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EXPERIMENTAL ARTICLES

The Role of Antioxidant Systems in the Response of *Escherichia coli* to Heat Shock

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Abstract—Shifting the temperature from 30 to 45°C in an aerobic *Escherichia coli* culture inhibited the expression of the antioxidant genes *katG, katE, sodA,* and *gor.* The expression was evaluated by measuring β-galactosidase activity in *E. coli* strains that contained fusions of the antioxidant gene promoters with the *lacZ* operon. Heat shock inhibited catalase and glutathione reductase, lowered the intracellular level of glutathione, and increased its extracellular level. It also suppressed the growth of mutants deficient in the *katG*-encoded catalase HPI, whereas the sensitivity of the wild-type and *sodA sodB* mutant cells to heat shock was almost the same. In the *E. coli* culture adapted to growth at 42°C, the content of both intracellular and extracellular glutathione was two times higher than in the culture grown at 30°C. The temperature-adapted cells grown aerobically at 42°C showed an increased ability to express the fused *katG–lacZ* genes.

Key words: *Escherichia coli*, heat shock, glutathione, catalase.

In nature, bacteria are always subject to various stresses, such as sharp shifts in temperature, osmotic pressure, pH, oxygen concentration, and solar radiation. In response, bacteria have evolved various mechanisms that allow them to tolerate stresses and to survive under unfavorable conditions. Investigations showed that a stressful factor may induce different cell responses, either specific or not. For instance, eukaryotic and prokaryotic cells may respond to heat shock by reactions that are typical of oxidative stress [1–3].

The present work was aimed at studying the role of antioxidant systems in the response of growing *E. coli* cells to heat shock.

MATERIALS AND METHODS

Experiments were carried out with eleven *Escherichia coli* strains: (1) AB1157 (the wild type); (2) JTG10 (the *gshA* mutant of AB1157); (3) 821 (the *gshA* mutant of AB1157); (4) MP180 (the wild type); (5) UM202 (the *katG* mutant of MP180); (6) GC4468 (the wild type); (7) QC909 (the *sodA sodB* mutant of GC4468); (8) JHC1092 (the *soxR* mutant of GC4468); (9) QC772 (carries the fused genes *sodA–lacZ*); (10) NM23 (carries plasmid pKT1033 with the gene fusion *katG–lacZ*); and (11) MB1417 (carries the fused genes *gor–lacZ*). Strains 1–3 were obtained from T. Ninoshiba (United States); strains 4 and 5 from P.C. Loewen (Canada); strains 6–8 from B. Demple (United States); strain 9 from D. Touati (France); strain 10 was constructed in our laboratory by transferring plasmid pKT1033 from strain RK4936 to strain AB1157 [4]; and strain 11 was obtained from A. Eisenstark (United States).

E. coli strains were grown in a minimal M9 medium [5] supplemented with 0.2% glucose, 0.2% casamino acids, and 10 μg/ml thiamine. In heat shock experiments, cells for inoculation were obtained by centrifuging 20 ml of an 18-h culture grown at 30°C. The precipitated cells were resuspended in 100 ml fresh medium in 250-ml flasks and cultivated at 30°C on to mid-exponential phase a shaker at 150 rpm. Culture growth was monitored by measuring the culture turbidity at 670 nm. Heat shock was achieved by elevating the culture temperature from 30 to 45°C for 10 min and maintaining it at this level for the rest of the experiment. Under the given cultivation conditions, *E. coli* cells could grow at 45°C, albeit at a slower rate than at 30°C. In experiments with *E. coli* cells adapted to 42°C, cells for inoculation were taken from an 18-h culture grown at the same temperature. The cultivation temperature was controlled directly in the cultivation flasks and in the surrounding liquid.

The reduced (GSH) and oxidized (GSSG) forms of glutathione were measured spectrophotometrically [6] as described earlier [7].

The catalase activity of intact cells was assayed by measuring the absorbance of the reaction mixture at 240 nm [8]. One unit of catalase activity was defined as the amount of enzyme catalyzing the breakdown of 1 μmole hydrogen peroxide at 25°C per minute.

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The activity of glutathione reductase was determined by measuring the decrease in the concentration of NADPH in the assay mixture at 340 nm. One unit of glutathione reductase activity was defined as the amount of enzyme reducing 1 μmole GSSG at 25°C per minute [9].

