ISSN 0006-2979, Biochemistry (Moscow), 2006, Vol. 71, No. 2, pp. 185-193. © Pleiades Publishing, Inc., 2006.
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Putrescine as a Modulator of the Level of RNA Polymerase σ^S Subunit in *Escherichia coli* Cells under Acid Stress

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Received February 10, 2005
Revision received March 1, 2005

Abstract—Metabolites accumulated in the culture medium of *Escherichia coli* cells induce expression of the *rpoS* gene encoding the alternative σ^S subunit of RNA polymerase, which controls adaptation of *E. coli* to acid stress during growth in glucose–mineral medium. The effect of acetate and succinate as end products of *E. coli* metabolism has been investigated on the levels of transcription, translation, and σ^S protein stability. These end products mainly influenced the stability of the RNA polymerase σ^S subunit. Under conditions of acid stress caused by acetate addition, the content of polyamines in the cells and medium decreased, whereas artificial *rpoS* gene switch-off by antisense RNA was accompanied by increase in polyamine level. Addition of polyamine to *E. coli* cells treated with acetate and especially with succinate caused a significant concentration-dependent stimulatory effect on *rpoS* expression. Thus, induction of the *rpoS* regulon depends on the combined action of the investigated metabolites determining adequate control of gene expression under conditions of acid stress. A scheme for metabolic pathways describing the role of putrescine in the maintenance of intracellular pH and polyamine pool homeostasis during *E. coli* adaptation to acid stress is proposed.

DOI: 10.1134/S0006297906020118

Key words: polyamines, acid stress, acetate, succinate, *rpoS*, expression

Colonization of the mammalian gastrointestinal tract with commensal and enteropathogenic strains of *Escherichia coli* is possible due to the existence of acid resistance systems in bacterial cells. These systems are responsible for survival of *E. coli* cells at extremely low pH values (1.5-2.0) and in the presence of short chain organic acids. There are three main systems responsible for acid resistance: 1) the oxidative system; its activity depends on the presence of the alternative σ^{38} subunit of RNA polymerase and activity of this system is controlled by cAMP and cAMP receptor protein (CRP); 2) glutamate decarboxylase, and 3) arginine decarboxylase systems [1]. The major consequence of cell exposure to low pH environment consists of acidification of the cytoplasm accompanied by a decrease in membrane electrochemical potential; this may induce energy deficit followed by cell death [2]. A similar effect can also be observed at almost neutral pH in the presence of short chain organic acids. In the undissociated state, they can cross the cell membrane and dissociate with release of protons and weak acid anions under weakly alkaline cytoplasmic pH [3]. The protection of microorganisms against acid stress is based on proton binding in the cytoplasm and subsequent release of the resultant products outside of the cells. The operation of this mechanism simultaneously solves two problems: a) proton neutralization, and b) restoration of membrane electrochemical potential. Proton binding also occurs in the decarboxylation reactions of amino acids, mainly glutamate and arginine [4, 5]. Besides the corresponding amino acid utilizing decarboxylases, these systems of acid resistance also include carrier proteins localized in the cell membrane, and these carrier proteins are responsible for exchange of extracellular substrates for protonated intracellular products of the decarboxylation reactions, γ-aminobutyrate and agmatine.

Organic acids are normal components present in the gut; they are formed during substrate cleavage by the commensal microflora and represent end products of carbohydrate fermentation; in batch cell culture significant amounts of organic acids are formed even under aeration conditions [6]. Weak organic acids, acetate, succinate, lactate, and formate are the end products accumulating in the culture medium of *E. coli*. In aerating defined culture medium (with glucose), such metabolites induce the

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oxidative type of acid response in *E. coli* cells [2]. Analysis of gene expression pattern revealed induction of many genes related to various functional groups (metabolism, transport, adaptation) where genes of the *rpoS* regulon represent a significant proportion [7]. Genes of this regulon are "pooled" together by a common transcription regulator, the alternative σ^{38}- or σ^{S}-subunit of RNA polymerase; under normal conditions of the batch culture of *E. coli* cells the content of this subunit in the cells is very low, but it significantly increases during transition to the stationary phase [8]. The regulon controlled by the σ^{S}-subunit includes genes encoding proteins that represent the adaptation factors to various kinds of stress including osmotic stress, oxidative stress, starvation, etc. This explains why the σ^{S}-subunit is referred to the class of regulators of general stress response. Under conditions of some kinds of stress (e.g., osmotic stress), increase in σ^{S}-subunit followed by *rpoS* regulon induction occurs in the exponential growth phase [9].

Under conditions of acidic stress, the functions of σ^{S}-subunit are not limited to the stationary growth phase, and induction of genes determining acid resistance (*rpoS* regulon genes) is also controlled by various transcription factors in the exponential phase [10, 11]. Complex transcription control of genes constituting this regulon provides their functional differentiation and specific response to various stressors. This occurs due to fine regulation of gene expression, which also involves metabolic factors [7]. For example, under conditions of osmotic shock potassium glutamate plays the role of such regulator [9], whereas under conditions of oxidative stress polyamines are involved in regulation of gene expression [12, 13].

