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Role of Putrescine in Regulation of the σ^S Subunit of RNA Polymerase in *Escherichia coli* Cells on Transition to Stationary Phase

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Abstract—Expression of the *rpoS* gene encoding the σ^S subunit of *E. coli* RNA polymerase was studied on transition to the stationary phase and under conditions of starvation. Polyamines, in particular putrescine, are involved in regulation of the *rpoS* expression by concentration-dependent stimulation at the levels of translation and stability of the protein σ^S. The stimulatory effect of exogenous putrescine inversely depended on its content in the cell and was the most pronounced in the beginning of the exponential growth. Possible mechanisms of the polyamine effect on the *rpoS* expression on post-transcriptional and post-translational levels are discussed.

Key words: polyamines, putrescine, stationary phase, starvation, *rpoS*, σ^S, expression, transcription, translation, protein stability

Existence of microorganisms in their natural environments is associated with constant changes in environmental conditions. Variously combined, these conditions provoke a response of the cell, which finally results in metabolic changes providing the best adaptation of the cell to the real situation. An adequate response to numerous signals entering the cell is based on various regulatory mechanisms, which form a sophisticated regulatory network with regulons as its basic elements. Genes which compose regulons are integrated into a system by transcriptional regulators (such as OxyR, SoxRS) or σ subunits of RNA polymerase which specifically bind to promoter or adjacent regions of target genes and thus stimulate their expression.

In *E. coli*, seven different species of the σ subunit of RNA polymerase have been described, and each of them recognizes a corresponding promoter [1]. The number of molecules and affinity of different σ subunits for the core enzyme of RNA polymerase are regulated by environmental factors, and this finally determines their inclusion into the holoenzyme interacting with the corresponding target genes of one or another regulon [1]. Under varied conditions, a vegetative σ^D is quantitatively predominant and determines the expression of the majority of genes responsible for basic metabolism of the cell. The content in the cell of any alternative σ subunit can significantly increase in response to specific environmental signals. And the regulation of the level of an individual σ subunit seems also to include convergence of different signals. In this connection, special attention should be given to the σ^S subunit, which controls expression of more than 50 genes responsible for the most significant functions in the course of *E. coli* adaptation for transition to the stationary growth phase [2].

Studies on the role of this subunit in regulation of metabolism of *E. coli* and its analogs in other microorganisms are now of increased interest because functions of this subunit concern not only the stationary growth phase, but also resistance to various stresses. It has been found that the σ^S subunit plays a significant role in response of *E. coli* exponential cultures to osmotic and acidic shocks, oxidative stress, and other exposures [2]. Because of its wide functional range, σ^S has been classified as one of the main regulators of *E. coli* metabolism. The response of σ^S to many signals is regulated by a very complicated system that involves many various factors, including normal products of the cell metabolism, in particular, polyamines.

Polyamines (putrescine, cadaverine, spermidine, spermine) are aliphatic cations, which are present in

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nearly all living organisms and necessary for normal cell growth [3]. Their involvement in various biosyntheses, first of all in biosynthesis of protein and nucleic acids, allowed these compounds to be referred to as universal cell regulators [4].

Many works have been recently published which show a significant role of polyamines in adaptation of microorganisms to various stresses [5-8]. In particular, these compounds are shown to modulate gene expression under conditions of oxidative stress [9-11]. The σ^S subunit is involved in the control of multiple stress resistance, and there is a question about the possible role of polyamines in the regulation of its level under stress conditions, in particular, associated with transition to the stationary phase. Therefore, attention should be given to the few works that have shown the stimulatory effect of polyamines on initiation of translation due to their influence on the secondary structure of mRNA [8] and also on binding of the initiation codon to the initiation tRNA that increased the synthesis of adenylate cyclase and, as a result, the content of the σ^F subunit of RNA polymerase in the cell [7]. Using polyamine-dependent mutants, the same authors have shown that polyamines stimulated the termination of translation of mRNA of the σ^S subunit, which could increase the content of this subunit in *E. coli* cells. However, this effect occurred only in the case of replacement of a base in the termination codon and was not recorded in wild type cells with undisturbed synthesis of polyamines [12].