β-Galactosidase activity was measured by Miller's method and expressed in arbitrary units calculated by the following formula:

$$(D_{420} - 1.75D_{550}/tVD_{600}) \times 1000,$$

where D_{420} and D_{550} are the turbidities of the reaction mixture measured at 420 and 550 nm; D_{600} is the turbidity of the cell suspension; t is the reaction time measured in minutes; and V is the culture volume measured in milliliters. The activity thus determined is proportional to an increase in the amount of *o*-nitrophenol formed in 1 minute per one bacterial cell [5].

The protein concentration was measured by the method of Lowry *et al.* [10], using bovine serum albumin (BSA) as the standard.

The results presented are the means of at least triplicate measurements.

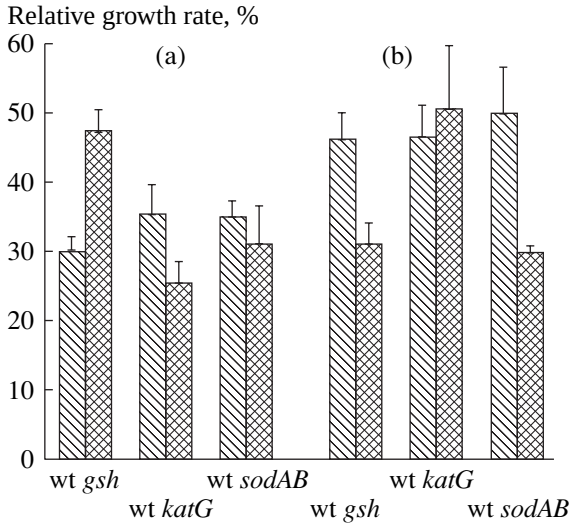

Fig. 1. The effect of (a) heat shock (the growth temperature shift from 30 to 45°C) and (b) ethanol on the aerobically grown wild-type and mutant *E. coli* cells. The bar height corresponds to the specific growth rate μ expressed as a percentage of the control (μ values at 30°C and in the absence of ethanol).

RESULTS AND DISCUSSION

The bacterium *E. coli* has at least two defense systems against oxidative stress: catalase–hydroperoxidase I (HPI) and Mn-, Fe-superoxide dismutases (Mn-SOD and Fe-SOD) [11]. The antioxidant role of glutathione in this bacterium still remains unclear [12–14].

To investigate the role of all these antioxidant systems in the adaptive response of *E. coli* to heat shock, we measured the specific growth rate of *E. coli* mutants impaired in these systems. For instance, strain UM202 has a mutation in the *katG* gene that leads to the inactivation of catalase HPI [15]. Heat shock reduced the growth rate of both the wild-type MP180 (*katG*$^+$) strain and its *katG* mutant, the inhibiting effect of the heat shock being more profound in the mutant (Fig. 1a).

At the same time, we failed to reveal any statistically significant difference in the effect of heat shock on the wild-type strain GC4468 (Fig. 1a) and its double *sodA sodB* mutant strain QC909, which lacks Mn-SOD and Fe-SOD activities [16].

To investigate the role of glutathione in the resistance of *E. coli* to heat shock, we used the *gshA* mutant strain JTG10 defective in GSH synthesis and found that this mutant is more thermoresistant than the wild-type strain AB1157 (*gshA*$^+$) (Fig. 1a).

The mutant strain JHC1092 carries a mutation in the regulatory *soxR* gene of the *soxRS* system, which controls the response of aerobically grown *E. coli* cells to the action of O_2^--generating agents [17]. The responses of this mutant JHC1092 (*soxR*$^-$) and its parent, strain GC4468 (*soxR*$^+$), to heat shock did not show any statistically significant difference.

It is known that heat shock and ethanol exert similar effects on prokaryotic and eukaryotic cells, which is presumably due to oxidative stress accompanying heat shock and the action of ethanol [1]. As can be seen from Fig. 1b, the effect of 5% ethanol may in fact be accompanied by oxidative stress. However, resistance to ethanol and heat shock is likely due to the functioning of different antioxidant systems, since ethanol resistance requires mainly the activity of catalase HPI, whereas thermoresistance requires mainly the activities of superoxide dismutase and glutathione.