Polyamines are the biogenic polycations with short linear carbon chains (C4-C10) and various number of amino groups (2-4) protonated at physiological pH. Polyamines have been found in many forms of organisms (from viruses to man). In *E. coli* these mainly include putrescine (1,4-diaminobutane) and also spermidine (3-aminopropyl-1,4-diaminobutane) and cadaverine (1,5-diaminopentane). The number of amino groups and the distance between them vary depending on the carbon chain of the polyamine molecule; this determines specificity of its interaction with negatively charged groups of all biopolymers such as nucleic acids and phospholipids [14]. Such interactions explain the effects of polyamines on various cellular processes, and so polyamines are considered as universal cell regulators [15]. There is increasing evidence that polyamines play an important role in adaptation of microorganisms to various kinds of stress [16, 17] including acid stress [18]. A positive role of polyamines in the regulation of acid resistance genes (*gadABC*) is determined by their negative regulation of intracellular concentration of cAMP, inhibiting expression of these genes; however, direct involvement of polyamines in regulation of *rpoS* expression was not demonstrated [18]. Nevertheless, polyamines may exert a concentration-dependent stimulatory effect on *rpoS* expression in batch *E. coli* culture during translation and at the posttranslational level [19].

In the present study, we have investigated the role of polyamines in regulation of gene expression of the *rpoS* regulon under conditions of acid stress induced by the end products of *E. coli* metabolism, acetate and succinate.

MATERIALS AND METHODS

***Escherichia coli* strains.** Table 1 lists the *E. coli* strains used in this study and their genotyping properties and sources.

For determination of *rpoS* gene expression, we used the principle of gene fusions, when the regulatory region and/or gene fragments of various lengths were fused with a promoterless part of a reporter gene (in our case *lacZ* encoding β-galactosidase). It is considered that activity of this enzyme in cells is proportional to expression of the gene studied. The fusion genes were obtained by the transduction method employing bacteriophage λ as a vector [20].

For *rpoS* expression switch-off, we used an *E. coli* strain carrying the pSOPR plasmid with inserted gene of the antisense RNA complementary to the 5′-site of the *rpoS* mRNA responsible for initiation of translation (the start codon and Shine–Dalgarno sequence) [21]. This blocks translation of *rpoS* mRNA. The structural part of the gene was fused with *lac*-promoter and so its expression was induced by adding of the non-metabolizing *lac*-inducer (0.3 mM isopropyl-β-thiogalactoside) to the culture of this strain at early exponential growth phase. This strain also carries a chromosome gene fusion of the target gene of the *rpoS* regulon, *osmY:lacZ*; its expression is controlled by the σ^{S}-subunit. Expression level of this gene reflected effectiveness of *rpoS* switch-off.

Table 1. *Escherichia coli* strains used in the study

Strains	Genotype	Reference
RO200	MC4100(λRZ5:*rpoS742::lacZ*)	[20]
RO90	MC4100(λRZ5:*rpoS379::lacZ*[hybr])	
RO91	MC4100(λRZ5:*rpoS742::lacZ*[hybr])	
MC4100	F^{-}Δ(*arg-lac*) *U169 araD139 rpsL150 ptsF25 flbB5301 rbsR deoC relA1*	
MC4100DE3Y (pSOPR)	MC4100DE3 (*osmY::lacZ*) transformed with pSOPR plasmid	[21]

Cultivation of microorganisms. *Escherichia coli* strains were inoculated into LB-broth containing 25 μg/ml streptomycin. After cultivation for 11 h at 37°C, cells were transferred to M-9 medium containing 25 μg/ml streptomycin and grown in 300 ml of the M-9 medium (using 500 ml flasks) at the same temperature and constant shaking (120 rpm). This culture was then used for subsequent inoculation into 100 ml of the M-9 medium (in 250 ml flasks) containing 0.13% glucose (limiting concentration) or 0.4% glucose (non-limiting concentration) and also the antibiotic, and the culture was cultivated under the same conditions.

The cultural liquid was separated by centrifugation of the isogenic culture at stationary growth phase (14 h) under sterile conditions (16,000*g* for 20 min at 0°C). After addition of various quantities of the supernatant (the cultural liquid), the glucose concentration was adjusted to final concentration of 0.4%.

Acid stress was induced by adding various concentrations of sodium salts of acetate or succinate to the medium.

Biomass was evaluated by absorbance (A_{600}) of the cell culture diluted with physiological saline.

Activity of β-galactosidase was measured in cells pretreated with a mixture of SDS (Sigma, USA) and chloroform by the method of Miller [22].

Polyamine content was determined by a fluorimetric assay. Cell culture (0.5 ml) was centrifuged at 16,000*g* for 1 min. Cells were extracted with 0.4 M $HClO_4$. After pH adjustment to 9.0 with 2 M Na_2CO_3, 100 μl extract was mixed with 100 μl of dansyl-chloride (5-[dimethylamino]naphthalene-1-sulfonyl chloride) (Sigma) in acetone (2.7 mg/ml) and incubated in darkness at 37°C for 2 h. The mixture was evaporated to dryness using an Eppendorf concentrator (Germany) and extracted with benzene. The benzene extracts were quantitatively applied onto silica gel plates for thin layer chromatography (Sorbfil, Russia), 100 × 100 mm, and sequentially separated in two systems: I) benzene–triethylamine (20 : 2 v/v); II) benzene–methanol (10 : 0.45 v/v). The dried plates were photographed using an Olimpus C-3040 digital camera (Japan) in ultraviolet light emitting green-blue fluorescence of dansyl-polyamine spots; sizes of these spots and their brightness were proportional to polyamine concentrations. Concentrations of polyamines were calculated after computer densitometry of photographs using the standard program Adobe Photoshop 5.0.

