Biochemistry (Moscow), Vol. 66, No. 9, 2001, pp. 973-978. Translated from Biokhimiya, Vol. 66, No. 9, 2001, pp. 1195-1201.

Role of Glutathione in the Response of *Escherichia coli* to Osmotic Stress

G. V. Smirnova*, T. A. Krasnykh, and O. N. Oktyabrsky

Institute of Ecology and Genetics of Microorganisms, Ural Branch of the Russian Academy of Sciences, ul. Goleva 13, Perm', 614081 Russia; fax: (3422) 646-711; E-mail: smirnova@ecology.psu.ru

Received December 13, 2000
Revision received February 12, 2001

Abstract—The growth of *Escherichia coli* mutants deficient in glutathione synthesis (*gshA*) and in glutathione reductase (*gor*) was suppressed in medium of elevated osmolarity. A mutant in γ-glutamyl transpeptidase (*ggt*) displayed better ability for osmoadaptation than the parental strain. The unfavorable effect of the *gsh* mutation on osmoadaptation of growing *E. coli* cells was more pronounced at low concentrations of K^+ in the medium. An increase in osmolarity caused an increase in the intracellular content of glutathione. Changes in the extracellular glutathione level were biphasic: the glutathione level rapidly decreased during the first stage of the response and increased during the second stage. The changes in glutathione levels suggest that under hyperosmotic shock the glutathione transport from the medium into the cell can contribute to the intracellular glutathione accumulation. Changes in the level of intracellular K^+ were similarly biphasic: a rapid increase in the K^+ level during the first stage of the response to hyperosmotic shock changed to a gradual decrease during the second stage. In mutant *gshA* cells adapted to osmotic shock, the intracellular K^+ level was markedly higher than in the parental strain cells. The possible role of glutathione in the response of *E. coli* to osmotic shock is discussed.

Key words: *Escherichia coli*, osmotic shock, glutathione, potassium, glutathione oxidoreductase, γ-glutamyl transpeptidase

Similarly to other enterobacteria, *Escherichia coli* during evolution developed systems responsible for regulation of the turgor pressure. The main function of these systems is the accumulation of intracellular osmolytes that do not inhibit enzyme activities and thus provide for bacterial growth in medium with high osmolarity [1]. Thus, during the initial stage of the response to osmotic shock, *E. coli* cells restore their turgor through accumulation of significant amounts of K^+ and glutamate. However, at elevated osmolarity of the medium, a high level of intracellular K^+ can cause disorders in activities of some enzymes; therefore, during the subsequent stages of the cell response to osmotic shock K^+ is replaced by such compounds as betaine, proline, and trehalose [2, 3].

Glutathione (GSH) is one of the *E. coli* metabolites that increases in concentration under osmotic shock [4, 5]. One of the functions of GSH in *E. coli* is potassium retention in the cytoplasm by regulation of K^+-release channels [6]. Since changes in the transmembrane flows of K^+ are significantly involved in the response of *E. coli* to osmotic shock, it was reasonably to expect that glutathione should be involved in osmoadaptation. Indeed, in medium of elevated osmolarity, *E. coli gsh*$^-$ mutants displayed decreased ability for osmoadaptation [5]. However, the authors of work [5] concluded that the favorable role of glutathione in osmoadaptation was not directly associated with its effect on K^+ retention.

Osmotic shock in *E. coli* is associated with reactions that are specific for oxidative stress [7]. This was concluded based on the decreased growth of *E. coli* cells deficient in the synthesis of superoxide dismutases SodA and SodB in medium of elevated osmolarity and also on the increased expression of *sodA* and *soxS* genes in response to osmotic shock. Both genes are involved in the response of *E. coli* to oxidative stress caused by superoxide anion [8]. On the other hand, in *E. coli* glutathione is suggested to be involved in the detoxication of O_2^- because treatment of cells with the O_2^- producer menadione was accompanied by a pronounced decrease in the levels of total and reduced glutathione concurrently with an increase in the level of oxidized glutathione (GSSG) [9]. Overall, the available data suggest that during osmoadaptation of *E. coli* cells glutathione should play other roles than those associated with its involvement in the regulation of K^+-release channels. In particular, GSH can be involved as an antioxidant in cell protection against oxidative damage during osmotic shock. The present work continues stud-

* To whom correspondence should be addressed.

