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EXPERIMENTAL ARTICLES

The Effect of Spo0A and AbrB Proteins on Expression of the Genes of Guanyl-Specific Ribonucleases from *Bacillus intermedius* and *Bacillus pumilus* in *Bacillus subtilis* Recombinant Strains

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Abstract—Guanyl-specific ribonucleases from *Bacillus intermedius* and *Bacillus pumilus* are actively secreted under phosphate starvation by recombinant strains of *Bacillus subtilis* with native regulatory systems and by strains defective in some proteins of the Spo0A phosphorylation pathway. The level of expression of ribonuclease genes has been shown to increase approximately sixfold in recombinant strains with mutation in the *spo0A* gene and threefold in the *spo0A/abrB* mutants, as compared with native strains. These results demonstrate that the Spo0A protein regulates the production of ribonucleases and thus acts as a repressor, while the AbrB protein is an activator of expression of the genes encoding ribonucleases from *Bacillus intermedius* and *Bacillus pumilus* in *Bacillus subtilis* cells.

Key words: bacilli, binase, Bpu RNase, regulation of biosynthesis.

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Extracellular ribonucleases from *Bacillus intermedius* (binase) and *B. pumilus* (Bpu RNase) are cyclizing guanyl-specific ribonucleases that cleave the phosphodiester bonds in RNA molecules and oligo- and polynucleotides after guanosine residues with the formation of intermediate nucleoside-2'-3'-cyclophosphates, followed by their hydrolysis to the corresponding ribonucleoside-3'-phosphates. These enzymes have similar physicochemical and catalytic characteristics [1], and the basic patterns of their biosynthesis are similar [2]. The synthesis of both RNases occurs under deficiency of inorganic phosphate in the medium and is regulated in *B. subtilis* cells at the level of transcription by the proteins of the PhoP–PhoR two-component signal transduction system, which controls the expression of the PHO regulon genes under phosphate starvation [3, 4]. It is known from the literature that the *phoPR* operon is influenced by other regulatory mechanisms (Fig. 1), in particular, the Spo0A phosphorelay system, AbrB protein, and the ResD–ResE two-component system [5]. The multicomponent and complex character of regulation of the Spo0A phosphorylation system, including several histidine kinases and phosphatases, is evidence of its key role in decision-making performed by a cell, timely and adequate to environmental conditions, concerning whether to develop in the direction of spore formation.

The goal of the present work was to establish the role of Spo0A and AbrB proteins in the expression of the PhoP-dependent genes of guanyl-specific ribonucleases from *Bacillus intermedius* and *Bacillus pumilus* in *B. subtilis* cells.

MATERIALS AND METHODS

The following strains were selected for the work: *Bacillus subtilis* JH642 (*pheAl trpC2*) possessing native regulatory systems; its derivatives with mutations in the *spo0A* and *abrB* genes *B. subtilis* JH646 (*pheAl trpC2 spo0A12*) and *B. subtilis* R15-13 (*pheAl trpC2 abrB23 spo0A12*); and strains deficient in KinA (*B. subtilis* MB170 (*kinA82*)) and Spo0E (*B. subtilis* JH647 (*pheAl trpC2 spo0E11*)), the proteins of the Spo0A phosphorelay (BGSC, Ohio State University, USA).

Plasmids pMZ55 and pMZ56 were used in the work; these plasmids carry complete genes of binase and Bpu RNase, respectively, attached to the gene of the intracellular inhibitor barstar protecting *B. subtilis* cells from the toxic effect of RNases [3]. The plasmids provide the expression of the genes of *B. intermedius* and *B. pumilus* RNases in *B. subtilis* cells.

