ISSN 1022-7954, Russian Journal of Genetics, 2018, Vol. 54, No. 11, pp. 1259–1265. © Pleiades Publishing, Inc., 2018.
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GENETICS
OF MICROORGANISMS

The New Role of CysB Transcription Factor in Cysteine Degradation and Production of Hydrogen Sulfide in *E. coli*

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Received March 29, 2018; in final form, May 24, 2018

Abstract—The paradoxical effect of deletion of the *Escherichia coli* genes *cysK* and *cysM* encoding cysteine synthase enzymes has been studied: such cysteine auxotrophs actively degrade the excess of cysteine transported from the medium to form H_2S. We have shown that deletions of any of the known genes controlling the degradation of exogenous cysteine, including the genes *aspC*, *mstA*, *cysK*, *cysM*, *tnaA*, *metC*, and *malY*, as well as the newly discovered genes *yciW*, *cyuA*, *cyuP*, and *cyuR*, do not deprive the cysteine auxotrophs Δ*cysK* Δ*cysM* of the ability to degrade cysteine. Cysteine degradation in the Δ*cysK* Δ*cysM* mutant is positively regulated by the products of the *cysB* and *cysE* genes. It is significant that the Δ*cysK* Δ*cysM* mutant shows an increased transcription of the genes opposing the oxidative stress (*sodA*, *catG*, *arcA*, and *cydD*). We assume that oxidative stress in cells of the Δ*cysK* Δ*cysM* mutant is provoked by restriction of cysteine resynthesis, while *cysB*-dependent degradation of exogenous cysteine and generated H_2S provide protection against oxidative stress.

Keywords: hydrogen sulfide production, degradation and transport of cysteine, CysB regulon, oxidative stress, *E. coli*

DOI: 10.1134/S1022795418110145

INTRODUCTION

Recent studies have shown that hydrogen sulfide (H_2S) in bacteria is an effective protector against oxidative stress and antibiotics [1]. The main source of H_2S in *E. coli* cells is cysteine, which is prone to desulfidation with the participation of genes *aspC* and *mstA*. We have found a close relationship between the generation of hydrogen sulfide and the expression of genes involved in the biosynthesis and transport of cysteine and controlled by the regulatory protein CysB [2]. In particular, we showed that when growing bacteria on the LB medium, the level of H_2S generation increases significantly as a result of constitutive expression of gene *tcyP*, which controls the transport of cystine/cysteine from periplasm to cytoplasm. These data indicate the important role of the exogenous cysteine contained in the LB medium in H_2S production.

One of the objectives of this work was to elucidate the role of endogenously synthesized cysteine in H_2S production. To this end, we inactivated genes *cysK* and *cysM*, which control the cysteine synthesis *de novo* [3], and studied the effect of the deletions obtained on H_2S generation. Unexpectedly, it turned out that bacteria defective for genes *cysK* and *cysM* not only retain the ability to produce H_2S but also, on the contrary, are characterized by its increased generation. We have shown that deletions of any of the known genes controlling the desulfohydrogenation of cysteine (*tnaA*, *metC*, and *malY*) [4], as well as new, recently discovered genes *yciW* [5, 6], *cyuA*, *cyuP*, and *cyuR* [7–9] involved in cysteine degradation, do not prevent H_2S formation by Δ*cysK* Δ*cysM* mutants. Also, we showed that the cysteine degradation and the H_2S formation in the Δ*cysK* Δ*cysM* mutant are suppressed by inactivation of genes *cysB* and *cysE*. Thus, a new role of the transcription factor CysB in the degradation of cysteine and the formation of H_2S has been revealed.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains of *Escherichia coli* used in this work and their genotypes are presented in Table 1. Deletion mutants were obtained by growing phage P1 on strains from the Keio collection [10] containing deletions of genes *cysK*, *cysM*, *cysB*, *cysE*, *tnaA*, *metC*, *malY*, *yciW*, *cyuA*, *cyuP*, and *cyuR* and their subsequent transduction into the genome of the *E. coli* strain MG1655. The preparation