To facilitate the observation of the expression of genes involved in the control of antioxidant systems, we used several mutant *E. coli* strains carrying the promoter fusions of the *katG*, *sodA*, *soxS*, *gor*, and *katE* genes with the *lacZ* operon encoding the synthesis of β-galactosidase. The products of the first three genes were mentioned above. The *gor* gene codes for the synthesis of glutathione reductase (GOR, EC 1.6.4.2), which catalyzes the reduction of GSSG to GSH. The *katE* gene codes for the synthesis of catalase HPII in the stationary-phase *E. coli* cells.

Experiments showed that the growth of *E. coli* at 30°C was accompanied by a gradual 20% increase in the expression of the fused *katG–lacZ* genes (Fig. 2a). Heat shock diminished the expression of *katG–lacZ* by a factor of 2.5 (Fig. 2a). The expression of the fused *katE–lacZ* genes changed in a similar way. These changes in the expression of the *katG* and *katE* genes agreed with the results of the measurements of catalase activity: after 60 min of incubation at 45°C, the catalase

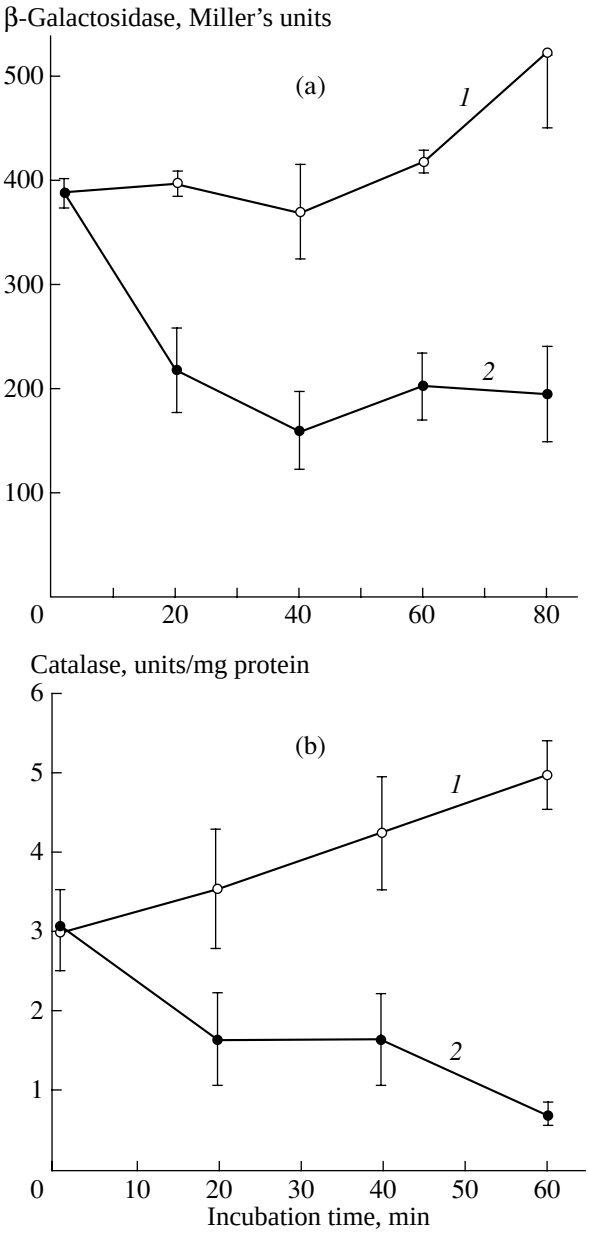

Fig. 2. The effect of heat shock on the expression of the fused *katG–lacZ* genes evaluated by the activity of (a) β-galactosidase and (b) catalase in the *E. coli* cells grown at (*1*) 30°C and (*2*) 45°C after elevating the growth temperature from 30°C.