Statistical treatment of results was carried out using Statistica for Windows 5.0 software (StatSoft, Inc., 1995) in the StatsGraph mode.

The induction degree was expressed as the difference of gene expression (Miller's units) in stressed and control cultures (in % of control).

3D-Graphs were plotted using the mean values of induction degree. Statistical significance of the data (at $p \le 0.05$) evaluated by Student's *t*-test is discussed in the text.

RESULTS AND DISCUSSION

The growth of *E. coli* cells on defined growth medium containing glucose was accompanied by acidification of the medium due to accumulation of metabolic products (Fig. 1). This can be attributed to the fact that the excess of substrate (always presenting in the batch culture) induces its partial fermentation followed by formation of end products even under conditions of intensive aeration (Crabtree effect) [6]. In the aerated defined cultivation medium low pH values or the presence of weakly dissociating short chain organic acids were shown to induce expression of acid resistance genes of *E. coli* (particularly genes constituting the *rpoS* regulon) [2]. Although induction of this regulon occurs mainly at stationary growth phase, similar response was also observed in exponentially growing cells under conditions of acid and osmotic stress [9, 10]. This is consistent with our results on investigation of *rpoS* expression in batch culture in the presence of sodium acetate (Fig. 1). In the control culture there was a single peak of this gene expression, which coincided with transition of the culture to stationary phase, whereas in the presence of sodium acetate there was an additional peak observed in the exponential growth phase. In this phase, acidic stress was the only reason for *rpoS* induction, whereas the increase in expression of this gene in the stationary phase was of multifactor nature. Due to the additive effect of these factors, sodium acetate

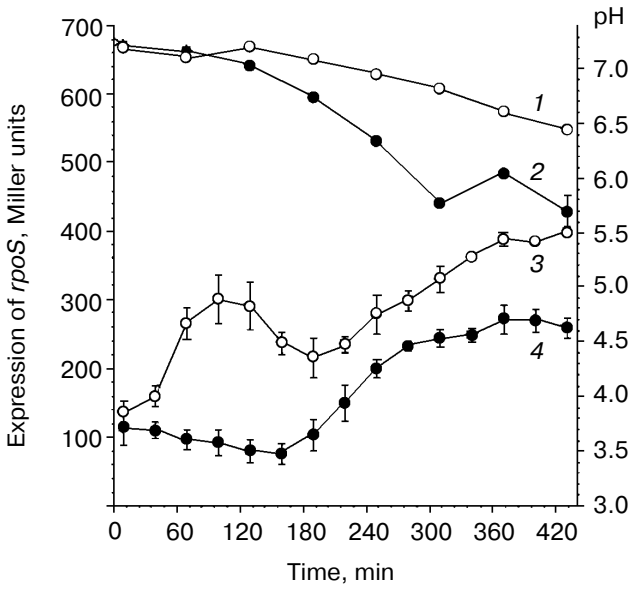

Fig. 1. Dependence of *rpoS* expression on the presence of 50 mM acetate in the cultivation medium of batch *E. coli* RO91 culture: *1*) pH value in the presence of acetate; *2*) pH value in the absence of acetate; *3*) *rpoS* expression in the presence of acetate; *4*) *rpoS* expression in the absence of acetate. Here and in the subsequent figures data represent mean of three independent experiments ($n \ge 3$) ± SD.

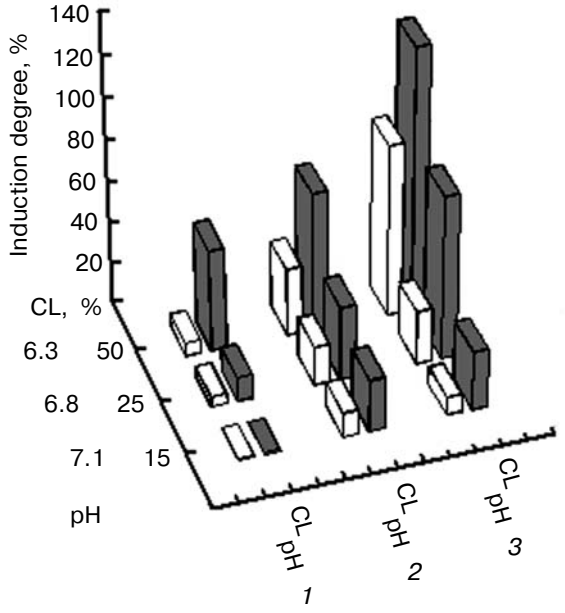

Fig. 2. Effect of metabolites accumulated in the cultivation liquid (CL) of *E. coli* on *rpoS* expression evaluated at the levels of: *1*) transcription (*E. coli* RO200); *2*) translation (*E. coli* RO90); *3*) protein stability (*E. coli* RO91).

added to the medium caused less pronounced changes in the expression level; the expression level became evident only at sufficiently high concentrations (in spite of the logarithmic increase in the amount of non-dissociating form of sodium acetate penetrating at lower pH value [2]).