Table 1. Genotype of *E. coli* strains used in this work

Strain	Genotype	Origin
MG1655	F^- wild type	Laboratory collection
AM3007	As MG1655, but Δ*mstA*	[2]
AM3009	As MG1655, but P_{tet}-*mstA*	[2]
AM4001	As MG1655, but Δ*cysK*	This work
AM4002	As MG1655, but Δ*cysM*	"
AM4005	As MG1655, but Δ*cysM* Δ*cysK*	"
AM4008	As AM4005, but Δ*mstA*	"
AM4011	As AM4005, but Δ*tnaA*	"
AM4013	As AM4005, but Δ*metC*	"
AM4014	As AM4005, but Δ*malY*	"
AM4015	As AM4005, but Δ*yciW*	"
AM4017	As AM4005, but Δ*cyuA*	"
AM4019	As AM4005, but Δ*cyuP*	"
AM4020	As AM4005, but Δ*cyuR*	"
AM4022	As AM4005, but Δ*cysB*	"
AM4023	As AM4005, but Δ*cysE*	"
AM4025	As AM4005, but Δ*tcyP*	"
AM4026	As AM4005, but Δ*tcyJ*	"
AM4028	As AM4005, but P_{tet}-*tcyP*	"
AM4029	As AM4005, but P_{tet}-*tcyJ*	"
AM4030	As AM4028, but Δ*cysB*	"
AM4032	As AM4029, but Δ*cysB*	"

of strains containing the genes *tcyP* and *tcyJ* under the control of the constitutive promoter P_{tet} is described in [2].

Media and culture conditions. The glucose-free LB medium was used as a complete nutrient medium for growing bacteria [11]. When necessary, 10 μg/mL chloramphenicol and 40 μg/mL kanamycin were added to the medium.

Determining the hydrogen sulfide production levels. The strains were grown in a complete LB medium for 18 h in test tubes under the caps of which a filter paper strip moistened with a 2% solution of lead acetate was attached. The level of H_2S production by the strains was evaluated visually by the intensity of blackening of the filter paper as a result of the formation of the PbS complex.

Isolation of total RNA. Total RNA was isolated from a culture of *E. coli* cells with an optical density of 0.4–0.6. RNA purification was carried out using the RNeasy Mini Kit (Qiagen) in accordance with the manufacturer's instructions. The resulting RNA preparations were treated with DNase I. The quality of total RNA was analyzed in a 1% agarose gel supplemented with formamide. The amount was determined spectrophotometrically by the absorption value at a wavelength of 260 nm.

PCR with product detection in real time. The *E. coli* cell cultures of the strains analyzed were grown to OD_{600} = 0.4–0.6, and then total RNA was isolated. Before carrying out the reverse transcription reaction, RNA samples were treated with DNase I (Thermo). The reverse transcription reaction was carried out in the presence of oligonucleotides specific to the genes under study using the SuperScript III First-Strand Synthesis Kit for RT-PCR (Invitrogen). Further, 1 μL of the volume of the entire reverse transcription reaction was used as a template for real-time detection PCR. Expression levels of genes *def* and *rpoA* were used for normalization. The analysis was carried out using a set of reagents for real-time PCR from the company Syntol. Amplification was carried out on a DTlite device (DNA-Technology). The reaction products were analyzed by electrophoresis in a 2% agarose gel to confirm that the products obtained had the expected size. Each reaction was set three times, where an average value was taken as a result. The level of transcription was determined from the values of the threshold cycle, taking into account that the concentration of the original specific DNA fragments increases approximately as 2^N, where N is the number of cycles.