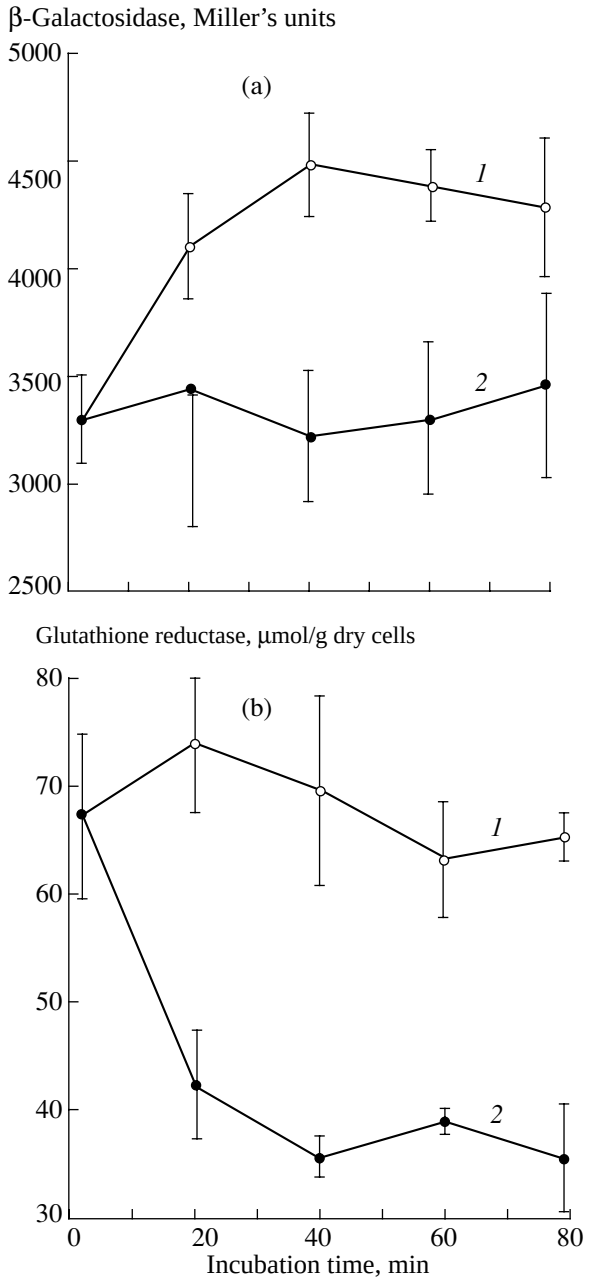

Fig. 3. The effect of heat shock on the expression of the fused *gor–lacZ* genes evaluated by the activity of (a) β-galactosidase and (b) glutathione reductase in the *E. coli* cells grown at (*1*) 30°C and (*2*) 45°C after the growth temperature shift from 30°C.

activity of *E. coli* cells was 5 times lower than that of the *E. coli* cells grown at 30°C (Fig. 2b).

In the process of *E. coli* growth at 30°C, the expression of the fused *gor–lacZ* genes noticeably increased. At the same time, after 40 min of incubation at 45°C, the expression of *gor–lacZ* decreased to comprise 72% of the level observed in the *E. coli* cells grown at 30°C (Fig. 3a). The activity of glutathione reductase in the *E. coli* cells grown at 45°C was twice as low as in the cells grown at 30°C (Fig. 3b).

The inhibitory effect of heat shock was most pronounced with respect to the expression of the fused *sodA–lacZ* genes. Indeed, during the growth of *E. coli* cells at 30°C, the activity of β-galactosidase increased by a factor of 2, whereas it decreased by a factor of 8 after heat shock (Fig. 4).

The expression of the fused *soxS–lacZ* genes in the *E. coli* cells grown at 45°C was 30% lower than in the cells grown at 30°C.

The intracellular level of the total glutathione in the *E. coli* cells grown at 30°C (GSH_{in} + $GSSG_{in}$) gradually increased by 30% (Fig. 5c). This increase was obviously due to an elevated content of intracellular GSH, since the intracellular level of GSSG in this case decreased twofold (Figs. 5b, 5c). As a result, the $GSH_{in}/GSSG_{in}$ ratio in the *E. coli* cells grown aerobically at 30°C increased more than twofold (from 145 to 315) (Fig. 5a). After 1 h of incubation at 45°C, the intracellular level of the total glutathione was two times as low as in the *E. coli* cells grown at 30°C, whereas the $GSH_{in}/GSSG_{in}$ ratio changed insignificantly (Fig. 5a).

Unlike the concentration of the total intracellular glutathione, the concentration of the total extracellular glutathione (GSH_{out} + $GSSG_{out}$) in the *E. coli* culture grown at 45°C gradually increased to comprise 145% of the level observed in the cells grown at 30°C (Figs. 6b, 6c). The increase in the concentration of the total extracellular glutathione was obviously due to the increased concentration of reduced extracellular glutathione, since the difference in the concentrations of $GSSG_{out}$ in the *E. coli* cultures grown at 45 and 30°C was insignificant. At both growth temperatures, the relative content of GSSG outside *E. coli* cells was an order of magnitude higher than inside cells. In this case, the $GSH_{out}/GSSG_{out}$ ratio remained at a level of 10 to 20.