Regulation of intracellular content of σ^S-subunit is a complex process that occurs at transcription, translation, and posttranslational levels. So the study of each of them involved three various types of gene fusions: one transcriptional (*E. coli* RO200) and two translational (*E. coli* RO90 and *E. coli* RO91) [20]. In the transcriptional fusion the expression of the reporter gene *lacZ* mainly depends on initiation of transcription of the *rpoS* gene promoter and consequently β-galactosidase activity reflects the transcriptional level. In the translational fusion of the first type, *rpoS* gene promoter (*E. coli* RO91) has *lacZ* insertion after base 742, and the sequence responsible for σ^S-subunit stability (the "turnover element") is translated into protein. This results in formation of a hybrid protein, which consists of σ^S-subunit fragment and β-galactosidase; and its stability (as well as β-galactosidase activity) is determined by the turnover element. With this type of fusion, β-galactosidase depends not only on the translation rate but also on protein stability. The second type of the translational fusion (*E. coli* RO90) differs from the first by lack of functional turnover element because the insertion *lacZ* takes place after the 379th base. The expression of this fusion is mainly determined by the rate of translation and is independent of protein stability.

Addition of metabolites (into the culture medium) accumulated in the stationary phase of the batch culture of *E. coli* to the fresh cultivation medium can induce the same effect at exponential phase of the culture growth as acetate; this effect has been observed at all levels of *rpoS* expression (transcription, translation, and posttranslational levels) (Fig. 2). The culture medium added caused pH shift, and this effect was proportional to the amount added; so in control cells we have investigated the effect of the same pH values which were generated in the fresh medium before inoculating the microorganism. The shift of pH caused *rpoS* induction; however, comparison of the effects of culture medium and pH effect revealed they were not identical. At the translational level (*E. coli* RO200), pH decrease did not cause proportional increase of the expression rate, but at the translational (*E. coli* RO90) and posttranslational (*E. coli* RO91) levels there was nearly proportional response. Comparison of the net metabolite effect (after subtraction of the effect of the pH component) on *rpoS* induction at various levels of gene expression revealed that it was maximal at the level of stability of σ^S-subunit and less pronounced at the levels of translation and transcription (Fig. 2). Each of these *rpoS* expression levels is controlled by proteins, RNA, and metabolites; this complex regulation yields adequate content of σ^S-protein and protein products of regulon genes providing cell adaptation to the external medium [8].

Autoclaving of the culture medium before addition to fresh medium did not influence the degree of *rpoS* induction in the exponential growth phase (data not shown). This suggests that such thermostable metabolites

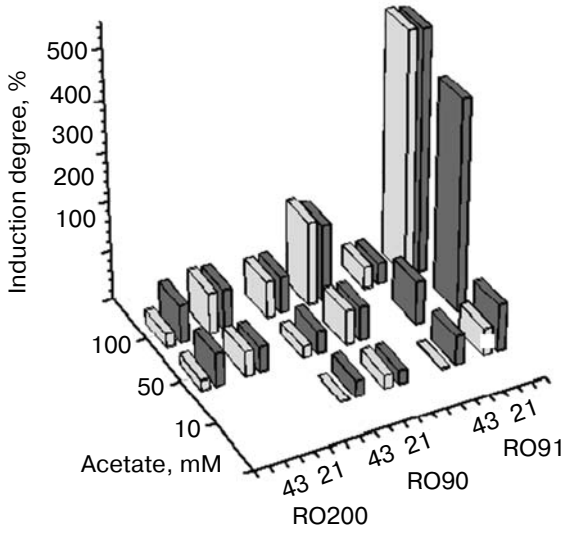

Fig. 3. Effect of acetate on *rpoS* expression in *E. coli* evaluated at the levels of transcription (RO200), translation (RO90), and stability of RpoS protein (RO91): *1*, *2*) exponential growth phase; *3*, *4*) stationary growth phase; *1*, *3*) cell cultures cultivated under glucose limited conditions (0.13%); *2*, *4*) cell cultures cultivated under glucose unlimited conditions (0.40%).

as organic acids (acetate and succinate) and also polyamines are the most probable factors involved in the regulation; the role of polyamines in the regulation of *rpoS* expression in batch *E. coli* culture has already been noted [19].

Study of the role of acetate in the *rpoS* induction revealed its concentration dependent effect at all levels of expression (Fig. 3). The most pronounced induction was found at the posttranslational level; this emphasizes its decisive contribution to regulation of σ^S-content under conditions of acid stress.