It is also known that the content of polyamines in wild type *E. coli* cells and in the environment markedly fluctuates under conditions of periodic culture [13], and this should affect phase-related changes in the σ^S level in the cells. And the problem of the possible regulatory effect of polyamines on stability level of the σ^S protein, which often determines the amount of this subunit in the cell, is still untouched. In *E. coli* periodic culture, phase-related changes in the intensity of transcription of the *rpoS* gene encoding σ^S are studied insufficiently, whereas this gene significantly determines the final content of its product in the cell, and the effect of polyamines on this process is quite intact.

The stationary phase in microorganisms can be conditioned by at least two qualitatively different physiological states, insufficiency of nutrition sources and high density of cells in the bacterial population. In the latter case, accumulation of final products of metabolism, changes in pH, oxygen deficiency, etc. are observed in the liquid culture, and these events mainly cause growth termination and transition of the cells to the stationary phase [2].

The purpose of the present work was to study the role of polyamines in regulation of expression of the *rpoS* gene on the levels of transcription, translation, and stability of the protein depending on the growth phase of *E. coli* culture and also in the course of transition to starvation.

MATERIALS AND METHODS

Biological materials. Strains of *E. coli* used in the present work are listed in Table 1 with data on their genotypic features and sources.

To determine the expression level of the *rpoS* gene, the principle of gene fusion was used when the regulatory region and/or fragments of different length of the gene under study were fused with the promoter-deprived part of the reporter gene, and in our case this role was played by *lacZ* encoding β-galactosidase. The activity of this enzyme in the cells is believed to be proportional to the expression level of the gene under study. The gene fusions were obtained by transduction, using bacteriophage λ as a vector [14].

Culture of microorganisms. Before the experiment, *E. coli* strains stored on a slant LB agar were inoculated into LB broth containing streptomycin (25 μg/ml). After culture for 11 h in a thermostat at 37°C, the cells were transferred onto M-9 medium containing the antibiotic in the same concentration and grown for 13 h in a 500-ml flask with 300 ml of medium M-9 on a thermostatted shaker (120 rpm). The grown culture was used as inoculate into 250-ml flasks containing 100 ml of medium M-9 with glucose in the limiting concentration of 0.13% or in the non-limiting concentration of 0.4% and also with the antibiotic and grown under the same conditions.

The cell biomass was estimated after a preliminary dilution of the culture in saline by optical density (OD_{600}) using an SF-46 spectrophotometer (LOMO, Russia).

Activity of β-galactosidase was determined in cells pretreated with a mixture of SDS (Sigma, USA) and chloroform by the method of Miller [15].

Contents of polyamines. The concentration of polyamines was determined fluorometrically. The culture aliquots (500 μl) were centrifuged for 1 min at 16,000*g*. The cells were extracted with 0.4 N $HClO_4$. The $HClO_4$-extract (100 μl) was adjusted to pH 9.0 with 2 M Na_2CO_3 and supplemented with 100 μl of dansyl chloride (1-dimethylamino-1-naphthalene-5-sulfonyl chloride, Sigma) in acetone (2.7 mg/ml) and incubated in the dark for 2 h at 37°C. The mixture was evaporated under a flow of