RESULTS

Detection of New Cysteine-Degrading Activity in E. coli Cells

According to our data [1], the main role in the H_2S generation in *E. coli* cells is played by the enzyme 3-mercaptopyruvate sulfotransferase (3-MST)

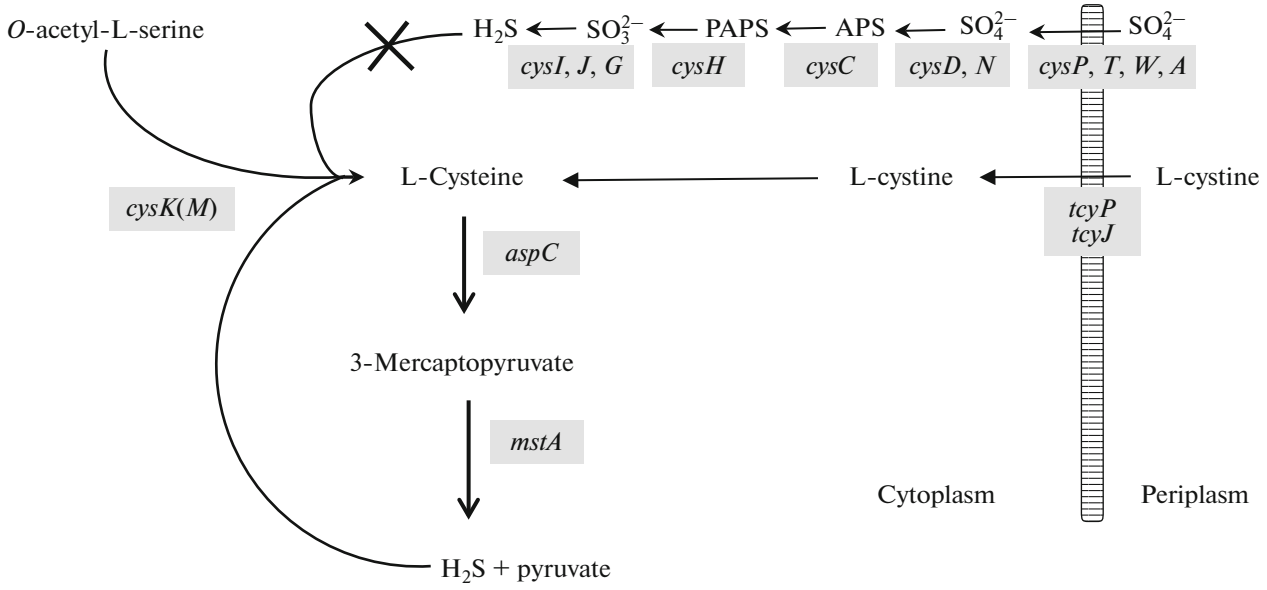

Fig. 1. The scheme of H_2S generation from cysteine with the participation of cysteine aminotransferase enzymes (gene *aspC*) and 3-mercaptopyruvate sulfotransferase (gene *mstA*) and cysteine synthesis from *O*-acetyl-L-serine and H_2S under the control of cysteine synthases (genes *cysK* and *cysM*). In addition, an alternative pathway for the synthesis of H_2S from exogenous sulfate is provided, which is not implemented when growing bacteria on LB medium. The localization of genes *tcyP* and *tcyJ* controlling the transport of cystine from periplasm to cytoplasm is shown.

encoded by gene *mstA* [12]. This enzyme catalyzes the conversion of 3-mercaptopyruvate into pyruvate and H_2S (Fig. 1). In turn, 3-mercaptopyruvate is formed during the transamination of L-cysteine by the cysteine aminotransferase enzyme (gene *aspC*) [13]. As for the synthesis of L-cysteine, it is formed by the condensation of H_2S and *O*-acetylserine in reactions catalyzed by two cysteine synthases under the control of genes *cysK* and *cysM* [3].