The initial expression level of the *katG* gene in the *E. coli* cells adapted to growth at 42°C was 2.5 times as low as in the cells grown at 30°C. After 80 min of aerobic incubation at 42°C, the expression of the *katG* gene in the *E. coli* cells adapted to this temperature was four times better than at the beginning of such incubation and two times better than in the cells grown at 30°C. These data agree with the results of the direct measurement of catalase activity: at the beginning of cultivation, the activity of catalase in the *E. coli* cells grown at 42°C was 30% lower than in the cells grown at 30°C, but at the end of cultivation, the catalase activity of the cells grown at 42°C was twice as high as of the cells grown at 30°C. The initial expression level of the *katE* gene in the *E. coli* cells adapted to 42°C was 38% lower than in the cells grown at 30°C. However, by the end of cultivation, the difference in the expression levels of this gene at these two temperatures was statistically insignificant. Like in the *E. coli* cells exposed to heat shock at 45°C, the expression level of the fused genes *sodA–lacZ* in the cells adapted to 42°C was low.

The intracellular concentration of the total glutathione in the *E. coli* cells adapted to 42°C was twice as high as in the cells grown at 30°C. At the same time, the differences in the $GSSG_{in}$ concentration and in the $GSH_{in}/GSSG_{in}$ ratio at these two temperatures were less profound. In the *E. coli* culture grown at 42°C, the concentration of extracellular glutathione was 2.5 times and the $GSH_{out}/GSSG_{out}$ ratio was two times greater than in the culture grown at 30°C.

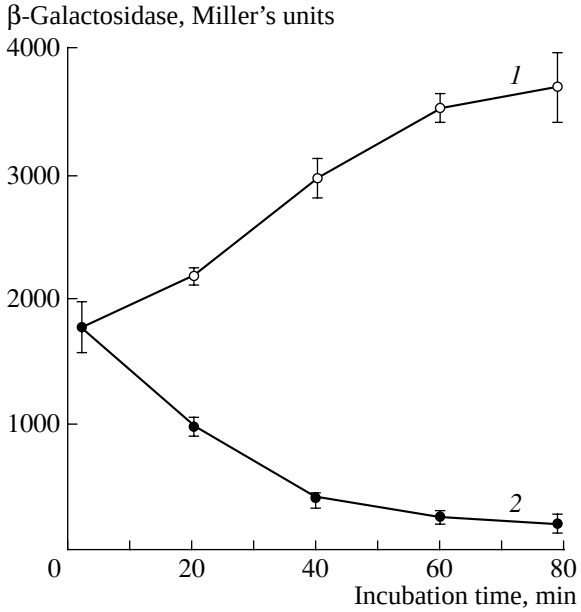

Fig. 4. The effect of heat shock on the expression of the fused *sodA–lacZ* genes evaluated by the activity of β-galactosidase in the *E. coli* cells grown at (*1*) 30°C and (*2*) 45°C after the growth temperature shift from 30°C.

It is known that active oxygen species (AOS) are normal cellular metabolites whose concentration in cells is maintained at a low level by various antioxidant systems. Under certain conditions, however, a balance between the rates of AOS formation and breakdown may be disturbed, resulting in oxidative stress.

Based on the kinetics and thermodynamics of chemical reactions, Mitchell and Russo [18] assumed that heat shock may enhance the formation of AOS and, therefore, antioxidant systems may play an important role in the thermoresistance of cells. Investigations showed that the intracellular concentration of glutathione rises in response to heat shock, suggesting that this compound is involved in protecting eukaryotic cells not only from oxidative stress but also from heat shock [18]. This suggestion is confirmed by the fact that pretreatment of cells with agents diminishing the concentration of intracellular glutathione increases the susceptibility of the cells to heat shock [18].

The role of glutathione in protecting prokaryotic bacterial cells from heat shock is less understood. The present study showed that heat shock decreased the intracellular concentration of glutathione and the $GSH_{in}/GSSG_{in}$ ratio in *E. coli* cells and that the deficiency of glutathione in (*gshA*$^-$) cells did not enhance their sensitivity to heat shock. Therefore, the roles of glutathione in the responses of eukaryotic and *E. coli* cells to heat shock considerably differ.