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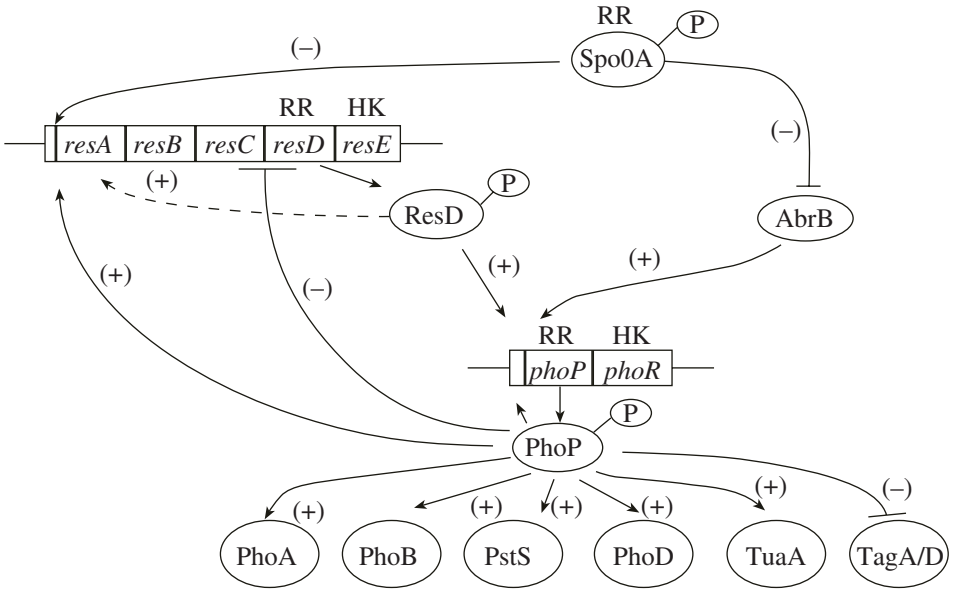

Fig. 1. The scheme of regulation of Pho regulon genes under phosphate starvation (from [5]).

The bacteria were cultivated at 30°C in 100-ml flasks (the medium/flask volume ratio, 1 : 7) on laboratory shakers at 200 rpm. The inoculum ratio (by volume) was the same for all experimental flasks.

Phosphorus-free synthetic medium (PFM) of the following composition (g/l) was used for cultivation: *Tris* (oxymethyl) aminomethane, 6.05; KCl, 5.0; NaCl, 1.0; $(NH_4)_2SO_4$, 2.0; sodium citrate, 1.0; $MgSO_4$, 0.2; glucose, 5.0; yeast extract, 0.5; pH 8.0. The concentration of inorganic phosphate in the medium was 4 μg/ml. For the cultivation of recombinant *B. subtilis* strains carrying plasmids pMZ55 and pMZ56, the nutrient medium was supplemented with kanamycin (10 μg/ml). The growth was monitored by measuring the optical density of the culture at 590 nm (OD_{590}).

B. subtilis cells were transformed by plasmid DNA according to the standard technique [6].

The stability of the plasmids in the obtained *B. subtilis* recombinants was determined as described in [7]. The number of generations (n) in the culture grown on L broth without the antibiotic was calculated from the equation $n = (\log N_K - \log N_0)/\log 2$, where N_0 and N_K are the initial and final cell quantity, respectively. For counting of the clones that have lost the plasmid during cultivation on the medium without the antibiotic, the culture was plated onto L agar with and without the antibiotic. The percentage of cells that had eliminated the plasmid was calculated.

The capacity of the transformants for ribonuclease synthesis was determined by the method of Jeffris; the clones were grown on petri dishes with PFM medium containing yeast RNA (5 mg/ml) and kanamycin [8]. RNase activity was detected by the presence of zones of RNA depolymerization around the colonies, which were revealed by the addition of 1N HCl.

Ribonuclease activity in the culture liquid was determined by the modified method of Anfinsen by the amount of acid-soluble hydrolysis products derived from the model substrate: high-polymer yeast RNA [9]. The amount of RNase which increased the optical density of the experimental samples by one optical unit after 1 h of incubation per 1 ml of enzyme solution (OD /(ml h)), as compared with the control, was taken as the unit of activity. Specific ribonuclease activity, an index of culture productivity with respect to enzyme synthesis, was calculated as the ratio of total enzyme activity to the quantity of biomass.

The results were statistically analyzed using the Excel 2003 software package. The standard deviation (σ) was calculated. The results were considered reliable at $\sigma \le 10\%$. The Student's criterion was used for calculation of reliability of the obtained differences, with $P \le 0.05$ as the reliable level of significance.