To determine the contribution of the endogenously synthesized cysteine to the H_2S generation, deletions of genes *cysK* and *cysM* encoding the synthesis of two cysteine synthases in *E. coli* cells were obtained. It is known that inactivation of gene *cysK* causes auxotrophy of bacteria for cysteine owing to disruption in the cysteine synthesis *de novo* [3]; growth of such bacteria in a complete LB medium is provided by cystine/cysteine contained in it. In addition, it was shown that both cysteine synthases simultaneously possess desulfhydrase activity, that is, the ability to degrade cysteine to produce H_2S [4]. On this basis, it could be expected that inactivation of genes *cysK* and *cysM* would lead to a loss of the ability of bacteria to produce H_2S. Figure 2 shows the results of determining the level of H_2S production in single mutants Δ*cysK* and Δ*cysM* and a double mutant Δ*cysK* Δ*cysM*. As controls in these experiments, we used the previously described strain containing the *mstA* gene deletion with disrupted synthesis of the 3-MST enzyme and characterized by a reduced ability to produce H_2S [1] and the P_{tet}-*mstA* mutant with constitutive expression of gene *mstA* with a high level of H_2S generation [2].

As follows from the data presented in Fig. 2, the Δ*cysM* mutant does not lose the ability to generate H_2S (Fig. 2, *4*), and the Δ*cysK* mutant produces it even in larger quantities (Fig. 2, *3*) than the wild-type strain. The level of H_2S generation in the double mutant *cysK* and *cysM* is even higher and close to that of the strain with constitutive expression of gene *mstA* (P_{tet}-*mstA*) (Figs. 2, *5* and *6*). One possible reason for the increasing capacity of strains with inactivated *cysK* and *cysM* genes to generate H_2S might be an increase in the efficiency of transport of cysteine or its derivatives comprising the LB medium into the cell and their subsequent degradation involving the *mstA* gene product. However, insertion of the *mstA* gene deletion into the genome of the Δ*cysK* Δ*cysM* double mutant resulted in a slight decrease in the ability of such a strain to gener-

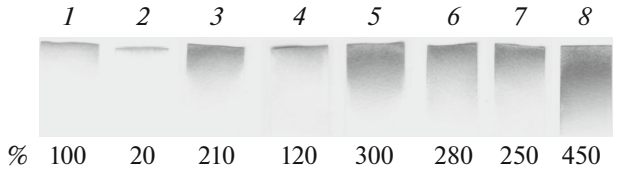

Fig. 2. Generation of H_2S when growing bacteria on LB medium with strains of different genotypes: (*1*) wt; (*2*) Δ*mst*; (*3*) Δ*cysK*; (*4*) Δ*cysM*; (*5*) Δ*cysK* Δ*cysM*; (*6*) P_{tet}-*mstA*; (*7*) Δ*cysK* Δ*cysM* Δ*mstA*; (*8*) Δ*cysK* Δ*cysM* + cysteine (500 μmol).

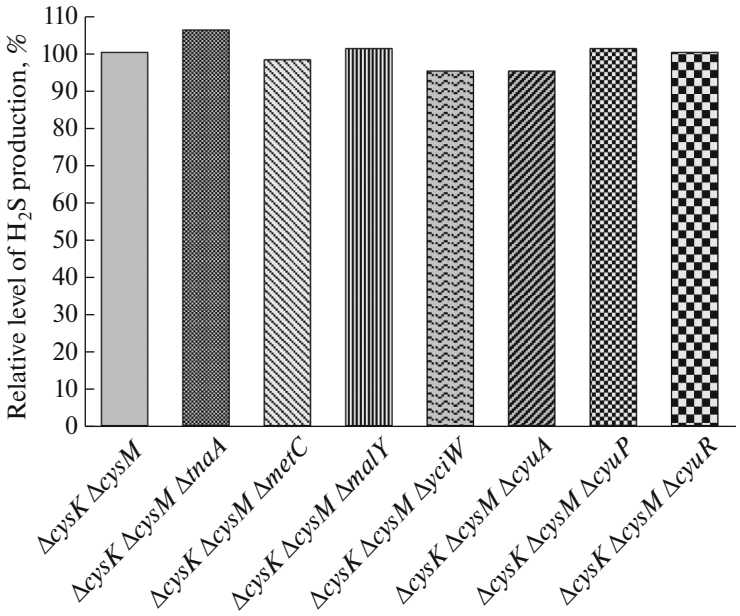

Fig. 3. Inactivation of genes *tnaA*, *metC*, *malY*, *yciW*, *cyuA*, *cyuP*, and *cyuR* does not affect the level of H_2S generation by the Δ*cysK* Δ*cysM* mutant.

ate H_2S (Fig. 2, *7*). At the same time, the addition of exogenous cysteine leads to an even greater increase in the level of H_2S production (Fig. 2, *8*).