0006-2979/01/6609-0973$25.00 ©2001 MAIK "Nauka/Interperiodica"

ies on the roles of glutathione and enzymes of its metabolism in the response of *E. coli* to osmotic shock.

MATERIALS AND METHODS

The following *Escherichia coli* strains were used: AB1157 (wt, wild type) and JTG10 (similar to AB1157, but *gshA*) were provided by B. Demple (USA); SH646 (wt) and SH641 (similar to SH646, but *ggt*) were provided by H. Suzuki (Japan); AE1318 (wt) and AE1319 (similar to AE1318, but *gor*) were provided by A. Eisenstark (USA).

The *Escherichia coli* cells were grown under aerobic conditions on minimal medium M9 [10] supplemented with 0.2% glucose, 0.2% casamine acids, and thiamine (10 μg/ml). After centrifugation, cells from a night culture were resuspended in 100 ml of the fresh medium and then grown at 37°C in 250-ml flasks on shakers at 150 rpm. The strains AE1318 and AE1319 were grown at 30°C. The cell growth was followed by changes in the light scattering at 670 nm. On studying the bacterial growth at varied K^+ concentrations in the medium, the insufficient to normal amount of K^+ was compensated by addition of Na^+ as necessary.

Concentrations of reduced (GSH), oxidized (GSSG), and total glutathione were determined spectrophotometrically using glutathione oxidoreductase [11] as described in [9]. Activities of the glutathione synthesis enzymes were determined as described in [12].

Intracellular K^+ was determined using a flame photometer in samples obtained by rapid filtration through a membrane filter [6].

Protein was determined by the Lowry method using bovine serum albumin (BSA) as the standard [13]. The cells were broken by ultrasonication at 0°C using six cycles, each of 30 sec. For each case, mean values are presented resulting from at least three independent experiments. All reagents for determination of GSH and GSSG, casamine acids, BSA, and thiamine were from Sigma Chemical Co. (USA). The other reagents were of chemical purity, domestic production.

RESULTS

To assess the osmotic shock effect, the growth rate of *E. coli* cells was determined during log phase after the addition of varied NaCl concentrations to the medium. The growth rate of *E. coli* JTG10 deficient in glutathione synthesis in the medium of high osmolarity was lower than the growth rate of the parental AB1157 strain. The unfavorable effect of the *gsh* mutation was more pronounced at higher concentrations of NaCl (Fig. 1a). This is consistent with findings on other *E. coli* strains grown in minimal medium K120 without addition of casamine acids with glucose as the osmolyte [5]. The effect of the *gsh* mutation on osmoadaptation of growing *E. coli* depended on the K^+ concentration in the medium, and