Table 1. Bacterial strains used in the present work

E. coli strain	Genotype	Reference
RO200	MC4100(λRZ5:*rpoS*742::*lacZ*)	[14]
RO90	MC4100(λRZ5:*rpoS*379::*lacZ*[hybr])	
RO91	MC4100(λRZ5:*rpoS*742::*lacZ*[hybr])	
MC4100	F^-Δ(*arg-lac*)*U169 araD139 rpsL150 ptsF25 flbB5301 rbsR deoC relA1*	

cold air and extracted with benzene. The benzene extracts were quantitatively placed onto Sorbfil (Russia) silica gel plates (100 × 100 mm) for thin layer chromatography and separated successively in two systems of solvents: I) benzene–triethylamine (20 : 2); II) benzene–methanol (10 : 0.45) The dried chromatograms were photographed using an Olympus C-3040 digital camera (Japan) in ultraviolet light which excited blue-green luminescence of dansyl-polyamine spots, the size and brightness of which were proportional to their concentration. The polyamine concentration was calculated by results of computerized densitometry of photograms using the standard Adobe Photoshop 5.0 program.

Results were processed statistically using the computer program Statistica for Windows 5.0 (StatSoft, Inc., 1995) in the StatsGraph regimen. Statistical figures and Table 2 present mean data from a series of similar experiments (not less than three), and vertical segments show values of mean square deviations.

RESULTS AND DISCUSSION

The σ^S content in the cell can be regulated on the levels of transcription, translation, and stability of the protein. To study every level, in the present work three types of gene fusions are used: the transcriptional one (*E. coli* RO200) and two translational fusions (*E. coli* RO90 and *E. coli* RO91). In the transcriptional fusion expression of the reporter gene *lacZ* mainly depends on initiation of transcription from the gene *rpoS* promoter; therefore, the activity of β-galactosidase characterizes the level of transcription. With the first type translational fusion (*E. coli* RO91), the gene *rpoS* had an insertion *lacZ* after base 742, and the sequence responsible for determination of the σ^S stability (the turnover element) was retained in the protein. As a result of expression of this fusion, a hybrid protein was produced consisting of the σ^S fragment and β-galactosidase, and the stability of this protein (and correspondingly, the activity of β-galactosidase) depended on conditions of the turnover element. The activity of β-galactosidase in the fusion of this type depended not only on the rate of translation but also on the protein stability. The second type of the translational fusion (*E. coli* RO90) was different from the first type by absence of the functional turnover element because of the *lacZ* insertion after the base 379. And expression of the fusion was mainly determined by the rate of translation and did not depend on the protein stability.

The expression level of three types of gene fusions was studied in *E. coli* periodic cultures. Transition to the stationary phase was studied in the course of culture to the maximal density under conditions of excess nutritional components in the medium or of limited provision with carbon and energy (starvation).

The *rpoS::lacZ* expression was activated on the level of transcription, which was determined by beginning of a stable increase in the activity of β-galactosidase even during the early exponential phase of the periodic culture of *E. coli* RO200, approximately at the optical density $OD_{600} = 0.5$ and the maximal specific rate of the growth (Fig. 1). These data are consistent with the finding that induction of the *rpoS* translation is controlled by an unusual sensory histidine kinase BarA, for which acetate accumulated in the culture even during the early exponential phase seems to be a signal [16]. The *rpoS::lacZ* expression in the unlimited culture flattened out approximately concurrently with transition to the stationary phase, and on transition to starvation its level continued to increase to values near to those of unlimited cultures (Fig. 2). The transcriptional activation of *rpoS* in our experiments with synthetic medium remained relatively