It remains unclear whether or not the aforementioned changes in the intracellular glutathione status in heat-exposed *E. coli* cells may reflect the involvement

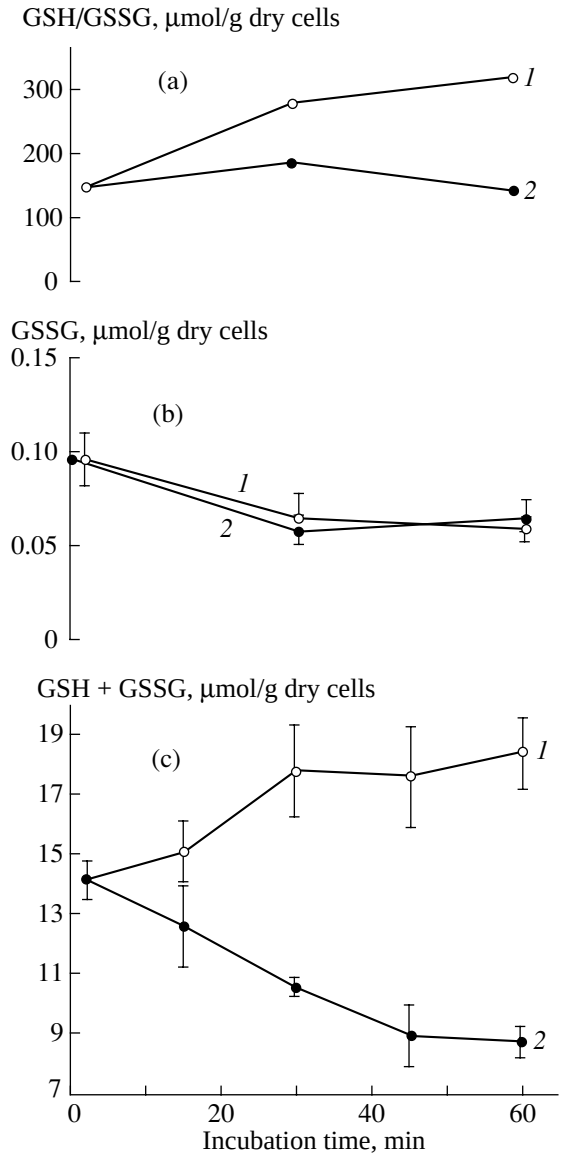

Fig. 5. Changes in the intracellular values of (a) the GSH/GSSG ratio, (b) the GSSG concentration, and (c) the total glutathione (GSH + GSSG) concentration in the *E. coli* culture grown at (*1*) 30°C and (*2*) 45°C after the growth temperature shift from 30°C.