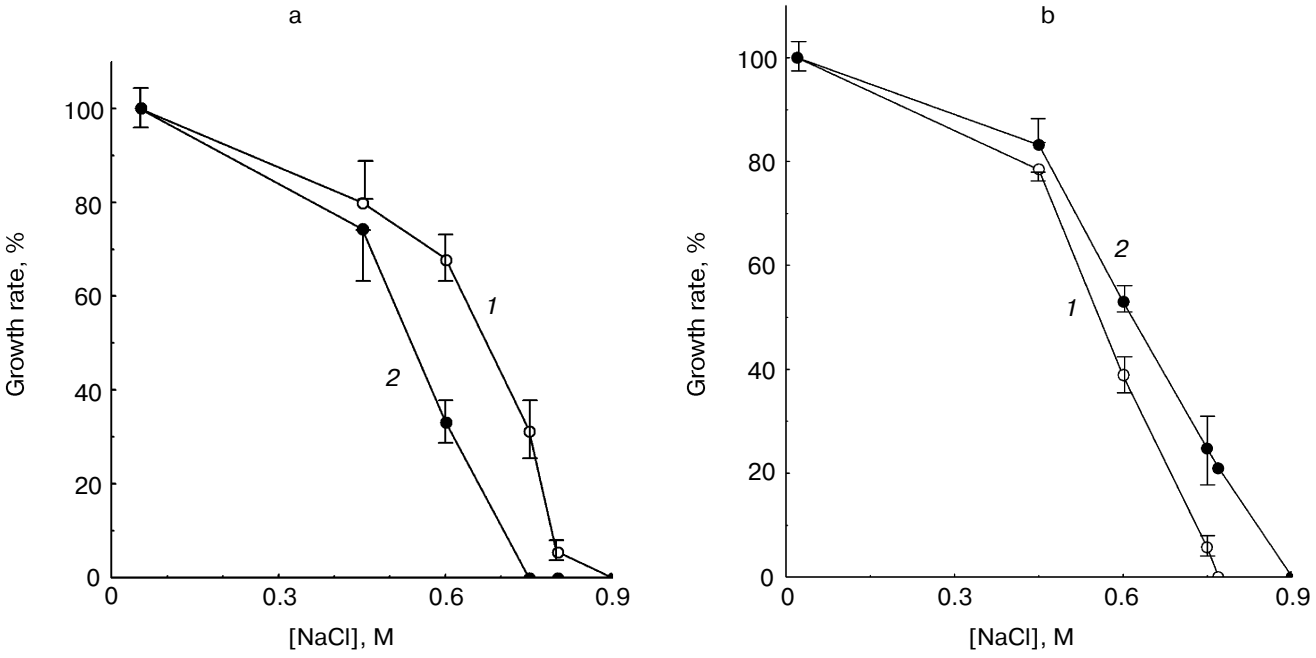

Fig. 1. Effect of the medium osmolarity on *E. coli* growth. a: *1*) AB1157 (wild type); *2*) JTG10 (*gshA*); b: *1*) SH646 (wild type); *2*) SH641 (*ggt*). The specific rate of cell growth in the medium of normal osmolarity is taken as 100%. The osmolarity of the medium was changed by addition of NaCl.

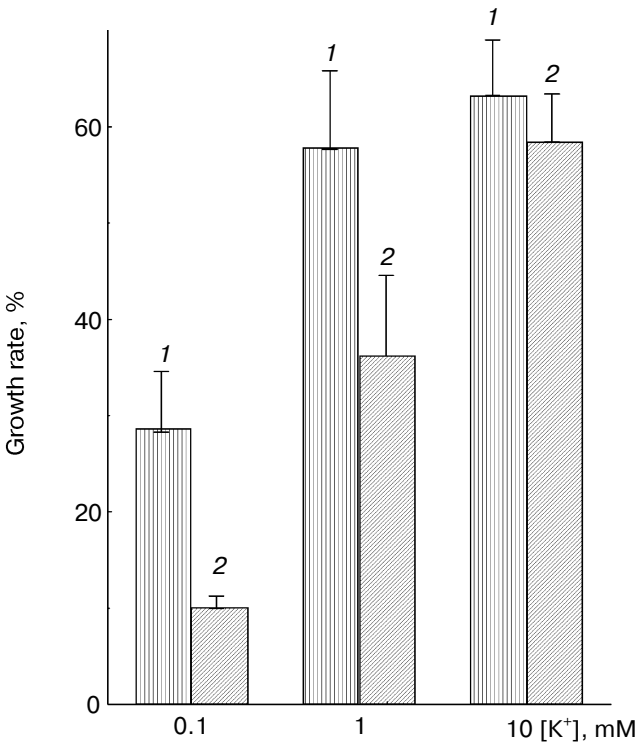

Fig. 2. Effect of intracellular K^+ concentration on *E. coli* growth in medium of elevated osmolarity: *1*) AB1157 (wild type); *2*) JTG10 (*gshA*).

the negative effect of the mutation was more pronounced at low concentrations of K^+ (Fig. 2).

