Microbiology, Vol. 70, No. 1, 2001, pp. 45–50. Translated from Mikrobiologiya, Vol. 70, No. 1, 2001, pp. 55–60.

EXPERIMENTAL ARTICLES

The Role of Antioxidant Systems in the Cold Stress Response of *Escherichia coli*

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Received July 21, 2000

Abstract—The response of aerobically grown *Escherichia coli* cells to the cold shock induced by the rapid lowering of growth temperature from 37 to 20°C was found to be basically the same as the oxidative stress response. The enhanced sensitivity of cells deficient in two superoxide dismutases, Mn-SOD and Fe-SOD, and the increased expression of the Mn-SOD gene, *sodA*, in response to cold stress were interpreted as both oxidative and cold stresses are due to a rise in the intracellular level of superoxide anion. The long-term cultivation of *E. coli* at 20°C was also accompanied by the typical oxidative stress response reactions—an enhanced expression of the Mn-SOD and catalase HPI genes and a decrease in the intracellular level of reduced glutathione (GSH) and in the GSH/GSSG ratio.

Key words: *Escherichia coli*, cold shock, superoxide dismutase, catalase, glutathione.

In nature, bacteria (for instance, *Escherichia coli* outside host organisms) often occur under conditions that are unfavorable for their life and reproduction. To cope with the stresses induced by drastic changes in the ambient temperature, pH, oxygen concentration, and solar radiation, bacteria have evolved various adaptive mechanisms that allow them to survive in hostile environments. One of these mechanisms is the synthesis of new proteins, which is regulated at the level of transcription of the respective regulatory genes. In *E. coli*, the gene *oxyR*, which controls the synthesis of catalase HPI and some other proteins, is induced in response to exposure to H_2O_2, while the multigene locus *soxRS* is induced in response to exposure to superoxide radical-generating compounds [1]. The exposure of *E. coli* to low temperatures induces the synthesis of cold shock proteins [2].

There is evidence that some stress factors can induce responses that are specific to other stresses. For instance, the exposure of eukaryotic and prokaryotic cells to heat can induce some reactions which are typical of oxidative stress [3, 4]. However, little is known about the relationship of responses induced by cold and oxidative stresses.

This work was undertaken to study the role of antioxidant systems, which are commonly induced by oxidative stress, in the cold stress response of *E. coli* cells.

MATERIALS AND METHODS

The *Escherichia coli* strains used in this study were as follows. (1) Strain AB1157 (wt, wild type), (2) strain JTG10 (the *gshA* derivative of AB1157), (3) strain MP180 (wt), (4) strain UM202 (the *katG* derivative of MP180, (5) strain GC4468 (wt), (6) strain QC909 (the *sodAsodB* derivative of strain GC4468), (7) strain JHC1092 (the Δ*soxR* derivative of GC4468), (8) strain QC771 (wt), (9) strain QC1732 (the Δ*fur* derivative of QC771), (10) strain QC722 (the *sodA–lacZ* derivative of QC771), and (11) strain NM23 (constructed in our laboratory by transferring the *katG–lacZ*-bearing plasmid pKT1033 from strain RK4936 [5] to strain AB1157). Strains 1 and 2 were obtained from T. Ninoshiba (USA); strains 3 and 4, from P. Loewen (Canada); strains 5–7, from B. Demple (USA); and strains 8–10, from D. Touati (France).

The *E. coli* strains were grown aerobically at 37°C in shaken 250-ml flasks (150 rpm) containing 100 ml of minimal M9 medium [6] supplemented with 0.2% glucose, 0.2% casamino acids, and 10 μg/ml thiamine. The medium was inoculated with cells from a 20-ml overnight culture. Growth was monitored by measuring culture turbidity at 670 nm.

Cold shock was induced by lowering the culture temperature from 37 to 20°C within 10 min. The temperature was measured in the culture and in the cooling liquid. During cultivation at 20°C, *E. coli* cells continued to grow, although at a low rate.