Thus, the obtained data make it possible to conclude that the *mstA*-independent cysteine-degrading activity leading to efficient generation of H_2S is revealed against the background of Δ*cysK* Δ*cysM* mutations in *E. coli* cells.

Generation of Hydrogen Sulfide by ΔcysK ΔcysM Mutants Does Not Depend on the Activity of the Known Desulfhydrase Genes

From published data, it is known that, in addition to *cysK*- and *cysM*-encoded cysteine synthases, at least three more proteins possess desulfhydrase activity: tryptophanase (gene *tnaA*), cystathionine-β-lyase (gene *metC*), and maltose transport protein (gene *malY*) [4]. In addition, recently, there have been reports about identification of new genes in *E. coli* involved in cysteine degradation. One of the genes, *yciW*, contains a binding motif for the CysB protein in the regulatory region, and its inactivation leads to the accumulation of intracellular cysteine content [5, 6]. The other two genes, *cyuA* (*yhaM*) and *cyuP* (*yhaO*), controlling the desulfidation of cysteine and its transport, respectively, form an operon that is prone to negative regulation by the CyuR (DecR) protein [7–9]. To determine the possible role of these desulfhydrases in the production of H_2S by the Δ*cysK* Δ*cysM* mutant, the genome of the latter was introduced with deletions of the corresponding genes, and the level of H_2S generation in the obtained triple mutants was checked (Fig. 3).

As shown in Fig. 3, none of the additional deletions affected the ability of the Δ*cysK* Δ*cysM* strain to produce H_2S. Thus, the obtained data allow us to conclude that, against the background of Δ*cysK* Δ*cysM* mutations, a new unknown cysteine-degrading activity starts functioning in *E. coli* cells, leading to efficient generation of H_2S.

The New Cysteine-Degrading Activity Depends on the Transcriptional Regulator CysB

Since genes *cysK* and *cysM* are part of the *cysB* regulon, the effect of the inactivation of genes *cysB* and *cysE* on the generation of H_2S by the Δ*cysK* Δ*cysM* mutant was investigated. Gene *cysB* encodes the synthesis of a transcription factor regulating the expression of a large group of genes involved in the metabolism of cysteine and sulfates and forming the *cysB* regulon [14]. The CysB protein, as a result of allosteric interaction with *N*-acetyl-L-serine, becomes active and activates or represses the transcription of target genes [15]. *N*-acetyl-L-serine is formed in the cell spontaneously from *O*-acetyl-L-serine, which is the product of a serine-acetyltransferase reaction under the control of gene *cysE*. Since the transcription of both genes *cysK* and *cysM* is under the positive control of the CysB protein, inactivation of genes *cysB* and *cysE* results in auxotrophy of the bacteria for cysteine. The results of determining the ability of the *cysK* and *cysM* mutants to produce H_2S after the insertion of deletions of genes *cysB* and *cysE* into their genome are presented in Fig. 4.