At the transcription level differences in the induction degree of exponentially grown cells in glucose limited (0.13%) and glucose unlimited (0.40%) medium were minimal; however, at the stationary phase the maximal induction of starving cells was significantly higher than in the unlimited culture (Fig. 3). These changes may be attributed to difference in cAMP concentration, which exerts negative effect on *rpoS* transcription in the exponential growth phase and positive effect in the stationary phase [23]. Such growth phase-dependent type of regulation is determined by the presence of two cAMP binding boxes in the main promoter of *rpoS*. One box is positioned upstream to this promoter, and it functions at the stationary phase; the other one is positioned downstream from this promoter and may act for suppression of transcription in the exponential growth phase [8]. Higher content of cAMP in the starving cells than in unlimited stationary cultures might explain differences in the induction degree (Fig. 3). A similar but less pronounced tendency was also noted in the stationary phase and both at the translational level and at the level of protein stability because differences in mRNA content influenced both translation process and amount of σ^S-subunit. Multifactorial nature of the peak of *rpoS* expression in the stationary phase (Fig. 1) is the major reason for the less pronounced induction degree of this gene in response to acetate addition than was noted during exponential growth. It is also possible that the value of gene expression in this particular phase induced as the resultant effect of all inducing factors reaches its physical maximum. Near this maximum, any effects on the level of this gene expression become less pronounced and reproducible.

It is generally accepted that the σ^S-dependent glutamate decarboxylase system is the main mechanism responsible for cell protection under conditions of acid stress; activity of this system is strictly controlled by and depends on intracellular cAMP content [10]. Amino acid decarboxylation is considered as one of the mechanisms protecting microorganisms against acid stress, because it is accompanied by incorporation of cytoplasmic protons into the reaction products and therefore this mechanism contributes to homeostasis of intracellular pH [24]. Decarboxylation reactions of such amino acids as ornithine and arginine leading to putrescine formation also play a key role in polyamine biosynthesis. So we investigated the role of polyamines in adaptation of *E. coli* cells to acid stress.

It has been previously shown that these compounds are involved in regulation of *rpoS* expression in batch *E. coli* cultures and the effects depend on their intracellular content [19]. So we postulated the involvement of polyamines in the response to acid stress; such involvement would not be limited by their role as end products of decarboxylation reactions, but might also include indirect regulation of acid response via *rpoS*-dependent protective mechanisms.

Acid stress-induced changes of intracellular content of polyamines may be considered as evidence for their involvement in protective mechanisms against stress (Fig. 4). There is a reverse dependence of putrescine concentration in the cell and in the cultivation medium on *rpoS* induction in the exponential growth phase reflecting potency of the stress treatment. Under these conditions, the decrease in total putrescine pool (cell + medium) in *E. coli* cultures suggests its catabolism during adaptation to acid stress; induction of the putrescine catabolism operon, *gabDTPC*, observed under some conditions seems to support (indirectly) the possibility of nonspecific induction of this operon [25]. Subsequent studies revealed that genes of this operon are components of a larger operon, *csiD-ygaF-gabDTP*, its expression being controlled by two σ^S-dependent promoters; consequently, it can be induced under various stress situations (including acid stress), which increase intracellular content of σ^S-subunit [26]. If this is correct, *rpoS* regulon

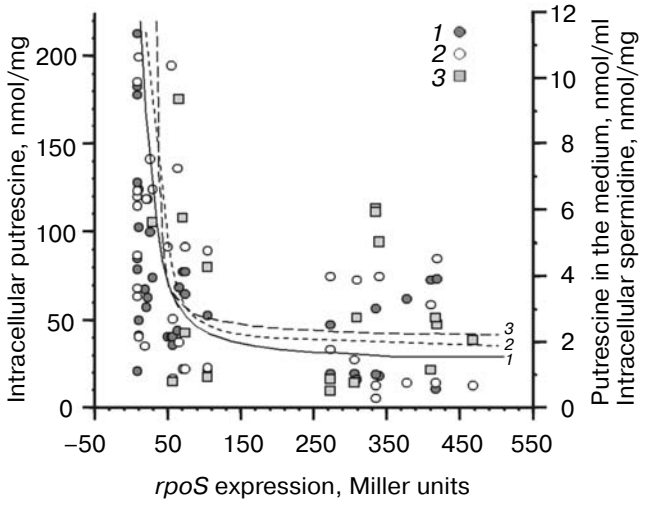

Fig. 4. Dependence of intracellular polyamine content on the level of expression of *E. coli rpoS*: *1*) intracellular putrescine; *2*) extracellular putrescence; *3*) intracellular spermidine. The significance of negative correlation between *rpoS* activity and putrescine content in the cell ($r = -0.4625$) and in the medium ($r = -0.4859$) and also spermidine in the cell ($r = -0.4927$) has been shown at $p \le 0.05$. Intracellular polyamine content was calculated per unit of absolutely dry biomass.