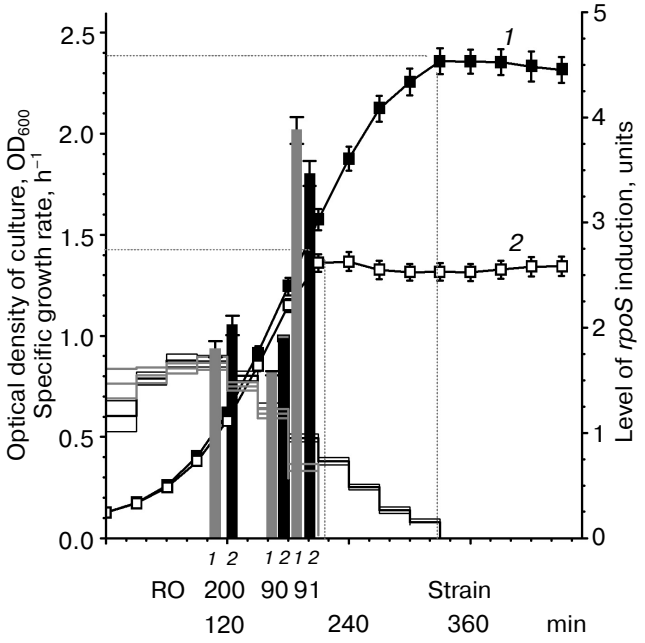

Fig. 1. Activation of the transcriptional (*E. coli* R0200) and translational (*E. coli* RO90 and *E. coli* RO91) gene *rpoS* fusions on transition of the culture to the stationary phase and starvation. *1*, *2*) Optical density (OD_{600}) of the unlimited (*1*) and limited culture (*2*). Broken lines show the mean specific growth rate ± *m* in the unlimited (dark line) and in the limited (light line) *E. coli* culture. Columns show the degree of activation in the limited (*1*) and unlimited (*2*) culture. Position of the columns with respect to the abscissa axis and their intersection points with the curves of OD_{600} and the specific growth rate characterize the corresponding time and physiological parameters of the culture at the start of activation of expression of the gene *lacZ* fusions. The degree of activation is the ratio of the maximal gene expression (Miller's unit) at the given point of the culture growth to its initial level. The microorganisms were grown on a thermostatted shaker (see "Materials and Methods") on medium M-9 supplemented with 0.4% glucose (unlimited culture) or 0.13% glucose (limited culture).

Table 2. Parameters of the *rpoS::lacZ* activation in *E. coli* periodic culture on transition to the stationary phase and starvation

Parameters of *rpoS* activation	Addition of putrescine, 5 mM	Content of glucose in the medium, %	*E. coli* strain		
			RO200	RO90	RO91
Beginning of activation, minutes before cell entry into stationary phase	—	0.13	105 ± 29	35 ± 9	18.5 ± 5.1
		0.4	225 ± 26	156 ± 17	133 ± 7
	+	0.13	105 ± 15	42 ± 7	22 ± 7
		0.4	225 ± 15	135 ± 9	135 ± 11
Degree of activation of the *rpoS::lacZ* gene fusion, units	—	0.13	1.8 ± 0.1	1.6 ± 0.0	3.9 ± 0.1
		0.4	2.0 ± 0.1	1.9 ± 0.0	3.5 ± 0.1
	+	0.13	1.6 ± 0.0	1.8 ± 0.0	4.5 ± 0.1
		0.4	1.8 ± 0.0	1.8 ± 0.0	5.7 ± 0.5
Effect of putrescine, %		0.13	absent	12.1*	17.0*
		0.4	absent	absent	64.5*

Note: The stimulatory effect of putrescine was calculated by mean values of the *rpoS* activation; asterisks indicate significance of data at $p < 0.05$.

low and was, on average, twofold higher than the starting values (Table 2). In complete nutritional media the expression of transcriptional fusions increased five-tenfold [17], and such media were used in the majority of studies on this problem. The few data obtained with synthetic nutritional media are contradictory and suggest either an insignificantly increased induction of transcriptional fusions, or its absence on transition of cultures to the stationary phase [18]. Data on putative factors involved in regulation of the gene *rpoS* expression on the level of transcription are similarly discrepant. In particular, the role of cAMP is unclear, which is thought either to inhibit or stimulate the *rpoS* transcription [2]. In our experiments the exhaustion of glucose in *E. coli* exponential cultures, which suggested an increase in cAMP in the cells not only failed to decrease the expression of the transcriptional fusion, but was accompanied by its further increase (Fig. 2).