As follows from the data presented in Fig. 4 (*3* and *4*), inactivation of genes *cysB* and *cysE* almost completely

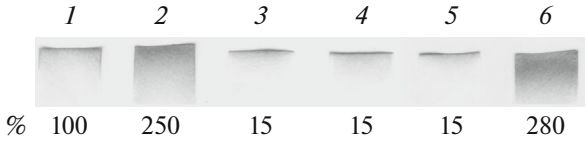

Fig. 4. The effect of inactivation of genes *cysB* and *cysE* on H_2S production by Δ*cysK* Δ*cysM* mutants. The level of H_2S generation is shown when growing bacteria on LB medium with strains of different genotypes: (*1*) wt; (*2*) Δ*cysK* Δ*cysM*; (*3*) Δ*cysK* Δ*cysM* Δ*cysB*; (*4*) Δ*cysK* Δ*cysM* Δ*cysE*; (*5*) Δ*cysK* Δ*cysM* Δ*cysB* + 5 mM *O*-acetyl-L-serine; (*6*) Δ*cysK* Δ*cysM* Δ*cysE* + 5 mM *O*-acetyl-L-serine.

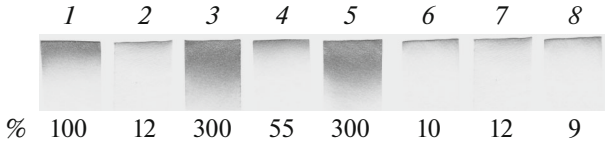

Fig. 5. The effect of cystine/cysteine transporters on H_2S production by Δ*cysK* Δ*cysM* mutants. The levels of H_2S generation are shown when growing bacteria on LB medium with strains of different genotypes: (*1*) Δ*cysK* Δ*cysM*; (*2*) Δ*cysK* Δ*cysM* Δ*tcyP*; (*3*) Δ*cysK* Δ*cysM* P_{tet}-*tcyP*; (*4*) Δ*cysK* Δ*cysM* Δ*tcyJ*; (*5*) Δ*cysK* Δ*cysM* P_{tet}-*tcyJ*; (*6*) Δ*cysK* Δ*cysM* Δ*cysB*; (*7*) Δ*cysK* Δ*cysM* Δ*cysB* P_{tet}-*tcyP*; (*8*) Δ*cysK* Δ*cysM* Δ*cysB* P_{tet}-*tcyJ*.

suppresses the generation of H_2S in the Δ*cysK* Δ*cysM* strains. The addition of exogenous *O*-acetyl-L-serine restores the ability of Δ*cysK* Δ*cysM* bacteria to generate H_2S against the background of the Δ*cysE* mutation (Fig. 4, *6*), but not in the case of inactivation of gene *cysB* (Fig. 4, *5*). The data obtained make it possible to make the following assumptions about the nature of the cysteine-degrading activity that we found. The negative effect of the *cysB* and *cysE* mutations on H_2S production may be due to the fact that expression of the gene encoding an unknown enzyme with desulfhydrase properties is under positive control of the active form of the CysB protein. Another explanation for the negative effect of the *cysB* and *cysE* mutations on H_2S production is based on the assumption that inactivation of these genes blocks the transport of cysteine, the main H_2S precursor, into the cell.

Effect of Cysteine Transporters on H_2S Production by ΔcysK ΔcysM Mutants

To study the effect of the TcyP and TcyJ transporters providing transport of cystine/cysteine from periplasm to cytoplasm of the cell [16, 17] on the ability of the Δ*cysK* Δ*cysM* strains to generate H_2S, deletions of genes *tcyP* and *tcyJ* were introduced into the chromosome of these strains. Since both of these genes are under the control of the CysB protein [19], the Δ*cysK* Δ*cysM* mutants were used to construct additional strains containing deletions of genes *tcyP* and *tcyJ* against the background of the inactivated *cysB* gene. In addition, strains with constitutive Cys-B-independent expression of genes *tcyP* and *tcyJ* were obtained by placing them under the control of the strong P_{tet} promoter. In the obtained isogenic strains, the level of H_2S generation was determined when growing bacteria on a standard LB medium (Fig. 5).