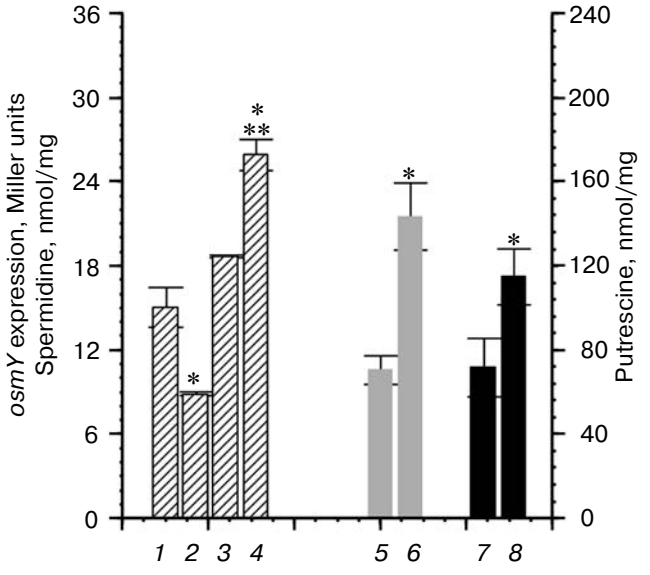

Fig. 5. Effect of *rpoS* switch-off on the level of *osmY* expression (*1-4*) and intracellular content of polyamines (*5-8*) in exponentially growing *E. coli* cells: *1*) control; *2*) *rpoS* switch-off; *3*) control + 5 mM putrescine (PT); *4*) the same + 10 mM PT; *5*, *6*) putrescine in control (*5*) or during *rpoS* switch-off (*6*); *7*, *8*) spermidine in control (*7*) or during *rpoS* switch-off (*8*). All calculations are expressed per unit of absolutely dry biomass. Statistical difference was evaluated using unpaired Student's criterion at $p \leq 0.05$: *, significance of differences between control and experiment; **, significance versus previous variant of the experiment.

switch-off should be accompanied by the increase in intracellular putrescine content due to lack of enzymes of its catabolism (representing protein products of *gabDTP*).

The *rpoS* expression switch-off by means of the antisense RNA complementary to the 5′-end of *rpoS* mRNA was carried out using induction of the gene engineering construct, which consisted of the gene of antisense RNA fused with *lac*-promoter and positioned within pSOPR plasmid using non-metabolizing inductor of *lac*-promoter, isopropyl-β-thiogalactoside [21]. The switch-off was controlled by a decrease in expression level of *osmY*, the target gene of the *rpoS* regulon (Fig. 5). Addition of the inducer to *E. coli* culture at early exponential phase was accompanied by significant increase in putrescine and spermidine pools and simultaneous decrease in *osmY* expression compared with the corresponding control. The latter indicates correctness of our hypothesis. At the same time, addition of putrescine to the control culture caused concentration-dependent increase in expression of the target gene; this suggests stimulatory effect of this diamine on the *rpoS* activity. Thus, the decrease in putrescine content in response to acid stress and its increase at the switch-off of the *rpoS* regulon (which contains genes of putrescine degradation) suggests involvement of polyamines in the mechanisms of protection of *E. coli* cells against acid stress.

Study of the putrescine effect on *rpoS* expression under conditions of acetate stress revealed that its transcription remains unchanged, but there was a tendency for some stimulation of translation, and putrescine also caused some stimulation of protein stability (Fig. 6). Thus, putrescine addition under conditions of acid stress may induce genes of its own catabolism: this occurs due to positive regulation of σ^S-subunit content. Protein products of these genes are involved in the regulation of intracellular pH and maintenance of polyamine homeostasis in the cells.

The key intermediate of putrescine degradation is γ-aminobutyrate, which is formed also in the glutamate decarboxylase reaction [26]. Putrescine participates in pH regulation indirectly, via γ-aminobutyrate, which is converted into succinate in the transamination reaction. Succinate, the end metabolite of *E. coli*, is accumulated in the medium; it may cause (as well as acetate) acid stress.

The study of *E. coli* cell response to the addition of succinate to the cultivation medium revealed a significant concentration-dependent effect of succinate on the *rpoS* expression in the exponential phase preferentially at the level of stability of the σ^S-subunit (Fig. 7). Under conditions of succinate-induced acid stress, putrescine significantly stimulated *rpoS* induction at this level (Fig. 8) without significant influence on transcription and translation of this protein (data not shown). Since succinate is the end product of putrescine degradation, it is relevant to suggest that succinate formation is primarily responsible

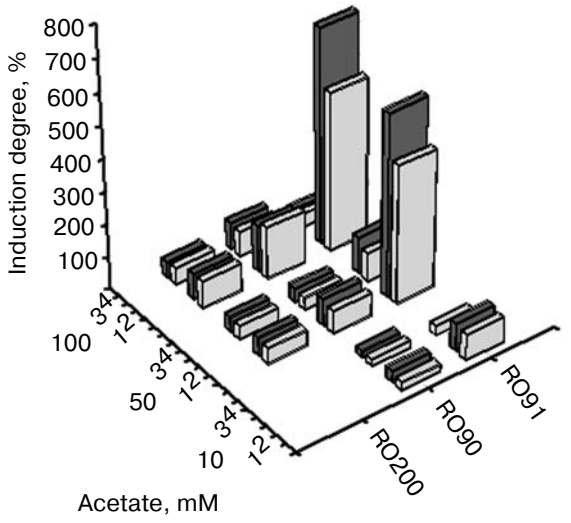

Fig. 6. Effect of putrescine (PT) on expression of *E. coli rpoS* evaluated by transcription (RO200), translation (RO90), and RpoS protein stability (RO91) under conditions of acid stress. Induction degree of *rpoS*: *1*, *2*) at the exponential phase; *3*, *4*) stationary phase; *1*, *3*) without PT additions; *2*, *4*) addition of 5 mM PT.