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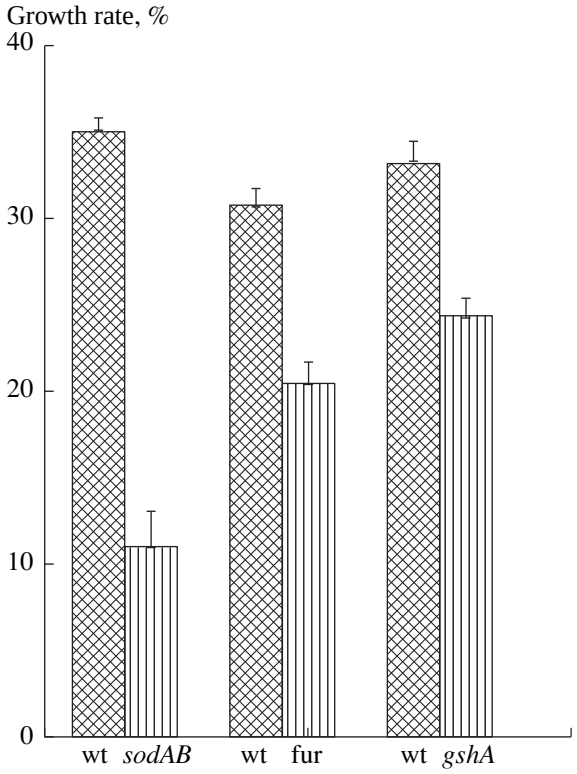

Fig. 1. Effect of cold stress (temperature shift from 37 to 20°C) on the growth rate of aerobic *E. coli* culture. The maximum specific growth rate (μ) at 37°C is taken as 100%. Designations: wt, wild-type (parent) strain; *sodAB*, SOD-deficient mutant; fur, mutant with the impaired regulation of iron assimilation; *gshA*, mutant deficient in glutathione synthesis.

β-Galactosidase was assayed as described in [6]. Catalase activity in intact cells was determined spectrophotometrically by measuring the absorbance at 240 nm [7]. One unit of catalase activity was defined as the amount of enzyme providing the breakdown of 1 μmol H_2O_2 per min at 25°C. Glutathione was determined spectrophotometrically with the use of glutathione oxidoreductase [8]. The amount of protein was quantified by the method of Lowry *et al.* [9] with bovine serum albumin (BSA) as the standard. The results presented are the means of experiments performed at least in triplicate.

RESULTS AND DISCUSSION

There are several enzymatic antioxidant systems operating in *E. coli*, the most important of which are catalase hydroperoxidase I (catalase HPI), Mn- and Fe-dependent superoxide dismutases (Mn-SOD and Fe-SOD), and reduced glutathione (GSH). To estimate the contribution of these systems on the bacterial response to cold shock, we measured the specific growth rate of parent bacteria and their derivatives carrying mutations in the genes that control the synthesis of the key components of these systems.

The *katG* mutation in *E. coli* UM202 cells leads to the loss of activity of the HPI catalase, which is involved in the breakdown of H_2O_2 in aerobically grown *E. coli* cells [10]. The *katG* gene is a component of a regulon, which is induced by H_2O_2 and controlled by the *oxyR* gene. In our experiments, the cold shock (37 ⟶ 20°C) slowed the growth rate of both the mutant strain UM202 and the parent strain MP180 (*katG*$^+$). The difference in the effect of cold shock on the growth rate of these two strains was statistically insignificant.

Mn-SOD and Fe-SOD, the products of the genes *sodA* and *sodB*, play an important role in the protection of *E. coli* from the superoxide radical (O_2^-). Strain QC909, which is a double *sodAsodB* mutant lacking Mn-SOD and Fe-SOD activities, exhibits enhanced sensitivity to the action of active oxygen species [11]. In our experiments, the growing culture of the SOD-deficient strain QC909 was found to be more sensitive to cold shock than the parent strain GC4468 (*sodA*$^+$*sodB*$^+$). The growth of strain QC909 was inhibited by cold three times more than that of the parent strain (Fig. 1).