Similarly to other organisms, oxidized glutathione in *E. coli* is reduced by an NADPH-dependent glutathione oxidoreductase (GOR, EC 1.6.4.2). To elucidate the role of GOR in the response of *E. coli* to osmotic shock, AE1319 cells with a mutation in the *gor* gene encoding the synthesis of this enzyme were used [14]. An increase in the osmotic pressure caused by addition of 0.6 M NaCl decreased the growth rate of the mutant cells to 23.2 ± 3.5% of their growth rate in the initial medium (M9 + casamine acids). Under the same conditions, the growth rate of the parental cells AE1318 decreased to 36.8 ± 3% ($p < 0.05$).

γ-Glutamyl transpeptidase (GGT, EC 2.3.3.2) is another enzyme that plays an important role in glutathione metabolism in various cells. In our experiments, SH641 cells [15] with a mutation in the locus that controls GGT synthesis were more resistant to osmotic shock than cells of the parental strain SH646 (Fig. 1b).

To study the role of glutathione in the vital activity of the cell, its intracellular concentration can be increased by addition of L-2-oxo-4-thiazolidine (OXO) to the incubation medium. In our experiments, pretreatment of cells with 1 mM OXO failed to affect the growth of *E. coli* AB1157 in the medium of elevated osmolarity (M9 + casamine cells + NaCl (0.6-0.75 M)).

An increase in the intracellular glutathione level was earlier found in *E. coli* under conditions of osmotic shock [4, 5]. In those works, the total glutathione (GSH + GSSG) concentration was determined. However, in many cases it is important to know the concentration of both reduced and oxidized glutathione and, correspondingly, the GSH/GSSG ratio. It is suggested that the latter parameter should determine the effect of glutathione on the activities of thiol-sensitive enzymes. In our experiments, increase in the osmotic pressure by addition of 0.6 M NaCl to the medium increased the intracellular glutathione (GSH_{intr}) level. Osmotic shock failed to significantly change the intracellular oxidized glutathione ($GSSG_{intr}$) concentration, which remained very low (Fig. 3a). The increase in the GSH concentration resulted in a 40% increase in the $GSH_{intr}/GSSG_{intr}$ ratio (from 230 to 320).

In response to osmotic shock, the GGT-mutant cells increase the intracellular glutathione concentration 1.5-fold faster than the wild-type cells. This seems to be due to the higher activities of the glutathione synthesis enzymes in the mutants compared to the wild-type cells (2.36 ± 0.2 and 1.54 ± 0.16 nmol/h per mg protein, respectively).

Escherichia coli cells are known to release micromolar amounts of glutathione into the medium [16]. Comparison of the data presented in Fig. 3 (a and b) shows opposite changes in the levels of intra- and extracellular GSH and GSSG in response to osmotic shock. During the first stage of the *E. coli* response to osmotic shock, the level of extracellular glutathione (GSH_{extra}) decreased by 40%, and 15 min after the addition of osmolyte its level was restored to the initial value (Fig. 3b). During the osmotic shock, the extracellular GSSG concentration decreased more than threefold and remained low during the observation time. As a result, the $GSH_{extra}/GSSG_{extra}$ ratio increased from 20 to 90.

Figure 4a shows the effect of the *gsh* mutation on changes in the intracellular K^+ in response to increase in the osmolarity of the medium. In response to osmotic shock, both strains (wt and *gsh*) displayed rapid accumulation of K^+ during the first stage of the response, with a subsequent partial release of K^+ during the second stage. These findings are consistent with previously obtained data for other strains of *E. coli* and under other conditions: the literature data indicate that the accumulation of K^+ was transitory, and 20-30 min after the hyperosmotic shock the K^+ accumulated was partially released from the cells [2, 17, 18]. The two strains were not significantly different in intracellular K^+ changes during the first stage of the response. However, the level of K^+ after adaptation to growth in the medium of elevated osmolarity (30 min after the addition of 0.6 M NaCl) was significantly higher in the mutant cells than in the parental strain cells.