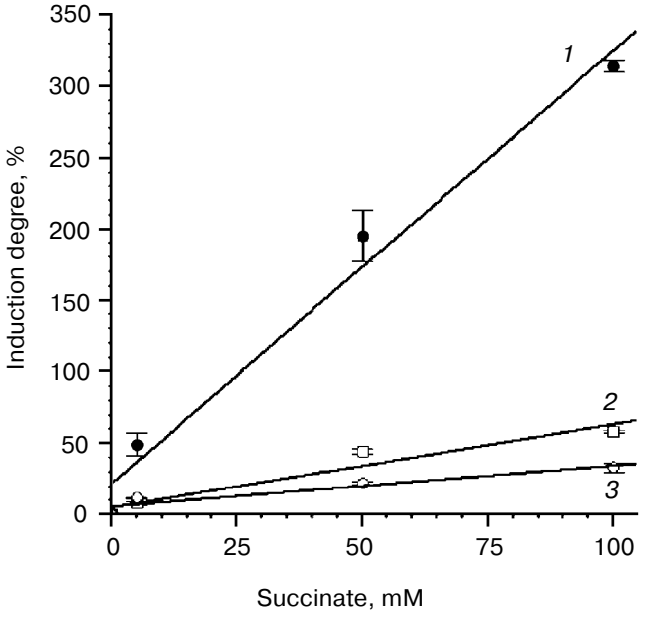

Fig. 7. Dependence of *rpoS* induction degree evaluated at protein stability (*1*), translation (*2*), and transcription (*3*) on succinate concentration in the medium of exponentially growing culture of *E. coli* cells.

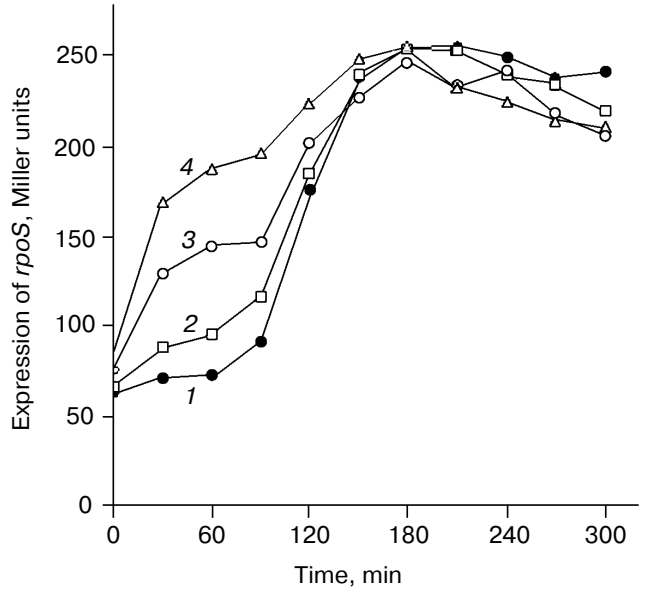

Fig. 8. Role of putrescine (PT) in the regulation of *rpoS* expression evaluated as RpoS protein stability under conditions of acid stress induced by succinate addition. The level of *rpoS* expression: *1*) control (without additions); *2*) in the presence of 5 mM succinate; *3*) 5 mM succinate + 5 mM PT; *4*) 5 mM succinate + 10 mM PT.

for mediation of the putrescine effect. However, comparison of proportionality of responses to the addition of these compounds revealed that in the presence of succinate putrescine caused more potent expression of *rpoS* than the independent effect of each compound added separately (Table 2). This suggests a regulatory nature of the putrescine effect; its regulatory potential as a transcriptional regulator has been described earlier [13]. However, it should be noted that putrescine-stimulated stability of σ^S-subunit was significantly higher under conditions of succinate stress (Table 2) than under conditions of acetate stress (Fig. 6). Perhaps these differences reflect structural features of these compounds. In fact, putrescine and succinate share the same length of carbon chain, which consists of four carbon atoms; these compounds also share symmetry of positioning of the terminal groups. It is also possible that this may underlie potent additive effect of these compounds on *rpoS* expression. The two-carbon structure of acetate (which significantly differs from putrescine and succinate) might explain the less effective influence of acetate on gene expression observed on combined addition with putrescine. Thus, the induction of the *rpoS* regulon is determined by the combined effect of all the metabolic factors determining adequate regulation of the gene expression under acid stress.