Although polyamines are known modulators of transcription [9, 11], putrescine was not found to cause any significant change in expression of the *E. coli* gene *rpoS* on the level of transcription on transition to the stationary phase and starvation (Table 2). However, considering the possible role of polyamines as regulators of the gene expression on the level of translation by stimulating the synthesis of adenylate cyclase [8] or influencing the secondary structure of mRNA [7], we studied the role of polyamines in regulation of the σ^S biosynthesis on the levels of translation and stability of the protein. These studies were reasonable because mRNA of the *rpoS* contains a secondary structure which includes the initiation codon [2] and also because of the complete absence of data on the possible role of polyamines in regulation of the σ^S stability.

Based on parameters describing physiological conditions of the cells (OD_{600}, μ), the start of activation of the *rpoS::lacZ* expression for both strains should be assigned

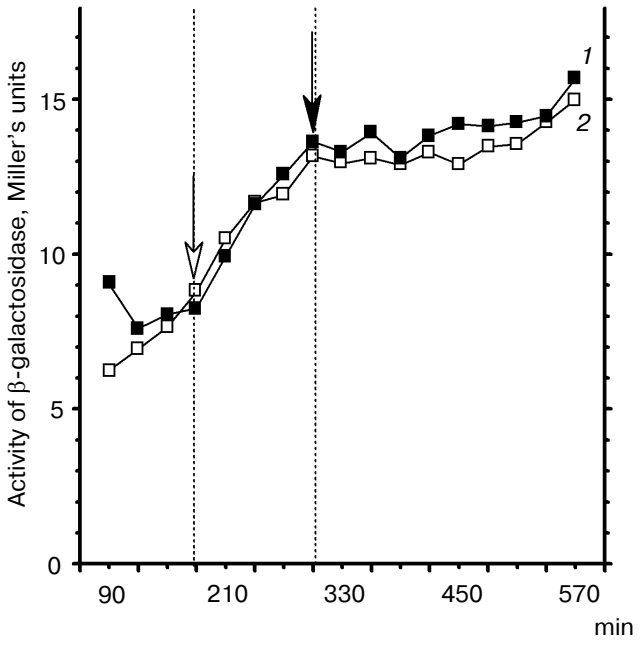

Fig. 2. Change in the expression level of the *rpoS* transcriptional fusion in the unlimited (*1*) and limited (*2*) *E. coli* RO200 culture. Arrows show transitions to the stationary phase of the limited (light arrow) and unlimited (dark arrow) culture. The culture conditions are shown in legend to Fig. 1.

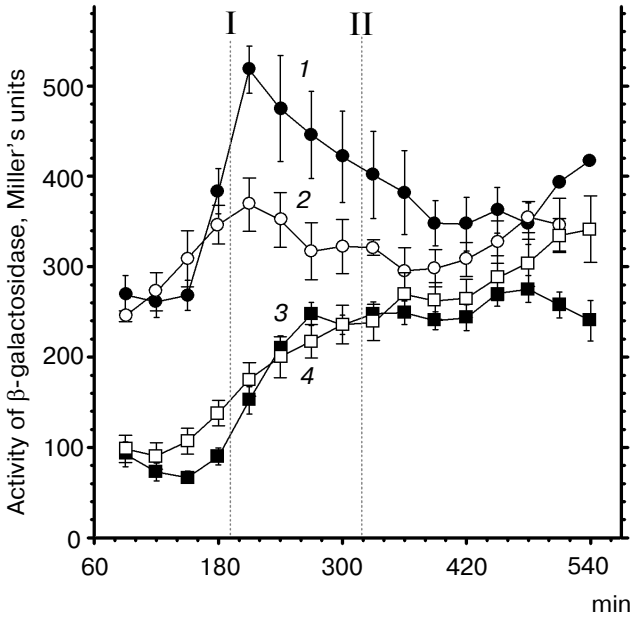

Fig. 3. Changes in the expression of the *rpoS* translational fusions in *E. coli* on transition to the stationary phase (*1*, *3*) or starvation (*2*, *4*) in the absence (*1*, *2*) and in the presence (*3*, *4*) of the turnover element. *1*, *2*) The *rpoS* expression level in *E. coli* RO90 cells; *3*, *4*) the *rpoS* expression level in *E. coli* RO91 cells. Vertical dotted lines show the entry of the culture into the starvation state (I) and into the stationary growth phase (II). The culture conditions are shown in the legend to Fig. 1.