RESULTS AND DISCUSSION

Obtaining of recombinant *B. subtilis* strains bearing plasmids with the ribonuclease genes To elucidate the role of the Spo0A phosphorelay system in gene expression regulation of the ribonucleases from *B. intermedius* and *B. pumilus*, we chose *B. subtilis* strains mutant in the *spo0A* gene, in the kinase *kinA* gene, and in the phosphatase *spo0E* gene, as well as a double *spo0A*/*abrB* mutant. Preliminarily, all the initial recipient strains were tested for the presence of extracellular ribonuclease. After 24 h of cultivation, the secreted RNase was present in trace amounts in the culture liquid of these strains. Its level was less than 1% of the

Table 1. Comparative analysis of ribonuclease activities in various *B. subtilis* strains

B. subtilis strain	Genotype	OD_{590}	RNase activity, OD/(ml h)
JH642	*pheAl trpC2*	2.77 ± 0.05	140 ± 3.82
JH646	*pheAl trpC2 spo0A12*	2.45 ± 0.03	128 ± 2.68
R15-13	*pheAl trpC2 spo0A12 abrB23*	2.69 ± 0.05	155 ± 2.95
MB170	*kinA82*	2.71 ± 0.02	73 ± 2.04
JH647	*pheAl trpC2 spo0E11*	2.83 ± 0.03	94 ± 1.75
JH642 pMZ-55	*pheAl trpC2*	1.98 ± 0.05	24074 ± 379
JH642 pMZ-56	*pheAl trpC2*	1.96 ± 0.03	42130 ± 576

activity of *B. subtilis* JH642 strains bearing plasmids pMZ55 and pMZ56 (Table 1).

The mutant strains were transformed by plasmids pMZ55 and pMZ56 carrying complete ribonuclease genes of *B. intermedius* and *B. pumilus*, respectively; the transformation was carried out according to the standard technique. Recombinants were selected by two features: resistance to kanamycin and presence of RNase activity. Ten clones of each variant were chosen for further analysis.

One of criteria of the aptitude of plasmids is their stability in recipient strains. Plasmids pMZ55 and pMZ56 were retained in the recombinant strains throughout the period of observation (Table 2). Very few clones (0–3%) lost the plasmid after 32–36 generations. Thus, the strains under study are stable and the obtained recombinant strains of *B. subtilis* can be used to study the expression of the binase and Bpu RNase genes.

The effect of Spo0A protein on expression of the genes of *B. intermedius* and *B. pumilus* ribonucleases in *B. subtilis* cells. The Spo0A protein is a component of the signal transduction system, the main function of which is to control the initiation of spore formation [10]. The level of Spo0A phosphorylation depends on the joint action of kinases and phosphatases; it determines the direction of cell development: at low Spo0A-P concentrations, adaptation to unfavorable conditions is achieved through development of competence, chemotaxis, and synthesis of antibiotics and lytic enzymes; at high Spo0A-P concentrations, the program of spore formation is initiated [11]. The role of the Spo0A protein in phosphate regulation has been demonstrated. This regulation is achieved by controlling the expression of the *abrB* and *resD* genes, the products of which are necessary for complete induction of the Pho regulon [12].

The *B. subtilis* strains carrying mutations in the *spo0A, kinA*, and *spo0E* genes were used to elucidate the possible role of the Spo0A protein in the regulation of the binase and Bpu RNase gene expression. The cells of the mutant *B. subtilis* strains contain different amounts of phosphorylated Spo0A and differ in their ability to form spores. The KinA protein is one of the main kinases receiving the signal that enters the Spo0A phosphorylation pathway [13]. Spo0E is a specific phosphatase of Spo0A required to recover the inactive form of Spo0A-P [14]. Mutation in the *kinA* gene reduces spore formation by 95% of the wild-type level [15], but this strain produces quite enough phosphorylated Spo0A for normal regulation of the *abrB* gene [13]. The *spo0E11* mutation induces the synthesis of a hyperactive protein, minimizing the amount of Spo0A-P in cells and preventing spore formation [16]. In the strain with mutation in the *spo0A* gene itself, the formation of a functional response regulator is almost impossible.