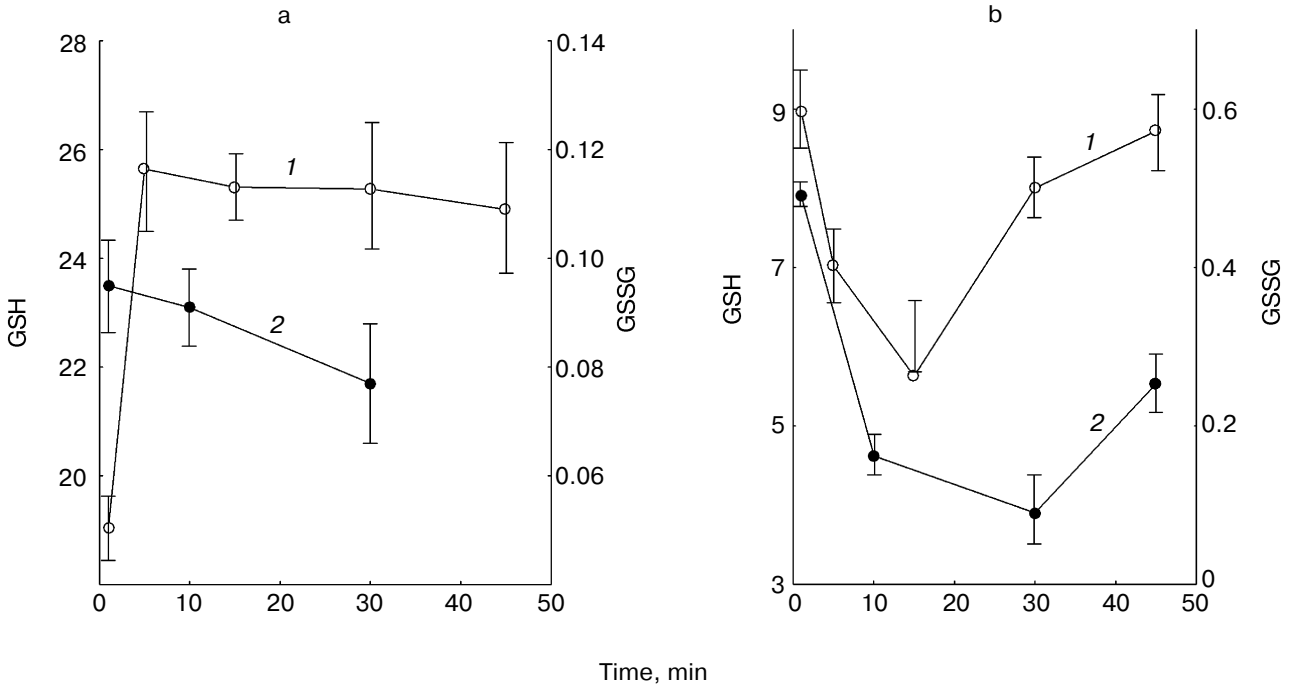

Fig. 3. Changes in the reduced (*1*) and oxidized (*2*) glutathione inside (a) and outside (b) growing *E. coli* cells during hyperosmotic shock. The culture was supplemented with 0.6 M NaCl at time $t = 0$. The GSH and GSSG concentrations are expressed in μmole per g dry cells.

