content of intracellular potassium decreased by 40, 25, and 52%, respectively.

In *E. coli*, the *sodA* gene codes for superoxide dismutase SodA, which is important in cell defense against superoxide anion. The pH downshift and 50 mM acetate enhanced the expression of the *sodA::lacZ* gene fusion by 50% 1 h after the treatment, whereas gramicidin S (10 μg/ml) augmented the expression by 100% (Fig. 4). It is known that the expression of *sodA* is controlled by the binary gene system *soxRS*, which modulates cell response to superoxide radical–generating compounds. The treatments used in this work had little influence on the expression of the *sodA* gene. In response to the action of 50 mM acetate, 10 μg/ml gramicidin S, and pH downshift, the β-galactosidase activity of the *E. coli* TN521 cells carrying the *soxS::lacZ* gene fusion increased by 24, 28, and 21%, respectively.

It is known that *E. coli* AB1157 cells detoxify hydrogen peroxide with the aid of two hydroperoxidases (catalases), HPI and HPII. The HPI protein, encoded by the *katG* gene, is a bifunctional catalase–peroxidase, whose induction by hydrogen peroxide depends on the OxyR transcription factor. HPII, encoded by the *katE* gene, is a monofunctional catalase regulated by the *rpoS* (*katF*)-encoded sigma factor RpoS. As was shown by other researchers, the incubation of *E. coli* cells with acetate enhances the expression of the *katG*, *katE*, and *katF* genes [3, 4]. In our experiments, 50 mM acetate enhanced the expression of the *katG::lacZ*, *katE::lacZ*, and *katF::lacZ* gene

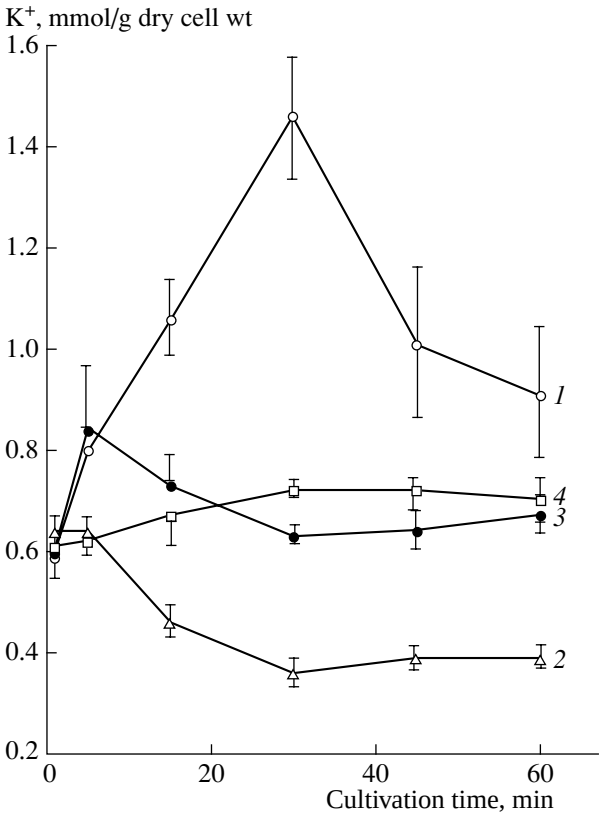

Fig. 3. The effect of (*1*) 50 mM acetate, (*2*) gramicidin S (10 μg/ml), and (*3*) pH downshift from 6.5 to 5.5 on the intracellular level of sodium in aerobically grown *E. coli* AB1157 cells. The cells were exposed to acetate, gramicidin S, and acidification stresses at time $t = 0$. Curve *4* refer to untreated cells. The other conditions are as described in the legend to Fig. 1.

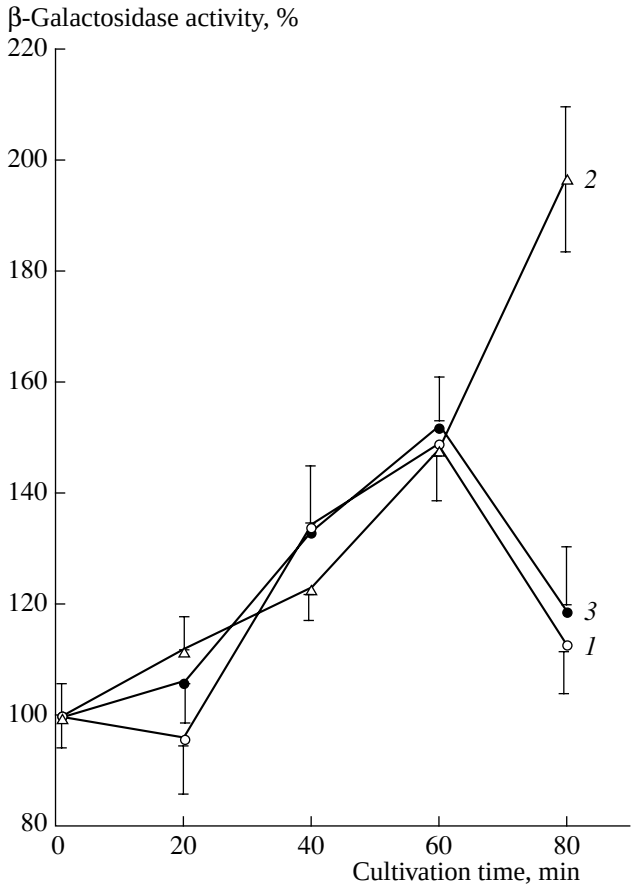

Fig. 4. The effect of (*1*) 50 mM acetate, (*2*) gramicidin S, and pH shift (*3*) on the expression of the *sodA::lacZ* gene fusion in aerobically grown *E. coli* QC772 cells. The cells were exposed to acetate, gramicidin S, and acidification stress at time $t = 0$. Other conditions are as described in the legend to Fig. 1.