to the late exponential growth phase (Fig. 1). However, expression in *E. coli* RO90 cells began to increase 15-20 min earlier than in the *E. coli* RO91 cells, under both limiting conditions and excess carbon and energy provision (Table 2). Values of the density and specific growth rate characteristic for the beginning of activation in the limited cultures of both strains indicated its being slightly shifted to the early exponential phase compared to the unlimited cultures. This seems to be due to the factor of starvation added to other inducing signals effective under conditions of excess carbon and energy provision.

The absence of the turnover element in the *E. coli* RO90 strain caused a significantly higher stability of the hybrid protein σ^S compared to the strain *E. coli* RO91 and resulted in a significant increase in its content and, correspondingly, the activity of β-galactosidase in the exponential phase of periodic culture (Fig. 3). And activation of the *rpoS::lacZ* expression in the *E. coli* RO90 strain on transition to the stationary phase was approximately twofold lower (Table 2), which suggested a weakened control of content of the hybrid protein σ^S in the absence of the turnover element. Disorder in the control of the σ^S stability caused the earlier start of activation of the *rpoS::lacZ* expression, but in the early stationary phase it was associated with a rapid decrease in amount of the hybrid protein to nearly the pre-stress level. This seems to be due to higher vulnerability of the imperfect protein to nonspecific effect of proteases. Surprisingly, the presence of the turnover element (*E. coli* RO90) provided the σ^S stability in the early stationary phase, at least under conditions of starvation when the content of the hybrid protein continued to increase. This seems to be due to lower vulnerability to proteases of the protein σ^S molecule because of its completeness (the presence of the turnover element) and, possibly, is also a consequence of dissociation with the response regulator RssB [19].

Comparison of parameters of the *rpoS::lacZ* activation in the *E. coli* strains RO90 and RO91 suggested that the activation during the exponential growth phase should be, first of all, conditioned by regulatory processes on the level of translation, whereas the high content of σ^S during the stationary phase was mainly due to regulation of the protein stability. This was also confirmed by the twofold increase in the activation degree in the presence of the turnover element (Table 2), while the mean degree of activation of the translational fusion was twofold and more higher than that of the transcriptional fusion, and absolute value of its expression was two orders higher (Figs. 2 and 3). Thus, although the contribution of transcription to the increase in the protein σ^S quantity on transition to the stationary phase was indisputable, the decisive events in the σ^S regulation in the cell under these conditions occurred on the level of translation and stability of the protein. These results fitted well with the universally adopted concept [2].

The secondary structure of mRNA of *rpoS*, i.e., the region of the paired double-stranded RNA including the initiation codon, is known to be mainly regulated on the post-transcriptional level. And factors which promote expansion of the secondary structure provide for the accessibility of the initiation codon and thus stimulate the translation [2]. Data on the possible role of polyamines as one such factor [8] reasoned the study of their effect on the level of expression of the translational *rpoS* fusion in *E. coli* periodic cultures.

The effect of putrescine on translational fusions of both types was studied, and its stimulatory effect on activation of the *rpoS::lacZ* expression was higher (independently of the substrate content in the medium) in the presence of the turnover element, whereas in its absence this effect was displayed only in limited cultures (Table 2). Thus, the effect of putrescine occurred on both the level of translation and (more markedly) on the level of the σ^S stability.

Addition of putrescine to the medium slightly shifted the beginning of the *rpoS::lacZ* activation to the early exponential growth phase (Table 2) that promoted the earlier response of the cells to transition to the stationary phase and seems to be a manifestation of the stimulatory effect of polyamines providing the faster adaptation of the cells to stress conditions.