As expected, inactivation of the *tcyP* and *tcyJ* transporters results in a significant suppression of H_2S production because of a decrease in the flow of exogenous cysteine entering the cell (Figs. 5, *2* and *4*), whereas the increase in expression of both transporters under the control of constitutive promoters causes a significant increase in its generation (Figs. 5, *3* and *5*). As noted above, inactivation of the *cysB* gene leads to the suppression of H_2S production, which may be a result of a decrease in the cysteine flux to the cell owing to the low activity of the TcyP and TcyJ transporters controlled by the CysB protein. Earlier, we showed that expression of the *tcyP* gene under the control of the P_{tet} promoter restores *mstA*-dependent generation of H_2S against the background of the *cysB* gene deletion [2]. However, as shown in Fig. 5 (*7* and *8*), the Δ*cysK* Δ*cysM* mutants containing copies of the *tcyP* and *tcyJ* genes under the control of the constitutive *cysB*-independent P_{tet} promoter show no amplification of H_2S production compared to the control strain defective for *cysB*. It follows that the transport of cystine/cysteine into the cell is not a bottleneck for the manifestation and implementation of the new cysteine-degrading activity. On this basis, it can be assumed that the observed dependence of the new cysteine-degrading activity on *cysB* is most likely due to the fact that the transcription of the gene coding for this activity requires activation with the participation of the regulatory protein CysB.

Inactivation of Genes cysK and cysM Leads to Partial Activation of CysB Regulon Genes and Oxidative Stress Protection Genes

Since the strain defective for *cysK cysM* should accumulate not only H_2S but also *O*-acetylserine (Fig. 1), which serves as the precursor of the inductor of the CysB regulon genes, we should expect an increase in their transcription level. By real-time PCR, we compared the level of transcription of several genes that make up the CysB regulon, as well as some genes involved in protecting the cells from oxidative stress, in the Δ*cysK* Δ*cysM* mutant and the wild-type strain. As follows from Fig. 6, the Δ*cysK* Δ*cysM* mutant exhibits an increased level of transcription of genes *cysP*, *tau*, and *tcyP*, which are under positive control of the regulatory protein CysB.

It should be noted that, in addition to the CysB regulon genes, the Δ*cysK* Δ*cysM* mutant shows an approximately 2- to 3-fold increase in the expression of genes *katG*, *sodA*, *arcA*, and *cydD*, indicating an increased level of formation of reactive oxygen species in cells of the Δ*cysK* Δ*cysM* mutants (Fig. 6).

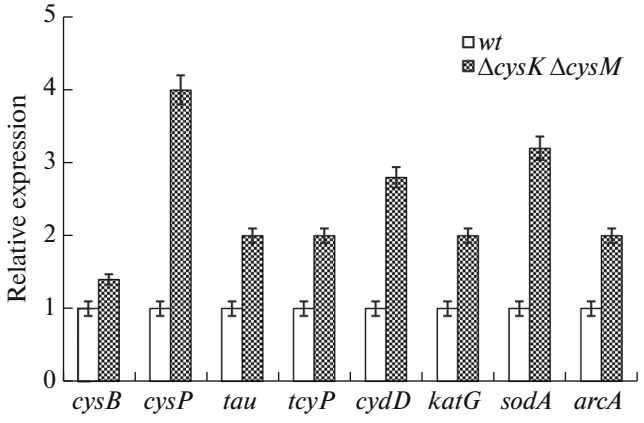

Fig. 6. Increase in the relative level of transcription of several genes in cells of the Δ*cysK* Δ*cysM* mutant.

It is known that the expression of gene *sodA* controlling the synthesis of manganese-dependent superoxide dismutase is activated in response to the formation of the superoxide anion in the cell [19, 20], while the induction of gene *katG* encoding catalase indicates an increase in the level of hydrogen peroxide [21]. These data indicate that the disruption of cysteine resynthesis as a result of the inactivation of the *cysK cysM* genes provokes a state of oxidative stress. The *arcA* gene product is the global regulator responsible for switching the cellular metabolism of bacteria as they move from aerobic to anaerobic growth conditions [22, 23]. Approximately under the same physiological conditions, the expression of terminal cytochrome oxidase *bd*-I is activated, a component of which is the *cydD* gene product [24, 25]. The increase in the expression level of genes *arcA* and *cydD* in the Δ*cysK* Δ*cysM* mutants indicates the important role of cysteine synthases in maintaining the redox balance of the cell and requires further research.