fusions by 200, 37, and 40%, respectively. The other treatments (pH downshift and incubation with gramicidin S) did not induce notable changes in the expression of the three genes. It should be noted that we are the first to show the enhanced expression of the regulatory gene *soxS* and the *sodA* gene controlled by the *soxRS* system in response to the action of acetate, gramicidin S, and pH downshift.

All of the treatments under study exert either a direct or an indirect effect on the intracellular pH and ionic balance in the cytoplasm and, hence, may influence the cellular processes that depend on these parameters. Reportedly, acetate acidifies the cytoplasm of *E. coli* cells [12–14] and elevates the membrane potential $\Delta\Psi$ [14]. At the physiological values of intracellular pH, acetic acid accumulates in the cytoplasm in the form of acetate anions, which disturbs the ionic balance in cells [12, 13]. In response to incubation with high acetate concentrations, the cells accumulate high amounts of potassium [12, 15, and this paper]. It should be noted that acetate is a normal cellular metabolite, but when accumulated in high amounts in fast-growing *E. coli* cells, it inhibits their growth [16].

Reportedly, the rapid acidification of the growth medium of *E. coli* decreases the intracellular pH and the cytoplasm volume [17, 18]. Of the stressful compounds used in the present work, gramicidin S increases the permeability of membranes to some ions, including potassium cations; valinomycin specifically increases the permeability of membranes to potassium cations; nigericin stimulates the K^+/H^+ exchange; and CCCP is a protonophore that efficiently uncouples oxidative phosphorylation.

Experimental data on the intracellular and extracellular status of glutathione are of great interest, since little is known about its role in cell response to acetate stress, pH shift, and exposure to ionophores. As shown in this work, the exposure of *E. coli* cells to acetate, gramicidin S, and pH downshift leads to a decrease in the content of intracellular GSH and the (GSH_{in} : $GSSG_{in}$) ratio. The decrease in the intracellular glutathione level is one of the responses of eukaryotic cells to oxidative stress. The enhanced expression of the antioxidant genes *katG* and *katE* in response to acetate stress and of the *soxS* and *sodA* genes in response to the treatments described above suggests that the intracellular level of reactive oxygen species is elevated.

Our earlier studies showed that the oxidative stress induced by the superoxide radical–generating compound menadione diminishes the content of intracellular GSH and the (GSH*in* : GSSG*in*) ratio [8]. Unlike the stresses studied in this work (Table 2), menadione lowered the level of GSH but augmented the level of GSSG in cells. This suggests that the stresses under study diminish the content of intracellular glutathione not only through the formation of reactive oxygen species but also through other mechanisms, such as the suppression of biosyntheses in response to the decrease in the intracellular pH, the activation of degradation processes, and the formation of conjugates. Gramicidin S likely induces the excretion of intracellular GSH into the medium.

At physiological values of pH, glutathione occurs in anion form and thereby may be involved in transmembrane ionic currents and in the maintenance of ionic balance. Roe *et al.* [13] showed that the accumulation of acetate anion in the *E. coli* cells subjected to acetate stress is accompanied by a decrease in the intracellular pool of glutamate and other negatively charged amino acids. This is in agreement with our finding that acetate stress induces a twofold decrease in the intracellular level of glutathione.

Glutathione may play a role in the redox regulation of not only eukaryotic but also prokaryotic cells. A deficiency of glutathione in the *gsh*-defective *E. coli* cells stimulates the expression of the antioxidant genes *soxS*, *katG*, and *katE* [19, 20]. The involvement of glutathione in the regulation of these genes can be due to its interaction with the redox-sensitive sites of regula-

tory proteins and due to its effect on the intracellular level of oxidants [20, 21]. These data allow the suggestion to be made that the activation of antioxidant genes observed in this work may be due to a decreased level of intracellular glutathione.

One of the known functions of glutathione in *E. coli* cells is its involvement in the control over gated potassium channels [10]. Changes in the intracellular potassium pool of *E. coli* cells in response to their exposure to acetate or gramicidin S stresses considerably differed (both qualitatively and quantitatively) in the wild-type and glutathione-deficient mutant cells.

Thus, the beneficial effect of glutathione on the tolerance of *E. coli* cells to acetate and gramicidin S stresses may be due to its global role as an antioxidant, redox regulator, and a component of the regulatory system of ionic balance. Investigations into the mechanism of the stimulating effect of gramicidin S, nigericin, valinomycin, and CCCP on the extracellular pool of glutathione are in progress in our laboratory.

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