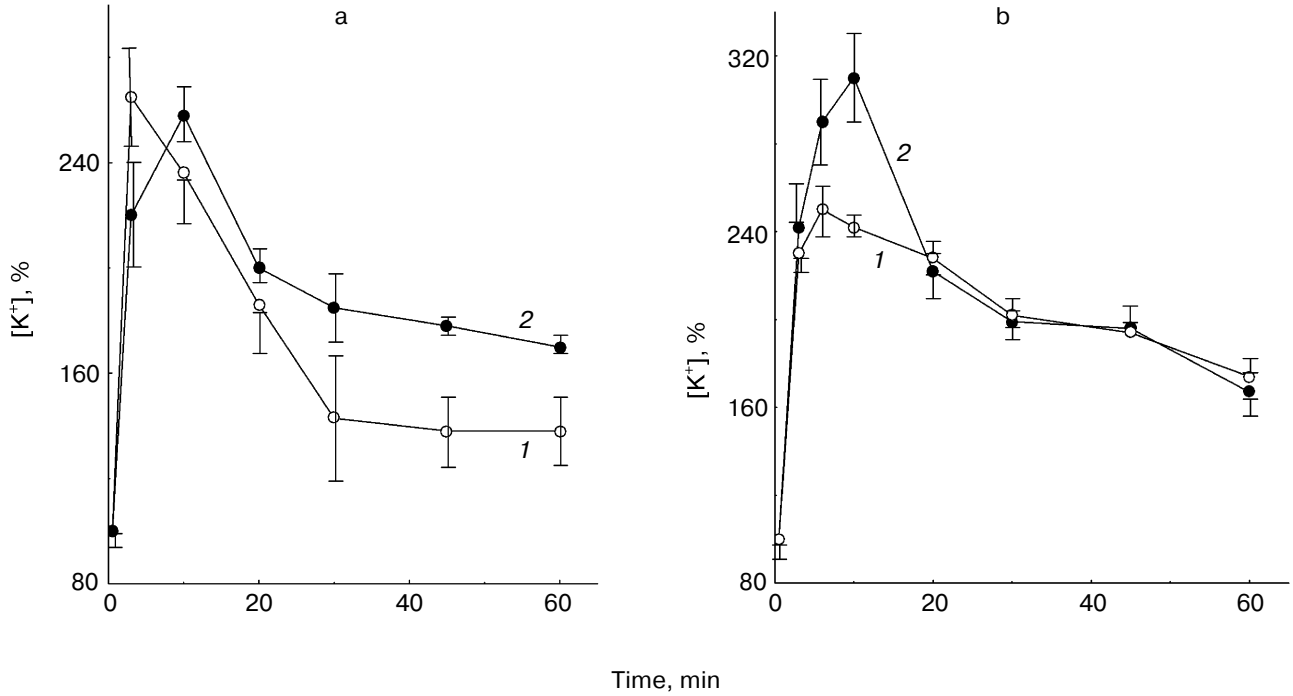

Fig. 4. Changes in intracellular K^+ in growing *E. coli* cells during hyperosmotic shock. a: *1*) AB1157 (wild type); *2*) JTG10 (*gshA*); b: *1*) SH646 (wild type); *2*) SH641 (*ggt*). The culture was supplemented with 0.6 M NaCl at time $t = 0$. The K^+ concentration before the addition of NaCl was taken as 100%. This value was 0.505 ± 0.03 for AB1157 (wild type), 0.4 ± 0.03 for JTG10, 0.55 ± 0.05 for SH646, and 0.48 ± 0.03 mmol per g dry cells in the case of SH641.

Immediately after the increase in osmolarity of the medium, the accumulation of K^+ by strain SH641 cells (*ggt*) was 25% higher than the parental strain cell accumulation; however, at a later stage of adaptation to osmotic shock the two strains did not differ in the accumulation of K^+ (Fig. 4).

DISCUSSION

Consider briefly the events in the growing *E. coli* cells under conditions of hyperosmotic shock. A sudden increase in the medium osmolarity causes a release of water from the cells, and this results in turgor pressure decrease, plasmolysis, and cessation of growth. The primary event in osmoadaptation [19] is rapid accumulation of K^+, and this promotes rehydration and turgor recovery. However, the accumulation of large amounts of K^+ is also associated with unfavorable consequences: an increase in the intracellular ionic strength, disturbance of ionic balance, decrease in the membrane potential, and increase in the cytoplasmic pH [1]. Overall, these factors have an unfavorable effect on the activities of macromolecules and prevent cell growth in medium of elevated osmolarity despite turgor recovery. The accumulation glutamate anions resulting from its synthesis, which accompanies the accumulation of K^+ [20], results in a partial recovery of ionic balance but fails to adequately neutralize the deleterious effect of high concentrations of K^+. Therefore, the *E. coli* cells recommence their growth only after K^+ is replaced by other compounds that maintain the turgor and thus provide a more favorable environment for the normal functioning of macromolecules [2, 17, 20, 21]. The available data suggest that the *E. coli* response to hyperosmotic shock should include a rapid accumulation of K^+ during the first stage of the response and a partial release of the accumulated K^+ during the second stage [2].