Natural fluctuations in the polyamine pool in *E. coli* cells during the development cycle of the periodic culture

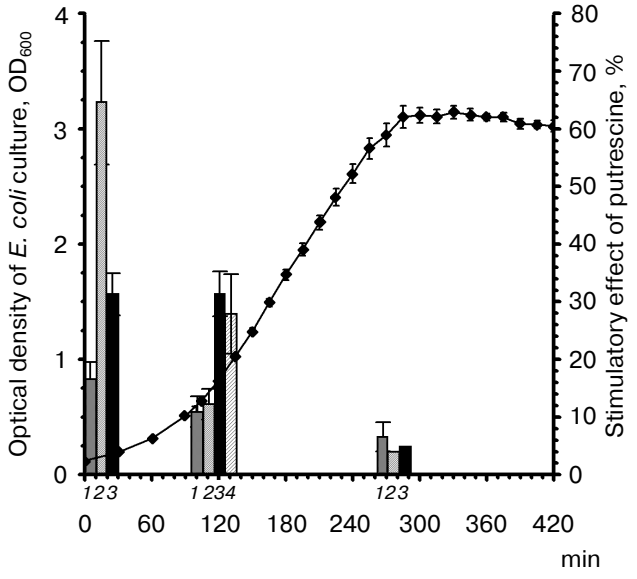

Fig. 4. Dependence of the stimulatory effect of exogenous putrescine on the growth phase of *E. coli* RO91 periodic culture. Columns show stimulatory effects of varied concentrations of putrescine on the activation degree of the *rpoS* expression (% of the control): *1*) 2 mM; *2*) 5 mM; *3*) 10 mM; *4*) 20 mM. The activation degree of the expression is the ratio of the maximal gene expression (Miller's units) at the given point of the culture growth to its initial level. Position of the columns with respect to the abscissa axis and their intersection points with the curves of OD_{600} and the specific growth rate characterize the corresponding time and physiological parameters of the culture on addition of putrescine. The culture conditions are described in "Materials and Methods".

[20] suggested a varied *rpoS* expression level in response to addition of putrescine during different growth phases. Addition of putrescine to the *E. coli* RO91 culture during the early growth phases stimulated the *rpoS::lacZ* expression more strongly than during the later stages (Fig. 4). Surprisingly, the polyamine content in the cells followed the inverse dependence, i.e., increases from the earlier to later growth phases [20]. Consequently, the effect of exogenous putrescine inversely depended on its content in the cell.

This regularity was fully confirmed by determination of putrescine content in the cells of cultures limited with various concentrations of glucose (Fig. 5). And the increase in putrescine content in the cell during the growth was accompanied by a proportional decrease in the stimulatory effect of exogenous putrescine on the *rpoS::lacZ* activation on exhaustion of the substrate.

The stimulatory effect of putrescine depended on its concentration, was maximal at 5-10 mM, and decreased along with further increase in the concentration (Fig. 4). It seemed that putrescine as it is failed to induce the *rpoS* expression, but its presence in the cell in a sufficient concentration by the beginning of activation promoted an increase in the response of adaptive systems of the cell to transition to the stationary phase. In particular, polyamines could influence the interaction of mRNA of the *rpoS* gene with nucleoid-binding proteins, such as Hfq or HU, which are believed to be main factors involved in expansion of its secondary structure [2]. This standpoint is indirectly supported by data on the stimulatory effect of polyamines on divergence of DNA palindrome sequences during formation of cross-shaped structures [21]. By analogy with this process, the electrostatic binding of polyamines, in particular putrescine, to the complementary polynucleotide regions of the secondary structure of the *rpoS* mRNA during their divergence seemed to promote formation of the linear structure of RNA and thus stimulate the initiation of translation.