It has been shown that binase and Bpu RNase are actively secreted on PFM both by recombinant strains with native Spo0A phosphorelay system and by mutant strains carrying plasmids with the RNase genes. It has

Table 2. Stability of plasmids pMZ55 and pMZ56 with the complete genes of ribonucleases from *B. intermedius* and *B. pumilus* in recombinant *B. subtilis* strains

B. subtilis strain	Number of generations	Quantity of clones retaining the plasmid, %
	pMZ55	
JH642	35.4	100
JH646	35.6	100
R15-13	35.0	99.1
MB170	36.2	98
JH647	34.5	99
	pMZ56	
JH642	32.5	100
JH646	33.0	97
R15-13	36.0	98
MB170	34.5	98
JH647	35.0	99

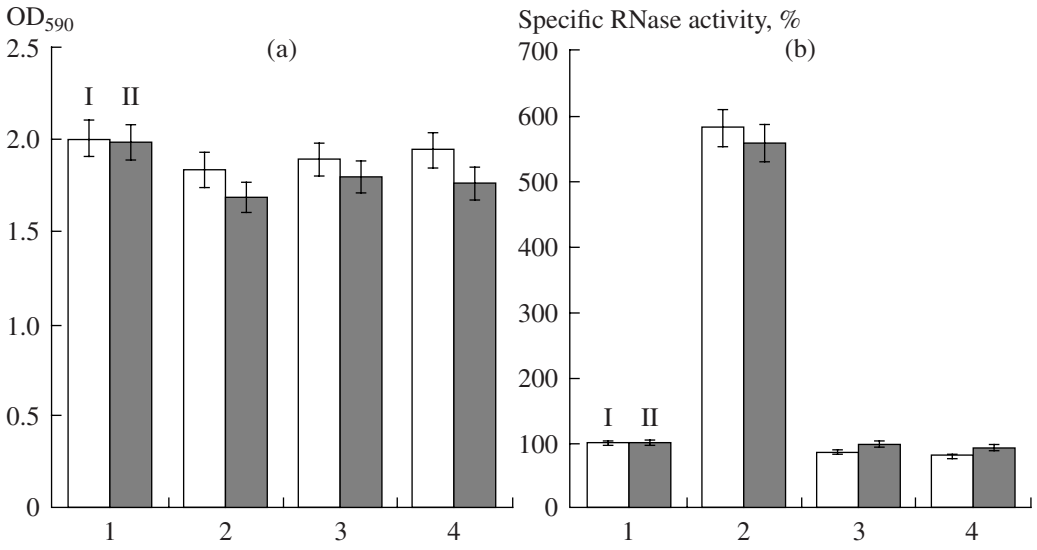

Fig. 2. Expression of the genes of ribonucleases from *B. intermedius* (**I**) and *B. pumilus* (**II**) in recombinant *B. subtilis* strains defective in the proteins of the Spo0A phosphorelay system: 1, *B. subtilis* JH642 (control); 2, *B. subtilis* JH646 (*spo0A*); 3, *B. subtilis* MB179 (*kinA*); 4, *B. subtilis* JH647 (*spo0A*); (a), growth of recombinant strains; (b), RNase activity. The level of enzyme activity in the recombinant *B. subtilis* strain with native Spo0A phosphorelay system was taken as 100%.

been shown that the level of RNase activity is higher in the strains defective in the Spo0A protein: in *B. subtilis* strains with mutation in the *spo0A* gene, the levels of specific activity of binase (*B. subtilis* JH646 pMZ55) and Bpu RNase (*B. subtilis* JH646 pMZ56) are 5.8- and 5.6-fold higher, respectively, than those in the control strains.

The strategy of experiments with the mutant strains is based on the following assumption: if the regulatory protein is needed for expression of some gene, its deletion will change the level of expression of the controlled gene. If the protein is a repressor, the level of expression of the genes controlled by this protein will be much higher; but if the protein is an activator, the expression of the regulated genes will be terminated in its absence.

Mutations in the *spo0A* gene result in hyperinduction of ribonucleases, which implies that the Spo0A protein regulates enzyme production, acting as a repressor.

The specific activity in the culture liquid of the strains carrying mutations in the *kinA* kinase and *spo0E* phosphatase genes corresponds to that of the control strain (Fig. 2); this finding can be explained by the presence of the minimal quantity of phosphorylated Spo0A, which represses the expression of the *abrB* gene.