E. coli JHC1092 is mutant in *soxR*, which is one of the regulatory genes of the two-gene system *soxRS* controlling the response of aerobic *E. coli* cells to the oxidative shock induced by O_2^- generators [1]. Experiments revealed no statistically significant difference in the responses of the strains JHC1092 (*soxR*$^-$) and GC4468 (*soxR*$^+$) to cold shock.

In aerobically grown cells, free iron ions in the cytoplasm may enhance the effect of oxidative stress through the formation of highly toxic hydroxyl radicals ($OH^\bullet$) in the Fenton reaction. The impaired regulation of iron assimilation in the *E. coli* Δfur mutants and, as a result, the high intracellular concentration of free iron, enhance the effect of oxidative stress [12]. In our experiments, cold shock inhibited the growth of the mutant strain QC7771 Δfur mutants 34% more than that of the parent strain QC1732 (*fur*$^+$) (Fig. 1).

To estimate the role of glutathione in the resistance of *E. coli* cells to cold shock, we used strain JTG10, which is deficient in GSH synthesis due to the *gshA* mutation, and found that this mutation decreased the resistance of the cells to cold shock, albeit to a lesser degree than the mutations *sodA*, *sodB* and Δ*fur* (Fig. 1).

The expression of the genes encoding the components of antioxidant systems was studied using the strains that bore the fused promoters of these genes with *lacZ* operon encoding β-galactosidase (Fig. 2). As can be seen from this figure, cold shock did not influence the expression of the gene fusion *katG–lacZ*. By

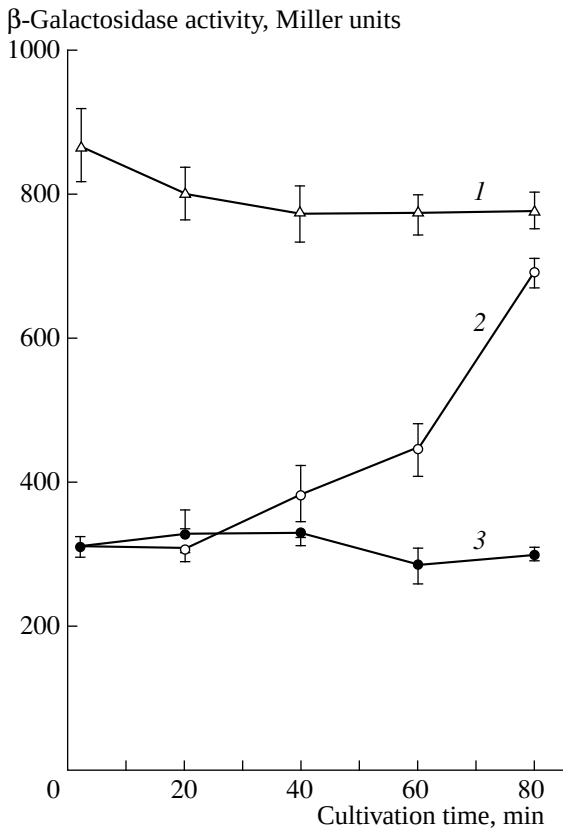

Fig. 2. Effect of cold stress on the expression of the *katG–lacZ* gene fusion: (*1*) cells preadapted to low temperature and grown at 20°C; (*2*) cells grown at 37°C; and (*3*) cells subjected to the cultivation temperature shift from 37 to 20°C at zero time.