The level of σ^S-subunit stability in the cell is determined by competitive interactions of many factors of peptide and non-peptide nature [27]. Short half-lifetime of σ^S-subunit (1-3 min) seen under normal conditions of development of *E. coli* cells in the minimal medium is attributed to the presence of so-called "turnover element" in the protein molecule. Binding of phosphorylated form of response regulator protein RssB at this element is a signal for involvement of ATP-dependent ClpXP protease in the proteolytic degradation of σ^S-subunit. If σ^S-subunit binds the RNA polymerase coreenzyme before RssB, it becomes inaccessible to proteases. This may be observed when σ^S-subunit content

Table 2. Involvement of putrescine in the regulation of *rpoS* expression at the level of protein RpoS stability during acid stress induced by succinate

Addition	Degree of *rpoS* induction*, %
Putrescine, 5 mM	17.30 ± 3.1
Succinate, 5 mM	41.86 ± 5.9 **
5 mM putrescine and 5 mM succinate	88.87 ± 9.1 ***

Note: Data represent mean of independent experiments ($n \geq 3$) ± SD. Significance of changes was evaluated using unpaired Student's *t*-test; differences were considered as statistically significant at $p \leq 0.05$.
* Increase of expression versus control.
** Significance of differences between control and experiment.
*** Significance of differences versus previous variant of the experiment.

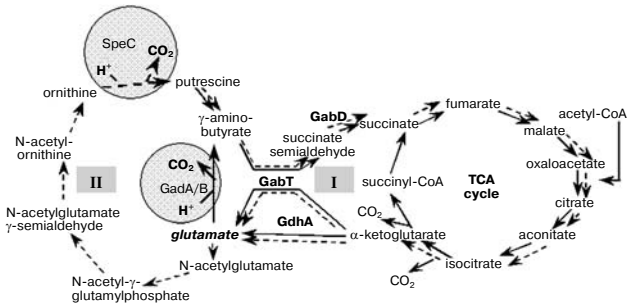

Fig. 9. Scheme showing involvement of catabolism and biosynthesis of putrescine in the metabolic pathways of adaptation of *E. coli* to acid stress. Solid arrows show cycle I (I), and dashed arrows show cycle II (II). All explanations are given in the text.

sharply increases as the result of increased protein biosynthesis (e.g., under conditions of hyperosmotic stress [9]) or increased affinity to RNA polymerase core enzyme induced by interaction with some factor such as ppGpp (under conditions of stringent response) or potassium glutamate (osmotic shock). Since acid stress is accompanied by increased *rpoS* expression assessed by σ^S-subunit stability (Fig. 3), it is relevant to suggest that the combined effect of putrescine and organic acids may be related with increased affinity of σ^S-subunit to RNA polymerase core enzyme as in the case of ppGpp [28].

On the basis of results of these studies and the literature data [19, 26], we propose the scheme illustrating metabolic pathways of adaptation to acid stress of microorganisms grown in mineral medium containing glucose (Fig. 9).

Glutamate plays the central role in adaptation; its decarboxylation catalyzed by two isoenzymes of glutamate decarboxylase (GadAB) is accompanied by binding of two cytoplasmic protons and formation of γ-aminobutyrate, an intermediate of putrescine catabolism [25]. In the absence of exogenous glutamate in the mineral cultivation medium, acid stress causes RpoS induction controlled by various factors including genes encoding enzymes of putrescine degradation, such as GabT and GabD and others. Protein products of these genes catalyze γ-aminobutyrate transamination followed by formation of glutamate from α-ketoglutarate and dehydrogenation of succinate semialdehyde yielding succinate. Succinate may be further metabolized in the tricarboxylic acid cycle, and one of its intermediates, α-ketoglutarate, closes the first cycle of proton binding (cycle I), forming glutamate. (This involves GabT or glutamate dehydrogenase; GdhA.)

Glutamate also functions in the other cycle of proton binding (cycle II). The latter differs from cycle I by the presence of an additional route, putrescine biosynthesis. The final step of putrescine biosynthesis catalyzed by ornithine decarboxylase (SpeC) is accompanied by cleavage of CO_2 and proton binding. This pathway is linked to cycle I by γ-aminobutyrate. In the case of significant *rpoS* induction under conditions of acid stress, this is accompanied by a decrease in putrescine content in cells and in the medium (Fig. 4) followed by reduction of biosynthetic processes and cell growth [14]. Results of artificial *rpoS* expression switch-off, which have demonstrated the increase in putrescine pool due to *rpoS*-dependent catabolic pathway switch-off (Fig. 5), support this interpretation.

Such metabolic pathways regulate intracellular pH; they also function as opposite regulatory components controlling putrescine homeostasis in the cell. At high cytoplasmic content putrescine acts as negative feedback regulator of enzymes involved in its biosynthesis; putrescine also plays the role of positive regulator of expression of the *gadA/B* genes by reducing concentration of their negative regulator, cAMP [18]. This results in the involvement of glutamate into cycle I and decrease in polyamine biosynthesis. This is accompanied by preferential decarboxylation of glutamate (instead of ornithine) with insignificant influence on adaptation processes to acid stress. In the case of decreased putrescine content in the cell, the reverse processes take place; this should result in the decrease in *gadA/B* expression and involvement of glutamate in putrescine biosynthesis.

Authors are grateful to Regina Hengge (Berlin University, Germany) for the generous gift of *E. coli* strains RO200, RO90, and RO91, and to Herb Schellhorn (MacMaster University, Hamilton, Canada) for the generous gift of *E. coli* strain MC4100DE3Y(pSOPR).

This work was supported by grants from the Russian Foundation for Basic Research (No. 05-04-48091 and No. 04-04-96062).

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