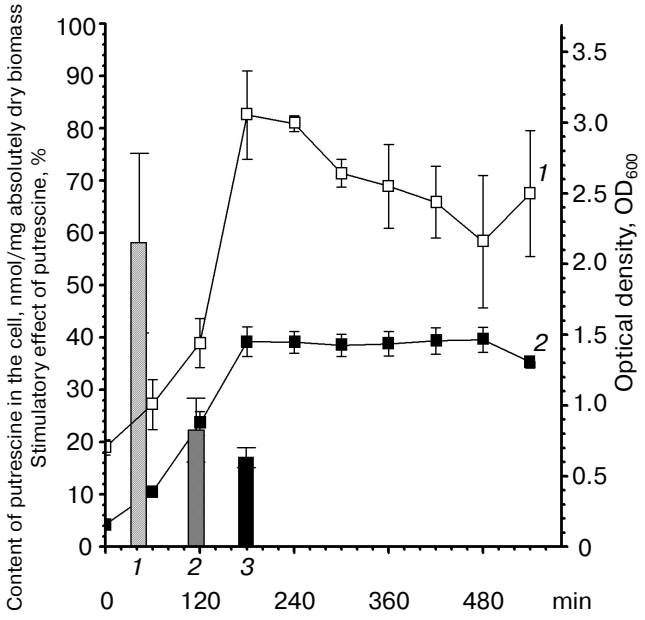

Fig. 5. Dependence of the effect of exogenous putrescine on its intracellular content during the growth of *E. coli* RO91 limited culture. *1*) Intracellular pool of putrescine; *2*) optical density (OD_{600}) of cells grown on the medium supplemented with 0.13% glucose. Columns show the stimulatory effect of putrescine on the *rpoS::lacZ* expression in cultures grown in the presence of limiting concentrations of glucose: *1*) 0.02%; *2*) 0.08%; *3*) 0.13%. Cells of *E. coli* RO91 were grown on medium M-9 (see "Materials and Methods") supplemented with 0.02, 0.08, and 0.13% glucose, in the absence (control) and presence (experiment) of 5 mM putrescine. During the growth and on transition to starvation the activity of β-galactosidase was determined in the cells, and its maximal value was used to calculate the mean value in similar experiments. The stimulatory effect of putrescine on the level of the *rpoS::lacZ* expression (in %) was calculated by the ratio of the mean maximal values of the β-galactosidase activity in the experimental and control samples. To simplify the understanding, the figure presents only the curves characteristic for cultures grown in the presence of 0.13% glucose, which coincided with the curves for other concentrations of glucose. Intersection points of the columns with the curves *1* and *2* correspond to maximal values of optical density and intracellular pool of putrescine on transition of the corresponding limited cultures to starvation.

Increase in the σ^S content in the cell on sudden stress conditions, such as carbohydrate starvation, is usually thought to be due to increase in the protein stability, which can be achieved for a few minutes [22]. However, the σ^S stability is known to be controlled by the response regulator RssB that in active phosphorylated state binds to the turnover element of σ^S and makes it accessible for degradation with the protease ClpXP [19]. Amounts of σ^S and RssB in the cell are in dynamic balance due to homeostatic mechanism of the feedback that maintains a stable rate of the σ^S proteolysis [23]. A sudden stimulation of the σ^S synthesis which occurs under stress conditions, such as starvation, acidification of the medium, or hyperosmotic shock [24], results in titration of RssB associated with decrease in its content in the cell and increase in the σ^S stability. The putrescine-caused increase in stability of the σ^S subunit of RNA polymerase described by us is likely to be associated with its effect on dissociation of RssB, possibly by interaction with the turnover element and functioning as a shield preventing the repeated binding of RssB to σ^S.

Thus, polyamines are involved in regulation of the σ^S content in *E. coli* on transition to the stationary phase on the level of translation and stability of the protein. The effect of exogenous putrescine is the most pronounced during the early growth phases of periodic culture when its concentration in the cells is minimal and decreases with increase in content of putrescine in the cell to the later growth phases.

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