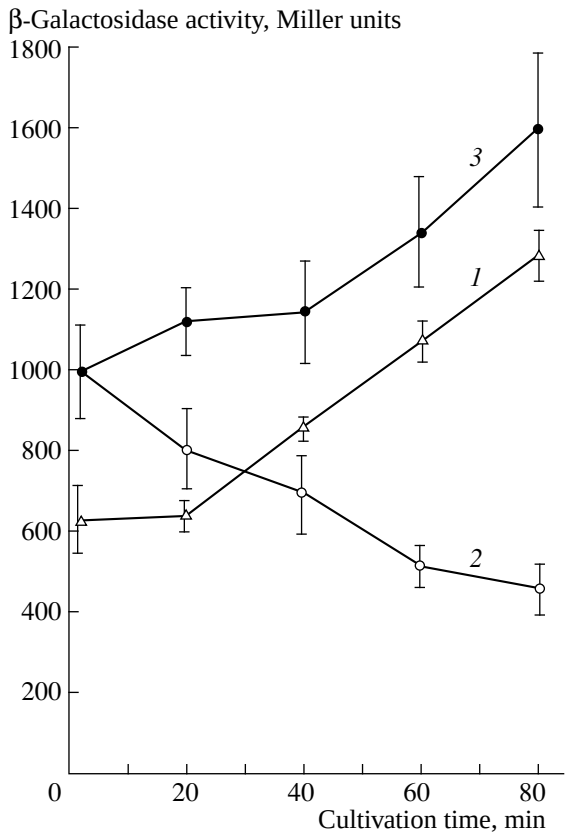

Fig. 3. Effect of cold stress on the expression of the *sodA–lacZ* gene fusion. For explanations, see the legend to Fig. 2.

comparison, Fig. 2 also presents the results of measurements of β-galactosidase activity in cells cultivated at 37°C in a batch mode. One can see a gradual increase in the level of the expression of the gene fusion *katG–lacZ* during such cultivation.

These data are in agreement with the results of the direct measurements of catalase activity, according to which catalase activity increased from 2.6 ± 0.17 to 8.0 ± 1.0 U ($P < 0.01$) during aerobic cultivation at 37°C in batch mode. Cold shock did not significantly influence catalase activity, which increased from 2.6 ± 0.17 to 3.2 ± 0.45 U ($P > 0.05$) 80 min after shock induction. Earlier, we showed that the increase in catalase activity and in the degree of *katG* expression during batch cultivation of *E. coli* at 37°C under aerobic conditions is due to the accumulation of H_2O_2 in cells [13]. All of these data are consistent with results that indicate the absence of the *katG* mutation effect on the growth of cold-stressed cells (Fig. 1).

We also estimated the degree of expression of the gene fusion *katG–lacZ* in the cells grown at 20°C (Fig. 2). In this experiment, cells grown for 18 h at 20°C were used as the inoculum. It should be noted that these cold-adapted cells, like those exposed to cold shock, did not exhibit any significant changes in the level of expression of *katG–lacZ* gene fusion during growth. However, the degree of the expression, in this case, was three times higher than in the cold-stressed cells and in the first hours of growth at 37°C. In the cells grown at 20°C in batch mode, catalase activity did not significantly change during cultivation, comprising 3.3 ± 0.16 U. Thus, we failed to observe a noticeable correlation between the degree of expression of the *katG–lacZ* gene fusion and the total catalase activity of the cells.

The expression of the *sodA* gene was studied by measuring the β-galactosidase activity of strain QC772 with the *sodA–lacZ* gene fusion [11]. The cold-stressed cells of this strain exhibited a 60% increase in the expression of the *sodA–lacZ* gene fusion (Fig. 3). The same degree of stimulation of the *sodA–lacZ* expression was observed in strain QC772 that was cultivated at 20°C in batch mode. It should be noted that the batch cultivation of this strain at 37°C led to a twofold decrease in the degree of expression of *sodA–lacZ* (Fig. 3). This indicates that the aerobic growth of *E. coli* at low temperatures is accompanied by a greater accumulation of superoxide anions than growth at optimal temperature (37°C). This suggestion is confirmed