The effect of AbrB protein on expression of the binase and Bpu RNase genes in *B. subtilis* cells. Activated Spo0A controls the expression of some genes directly, by binding to their promoters, and of other genes indirectly, by affecting the transcription of regulatory proteins, in particular, ArbB protein. The ArbB protein regulates the competence and synthesis of antibiotics and lytic enzymes, represses transcription of some genes essential for spore formation, and is an activator of the Pho regulon [12, 17–19]. A minor quantity of phosphorylated Spo0A is sufficient for AbrB protein repression [11]. Using the strain with mutation in the *spo0A* gene and a double *spo0A/abrB* mutant, it is possible to track both pathways of Spo0A functioning in a cell.

It has been shown that the levels of binase and Bpu RNase in recombinant strains defective in the *Spo0A* and *AbrB* regulatory proteins, as well as in single *spo0A* mutants, are higher than in native strains. However, there are quantitative differences in these enzyme activities between *B. subtilis* strains defective in the *spo0A* and *abrB* genes and *B. subtilis* strains mutant only in the *spo0A* gene. The levels of expression of *B. subtilis* binase and Bpu RNase are 319% and 313%, respectively, in the double *spo0A/abrB* mutant strains and 579% and 556%, respectively, in the strains with mutation in the *spo0A* gene (as compared with that in the initial strain). Thus, the role of ArbB is opposite to the role of Spo0A; i.e., ArbB is an activator of the binase and Bpu RNase genes. Since we used strains mutant in both genes (repressor and activator), expression of the ribonuclease genes was possible but its level was 1.8-fold lower as compared with that in the strains with mutation in the *spo0A* gene.

The fact that the expression of the genes of ribonucleases from *B. intermedius* and *B. pumilus* in the double mutant strains of *B. subtilis* (*spo0A/abrB*) is maintained at a 60% level of the expression of these genes in the *spo0A* gene mutants is evidence of the parallel pathway of regulation of the RNase genes. Taking the literature data into consideration, it can be assumed with

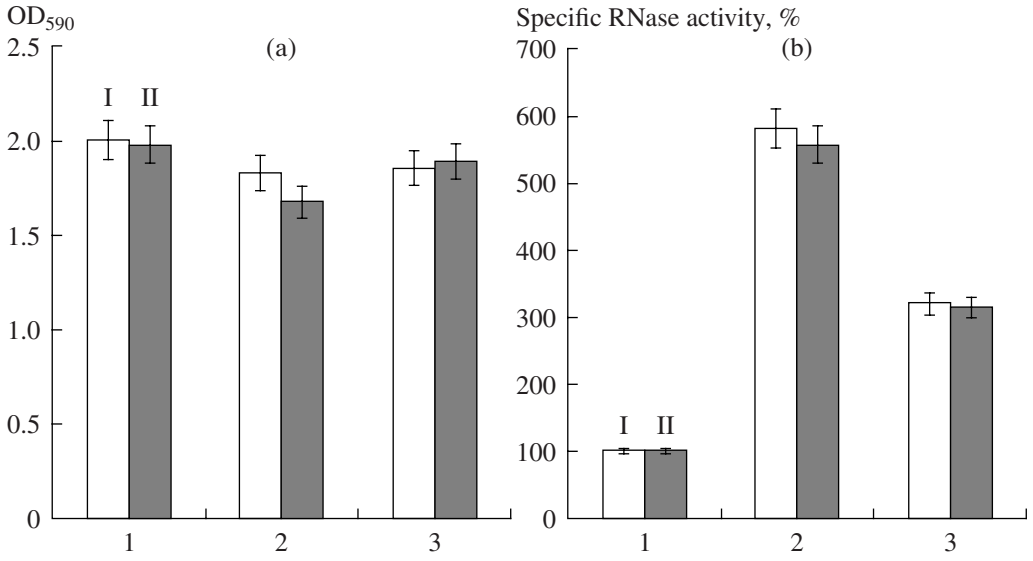

Fig. 3. Expression of the genes of ribonucleases from *B. intermedius* (**I**) and *B. pumilus* (**II**) in recombinant *B. subtilis* strains defective in Spo0A and AbrB proteins: 1, *B. subtilis* JH642 (control); 2, *B. subtilis* JH646 (*spo0A*); 3, *B. subtilis* R15-13 (*spo0A/abrB*); (a), growth of recombinant strains; (b), RNase activity. The level of enzyme activity in the recombinant *B. subtilis* strain with native regulatory proteins was taken as 100%.

high probability that this pathway is controlled by the ResD-ResE system.

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