How does glutathione favorably influence osmoadaptation? Because the intracellular glutathione is increased in response to an increase in osmotic pressure, the possibility of its direct involvement in the maintenance of turgor pressure was considered. However, the calculations have shown that the glutathione concentration is rather low compared to other intracellular osmolytes and that even at the highest accumulation glutathione cannot significantly contribute to the maintenance of the turgor pressure under conditions of osmotic shock [4, 5].

One of the known functions of glutathione in *E. coli* is related to its involvement in the regulation of K^+-release channels. The rate of K^+ release in *E. coli* gsh^- mutants is increased [6]. In work [5], a possible relation was studied between GSH-dependent osmoadaptation and the rate of K^+ release and its accumulation by cells. In these experiments, various strains of *E. coli* were used with mutations in the *gsh*, *kefB*, and *kefC* genes that control the GSH-dependent K^+-release channels. The authors compared the rates of K^+ release on the transfer of such cells into potassium-free medium with the growth rates of the corresponding strains in medium of elevated osmolarity and concluded that the role of glutathione in osmoregulation was not related to its involvement in the retention of K^+ [5].

In the present work, we directly measured the intracellular K^+ in *E. coli* under conditions of osmotic shock and found that the capacity of the gsh^+ cells for releasing K^+ during the second stage of the response was greater than the capacity of the gsh^- mutants (Fig. 4a). This property is suggested to determine the higher capacity for osmoadaptation in the cells with the normal level of GSH.

The data on changes in the glutathione status on either side of the cytoplasmic membrane after the increase in the medium osmolarity are especially interesting. Since during the first stage of the response to shock the extracellular glutathione level was decreasing concurrently with the increase in the level of intracellular glutathione, it was suggested that glutathione was transferred from the medium into the cell. The intracellular glutathione level could also be increased due to increase in activities of enzymes responsible for its biosynthesis as a result of increase in cytoplasmic pH during the hyperosmotic shock [2, 22, 23]. In the range of physiological pH values (7-8), the activities of glutathione biosynthesis enzymes increase nearly linearly with the increase in pH [24]. The decrease in the extracellular glutathione level found by us was accompanied by a concurrent release of putrescine from the cells [4]. Since at physiological pH values glutathione is an anion and putrescine is a cation, the transmembrane exchange of these substances will promote the recovery of ionic balance.

Thus, we suggest that the changes in glutathione status inside and outside the cell observed under conditions of osmotic shock are caused by changes in such important cell parameters as turgor pressure, ionic balance, pH, and membrane potential. Since glutathione has features of a redox-mediator and antioxidant, changes in its concentrations inside and outside the cell can directly or indirectly influence osmoadaptation (e.g., due to changes in redox conditions more favorable for osmolyte synthesis or due to an effect on the redox-sensitive membrane transport systems).

Osmotic shock in *E. coli* is accompanied by reactions characteristic of oxidative stress [7]. The favorable effect of glutathione and GOR on the osmoadaptation of *E. coli* may be associated with the antioxidant properties of glutathione (see "Results").

The presence of γ-glutamyl transpeptidase in *E. coli* cells is more likely to be unfavorable than favorable for osmoadaptation (Fig. 1b). Little is known about the functions of GGT in *E. coli*. The strains of *E. coli* deficient in

the GGT synthesis increased the amount of glutathione outside the cells [25]. However, although in our experiments the *ggt*-mutant cells accumulated glutathione in the medium, they had more active enzymes of its synthesis, this seeming to provide the mutants with 1.5-fold faster increase in the intracellular glutathione concentration in response to osmotic shock compared to the wild-type cells (Fig. 4b). This is perhaps one of the causes for the increased resistance of the cells mutant in *ggt* to osmotic shock.