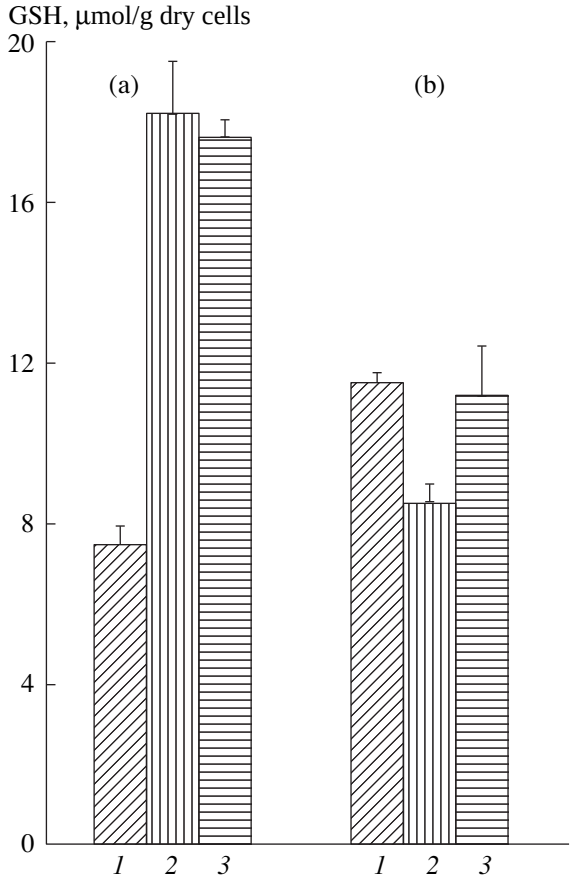

Fig. 4. Effect of cold stress on the (a) intracellular and (b) extracellular concentrations of GSH: (*1*) cells preadapted to low temperature and grown at 20°C; (*2*) cells grown at 37°C; and (*3*) cells subjected to the cultivation temperature shift from 37 to 20°C at zero time.

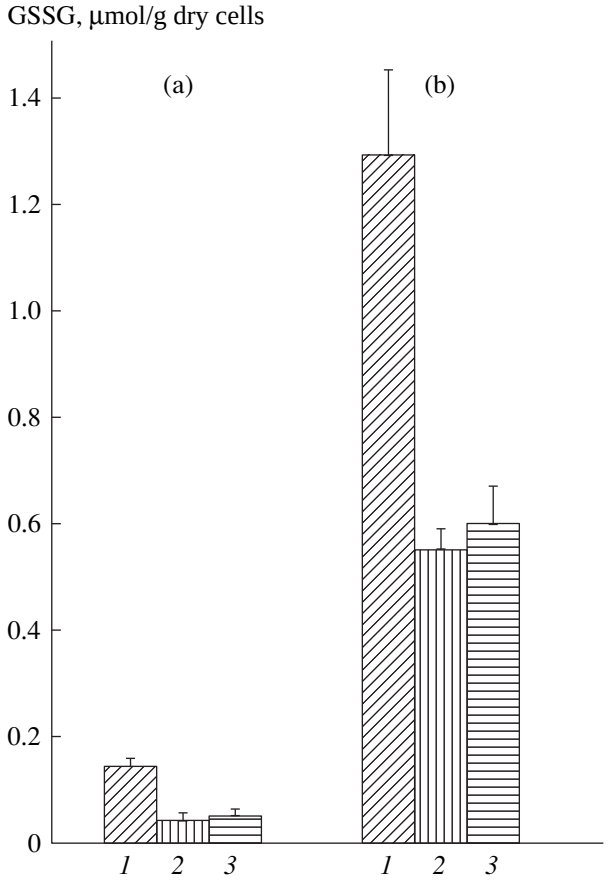

Fig. 5. Effect of cold stress on the (a) intracellular and (b) extracellular concentrations of GSSG. For explanations, see the legend to Fig. 4.

by the data showing that QC909 cells, which are deficient in the synthesis of cytoplasmic superoxide dismutases, are distinguished by enhanced sensitivity to cold shock (Fig. 1).

The exposure of *E. coli* cells grown at 37°C to cold shock did not induce any noticeable changes in the intracellular level of reduced glutathione (GSH_{in}) (Fig. 4a). However, in the cells preadapted to low temperature and grown at 20°C, the concentration of $GSH_{in}$was 2.4 times lower than in the cells grown at 37°C. In this case, cells grown at 20°C exhibited an enhanced level of oxidized glutathione ($GSSG_{in}$) (Fig. 5a). Reportedly, the low $GSH_{in}/GSSG_{in}$ ratio in eukaryotic cells implies that they are subject to oxidative stress. In our experiments, this ratio was found to be 423 for *E. coli* cells grown at 37°C (cold stress decreased the $GSH_{in}/GSSG_{in}$ ratio to 352) and 51 for the low-temperature adapted cells grown at 20°C (Figs. 4 and 5).