DISCUSSION

It is generally believed that the reduced low-molecular-weight sulfur-containing metabolites (cysteine, glutathione, etc.), along with catalases and dismutases, are the main antagonists of reactive oxygen species (ROS). However, these tools have by now received a serious addition in the form of H_2S, which is formed in cells of microorganisms and eukaryotes in the processes of transulfurization and degradation of cysteine. Earlier, we showed that the degradation of cysteine and the formation of H_2S under the control of *aspC* and *mstA* genes of *E. coli* are effective conditions for inhibiting the most toxic form of ROS, the hydroxyl radical of the Fenton reaction [2]. These ideas about the priority of H_2S as an effective protector against oxidative stress are fully supported by the data of this work. We have studied the paradoxical effect of deletions of genes *cysK* and *cysM* encoding cysteine synthase enzymes: such cysteine auxotrophs actively degrade cysteine to form H_2S. It is noteworthy that the generation of H_2S by the Δ*cysK* Δ*cysM* mutants is carried out without the participation of the canonical enzymes AspC and MstA. We have shown that deletions of any of the known genes controlling the degradation of exogenous cysteine, including the genes *cysK*, *cysM*, *tnaA*, *metC*, and *malY*, as well as the new genes *yciW*, *cyuA*, *cyuP*, and *cyuR*, do not deprive the cysteine auxotrophs Δ*cysK* Δ*cysM* of the ability to degrade cysteine and produce H_2S. Thus, the data testify to the existence of a new cysteine-degrading activity leading to intense production of H_2S. The nature of this activity remains unclear, but the search for an individual desulfhydrase may not be productive, since the total level of H_2S production may be the result of many processes. Since in the growth of the Δ*cysK* Δ*cysM* mutants on the LB medium the only source of H_2S is cysteine of the medium, it can be assumed that the high level of H_2S generation is due to the absence of cysteine resynthesis, which is normally carried out by CysK and CysM cysteine synthases.

An unexpected result of this work is the discovery of the role of the regulatory protein CysB in the cysteine degradation and H_2S production. It turned out that the inactivation of gene *cysB*, which encodes the CysB transcriptional regulator, or gene *cysE*, whose product *O*-acetylserine is necessary for the activation of protein CysB, completely suppresses the H_2S production in the Δ*cysK* Δ*cysM* mutant. Thus, we discovered a new role of the CysB transcription factor as a positive regulator of cysteine degradation with the formation of H_2S. At the same time, the activity of the CysB factor in the cysteine auxotroph leads to an increase in the level of transcription of several genes that make up the CysB-regulon (*cysP*, *tau*, *tcyP*). An important characteristic of the Δ*cysK* Δ*cysM* mutants is an increase in the level of transcription of the genes that protect against oxidative stress (*katG*, *sodA*, *arcA*, and *cydD*). This indicates the involvement of cysteine synthases, especially CysK, in maintaining the cell redox balance, which was not described previously: their inactivation provokes oxidative stress. The paradox of the situation is that the exogenous cysteine, which is necessary to maintain the growth of auxotrophs Δ*cysK* Δ*cysM* and synthesis of the antioxidant glutathione, undergoes intense degradation with the formation of H_2S. From this, it follows that H_2S is a more preferred antioxidant than cysteine and its derivatives and corresponds to our notions about the decisive role of H_2S in protecting cells from oxidative stress [2] and antibiotics [1, 26].

ACKNOWLEDGMENTS

We are grateful to E.A. Nudler for valuable comments in discussing the results of this work.

The results of the studies presented in Figs. 2 and 3 were obtained in the framework of the Program of Fundamental Research of the State Academies of Sci-

ences for 2013–2020 (topic no. 01201363822). The results of the studies presented in Figs. 4–8 were obtained using funds of the Russian Science Foundation (project no. 17-74-30030).

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Translated by K. Lazarev