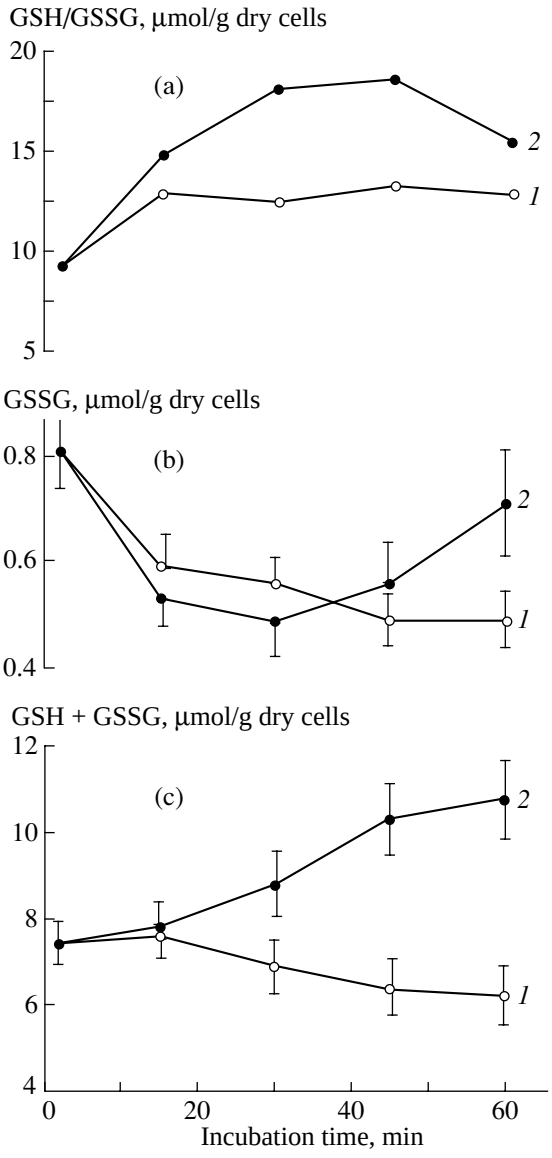

Fig. 6. Changes in the extracellular values of (a) the GSH/GSSG ratio, (b) the GSSG concentration, and (c) the total glutathione (GSH + GSSG) concentration in the *E. coli* culture grown at (*1*) 30°C and (*2*) 45°C after the growth temperature shift from 30°C.

of oxidative stress in the cell response to heat shock. A comparison of the results presented here and in our previous work [7] shows that the heat shock–induced changes in the glutathione status in *E. coli* cells are very similar to those induced by hydrogen peroxide in cells deficient in catalase HPI and by menadione in cells deficient in superoxide dismutase. At first sight, this testifies to the involvement of oxidative stress in the cell response to heat shock, the more so that heat shock inhibits the activity of catalase HPI and superoxide dismutase in the wild-type cells and the phenotype of these cells exposed to heat shock is close to that of the *katG* and *sodA* mutants.

However, the problem will appear more sophisticated if the heat-induced changes in the status of glutathione are considered simultaneously inside and outside the cells. The data presented in Figs. 5 and 6 show that heat shock was accompanied by a considerable rise in the concentration of extracellular glutathione; therefore, the drop in the concentration of intracellular glutathione may be due to its secretion into the medium. It should also be noted that heat shock did not raise the intracellular concentration of GSSG and the elevated concentration of extracellular glutathione was primarily due to GSH. These data do not allow the heat shock–induced decrease in the intracellular concentra-

tion of glutathione to be explained through the direct oxidation of reduced glutathione inside cells.

In *E. coli*, glutathione is not an essential antioxidant component protecting this bacterium from hydrogen peroxide and superoxide-generating agents [12, 14]. Moreover, glutathione was found to inhibit the expression of the *soxS* gene induced by paraquat [19] and the expression of the *katG* gene induced by hydrogen peroxide [13]. These data suggest that the beneficial effect of the *gshA* mutation on the heat resistance of *E. coli* may be related to a GSH deficiency in the *gshA* mutant, due to which it is fairly susceptible to the induction of catalase activity.

It is believed that oxidative stress in *E. coli* is accompanied by the induction of genes responsible for the synthesis of antioxidant enzymes [11, 17]. On the other hand, as was shown above, heat shock not only fails to induce antioxidant genes but even inhibits their expression, presumably due to the thermolability of some components involved in this expression. The thermosensitivity of mutants deficient in catalase HPI may indicate that heat shock raises the concentration of active oxygen species, particularly H_2O_2, in growing *E. coli* cells. It is likely that the heat shock–induced production of AOS in *E. coli* cells, which is also typical of oxidative stress, does not follow from the temperature rise but rather is related to the inhibition of antioxidant enzymes.

The experiments with the *E. coli* culture adapted to 42°C showed that growth at this temperature is accompanied by an increase in the concentration of AOS and by the induction of catalase at the level of its transcription. The high pools of intracellular and extracellular glutathione in such culture can be regarded as an adaptive response to growth at elevated temperatures.

The expression of the *sodA* gene did not increase in response to upshifting the growth temperature from 30 to 45°C, nor could the *sodA* and *sodB* mutations considerably influence the resistance of *E. coli* cells to heat shock. According to the observations of other researchers, the SOD^+ and SOD^- *E. coli* strains do not differ in their sensitivity to 53°C [2, 20]. At the same time, after elevating the growth temperature from 37 to 45–48°C, the survival rate of the *E. coli sodA sodB* cells was considerably lower than that of the wild-type cells [2]. Some disagreement between the results of those and the present study may be due to the use of different temperature shifts and different methods for evaluating their effects. In our experiments, the effect of heat shock was evaluated with respect to its influence on bacterial growth, whereas in the studies discussed [2, 20], this effect was evaluated with respect to its influence on bacterial survival.

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