The authors are grateful to B. Demple (Harvard University), A. Eisenstark (Missouri University), and H. Suzuki (Kyoto University) for the bacteria strains provided. This work was supported by the Russian Foundation for Basic Research (grant No. 98-04-49053).

REFERENCES

1. Wood, J. M. (1999) *Microbiol. Mol. Biol. Rev.*, **63**, 230-262.
2. Dinnbier, U., Limpinsel, E., Schmid, R., and Bakker, E. P. (1988) *Arch. Microbiol.*, **150**, 348-357.
3. Larsen, P. I., Sydnes, L. K., Landfald, B., and Strøm, A. R. (1987) *Arch. Microbiol.*, **147**, 1-7.
4. Munro, G. F., Hercules, K., Morgan, J., and Sauerbier, W. (1972) *J. Biol. Chem.*, **247**, 1272-1280.
5. McLaggan, D., Logan, T. M., Lynn, D. G., and Epstein, W. (1990) *J. Bacteriol.*, **172**, 3631-3636.
6. Meury, J., and Kepes, A. (1982) *EMBO J.*, **1**, 339-343.
7. Smirnova, G. V., Muzyka, N. G., and Oktyabrsky, O. N. (2000) *FEMS Microbiol. Lett.*, **186**, 209-213.
8. Demple, B. (1991) *Annu. Rev. Genet.*, **25**, 315-337.
9. Smirnova, G. V., Muzyka, N. G., Glukhovchenko, M. N., and Oktyabrsky, O. N. (2000) *Free Rad. Biol. Med.*, **28**, 1009-1016.
10. Miller, J. H. (1972) *Experiments in Molecular Genetics*, Cold Spring Harbor, N. Y.
11. Tietze, F. (1969) *Analyt. Biochem.*, **27**, 502-522.
12. Alonso-Moraga, A., Bocanegra, A., Torres, J. M., López-Barea, J., and Pueyo, C. (1987) *Mol. Cell. Biochem.*, **73**, 61-68.
13. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) *J. Biol. Chem.*, **193,** 265-275.
14. Becker-Hapak, M., and Eisenstark, A. (1995) *FEMS Microbiol. Lett.*, **134**, 39-44.
15. Hashimoto, W., Suzuki, H., Yamamoto, K., and Kumagai, H. (1997) *Biosci. Biotech. Biochem.*, **61**, 34-39.
16. Owens, R. A., and Hartman, P. E. (1986) *J. Bacteriol.*, **168**, 109-114.
17. Ohwada, T., and Sagisaka, S. (1988) *Agric. Biol. Chem.*, **52**, 313-319.
18. Oktyabrsky, O. N., and Smirnova, G. V. (1993) *Biochem. Mol. Biol. Int.*, **30**, 377-383.
19. Epstein, W., and Schultz, S. G. (1965) *J. Gen. Physiol.*, **49**, 221-234.
20. Measures, J. C. (1975) *Nature*, **257**, 398-400.
21. Le Rudulier, D., Strøm, A. R., Dandekar, A. M., Smith, L. T., and Valentine, R. C. (1984) *Science*, **224**, 1064-1068.
22. Tempest, D. W., Meers, J. L., and Brown, C. M. (1970) *J. Gen. Microbiol.*, **64**, 171-185.
23. Castle, A. M., Macnab, R. M., and Shulman, R. G. (1986) *J. Biol. Chem.*, **261**, 7797-7806.
24. Apontoweil, P., and Berends, W. (1975) *Biochim. Biophys. Acta*, **399**, 1-9.
25. Suzuki, H., Kumagai, H., and Tochikura, T. (1987) *J. Bacteriol.*, **169**, 3926-3931.