The level of reduced extracellular glutathione (GSH_{ex}) in the cold-stressed and cold-adapted cultures grown at 20°C was appreciably higher than in the culture grown at 37°C (Fig. 4b). In all cases, the concentration of oxidized extracellular glutathione ($GSSG_{ex}$) was an order of magnitude higher than its concentration in the cells (Fig. 5). Accordingly, the $GSH_{ex}/GSSG_{ex}$ ratio was very low (from 11 to 19) and did not appreciably depend on the cultivation temperature.

It should be noted that the observed changes in the extracellular concentration of GSH in the *E. coli* culture exposed to cold stress probably represent a common and universal response of *E. coli* to various kinds of stresses. Earlier, we reported an increase in the level of extracellular low-molecular-weight thiols in response to starvation, hypoosmotic shock, acidification of the cytoplasm, and in the presence of antibiotics inhibiting protein synthesis [14, 15].

The exposure of *E. coli* to cold shock induces the synthesis of new proteins, some of which are specific to this type of stress [2, 16]. In this case, the temperature shift from 37 to 10°C resulted in that *E. coli* cells could not grow over a period of 4 h [2]. In the present study, the cultivation temperature was decreased from 37 to 20°C. After this mildly stressful change, the wild type

E. coli cells continued to grow, albeit at a rate about three times lower than that observed at 37°C. It should be noted that such temperature shifts are common in the life cycle of enterobacteria.

The results obtained in the present study indicate that the response of aerobically grown *E. coli* cells to cold shock induced by a shift in cultivation temperature from 37 to 20°C within 10 min is basically the same as the response to oxidative stress. The increase in the sensitivity of cells that are deficient in two superoxide dismutases, Mn-SOD and Fe-SOD, and in the degree of expression of the *sodA* gene, which encodes Mn-SOD in response to cold shock, suggests that it enhances the intracellular level of superoxide anion rather than of H_2O_2. This inference is indirectly confirmed by the decrease in the intracellular concentration of GSH and in the GSH/GSSG ratio in the cells grown at 20°C. As shown previously, the exposure of the aerobically grown *E. coli* cells to menadione (a generator of superoxide anions), but not to H_2O_2, leads to a decrease in the intracellular level of total glutathione and in the $GSH_{in}/GSSG_{in}$ ratio [17].

Thus, the data presented show that the long-term cultivation of *E. coli* at 20°C is accompanied by metabolic changes that are also typical of oxidative stress. However, the role of particular antioxidant systems in the cell response to these stresses remains unclear.

One of the reasons for this phenomenon may be the change in the concentration of dissolved oxygen in aerobic *E. coli* culture, which is induced by the temperature shift from 37 to 19–22°C. Due to a decreased growth rate at low cultivation temperatures, the rate of oxygen consumption diminishes, while the rate of oxygen that dissolves in the colder cultivation medium increases. As a result, the steady state concentration of dissolved oxygen in the cultivation medium rises [18].

Another reason for the similarity of cell responses to oxidative and cold stresses may be the enhanced production of active oxygen species in the respiratory chain, since there is evidence that lowering the cultivation temperature from 35 to 20°C substantially alters the properties of bacterial membranes [19]. Furthermore, the membrane components of the respiratory chain can produce active oxygen species in the aerobically grown *E. coli* [20]. The nature of these factors is such that they may act concurrently.

ACKNOWLEDGMENTS

We are grateful to T. Ninoshiba, P. Loewen, B. Demple, D. Touati, and K. Tao for providing bacterial strains.

This work was supported by the Russian Foundation for Basic Research, project no. 98-04-49053.

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