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EXPERIMENTAL ARTICLES

Biosynthesis of Secreted Ribonucleases of *Bacillus intermedius* and *Bacillus circulans* under Nitrogen Starvation

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Abstract—The level of biosynthesis of secreted guanyl-specific ribonucleases (RNases) of *Bacillus intermedius* (binases) and *Bacillus circulans* (RNases Bci) by recombinant *B. subtilis* strains increases under nitrogen starvation. The promoter of the binase gene carries the sequences homologous to the recognition sites of the regulatory protein TnrA, which regulates gene expression under growth limitation by nitrogen. Using the *B. subtilis* strain defective in protein TnrA, it has been shown that the regulatory protein TnrA is involved in the regulation of expression of the binase gene and the gene of RNase Bci. The TnrA regulation of expression of the RNase Bci gene is indirect, probably by means of the regulatory protein PucR. Thus, it has been established that at least two regulatory mechanisms activate the expression of the genes encoding the secreted RNases of spore-forming bacteria: a system of proteins homologous to the *B. subtilis* PhoP–PhoR, and regulation by a protein similar to the *B. subtilis* TnrA regulatory protein.

Key words: bacilli, binase, RNase Bci, biosynthesis regulation.

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Representatives of the genus *Bacillus* are gram-positive, spore-forming bacteria; their habitats often contain insufficient quantities of available nutrients. At the same time, spore formation is not the only survival strategy for bacilli under the shortage of sources of carbon, phosphorus, nitrogen, and other necessary substances. Prior to initiation of sporulation, bacteria make an attempt to accomplish a number of adaptive approaches promoting the resumption of vegetative growth (the synthesis of extracellular degradation enzymes and antibiotics, increase of motility, chemotaxis) [1]. The synthesis of degradation enzymes, including various hydrolases, can be considered as one of the methods of bacterial adaptation to environmental conditions. For example, expression of a number of genes, which comprise a single phosphate (Pho) regulon and are under the same physiological and genetic control, is induced in bacterial cells under phosphate starvation. The products of these genes participate in assimilation of exogenous phosphate [2]. Our studies demonstrated that in many species of bacilli *(B. intermedius, B. pumilus, B. thuringiensis, B. circulans*) the biosynthesis of secreted ribonucleases is induced under deficiency of inorganic phosphate in the medium, is coregulated with the biosynthesis of alkaline phosphatases, and with the exception of *B. circulans* RNase, is accomplished by proteins of the two-component signal transduction system PhoP–PhoR, similar to the genes of *B. subtilis* Pho regulon [3–6].

Along with phosphate, nitrogen is an important macroelement necessary for the existence of living organisms. The synthesis of nucleic acids, amino acids, proteins, and other nitrogen-containing molecules requires the presence of amino groups in the cytoplasm; they are integrated into molecules through amination or transamination. Bacilli can quickly metabolize various nitrogen sources. One of the most preferable sources is ammonium, which is transported into *B. subtilis* cells by proteins NrgA and NrgB [7]. Under nitrogen deficiency, the action of regulatory protein TnrA in *B. subtilis* results in an increased level of expression of some genes involved in utilization of secondary nitrogen sources, e.g., the genes of nitrate assimilation enzymes (*nasABCDEF*), asparaginase, permease of γ-aminobutyric acid (*gabP*), urease (*ureABC*) [8, 9]. *B. subtilis*, like many bacteria, can utilize the nitrogen of purine and pyrimidine bases [10–14]. The presence of available bases in the environment is largely associated with the functioning of extracellular hydrolytic enzymes degrading nucleic acids.

In view of the above, it can be supposed that bacillar secreted ribonucleases are not only involved in the system of phosphate regulation but are also necessary for the activity of spore-forming bacteria under nitrogen starvation.

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Promoter of the gene of binase (*B. intermedius* 7P, AN X53697)

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           C                                                               A          A  A                 –10
TATTTCATCAGAAGGTTATCAGGAAAAAGCCTCATTTTAGCAAAGAACCTGTTTCTTTACATTTCCTTCATGTTCGGGTGCTATAATATGAGGTA
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Promoter of the gene of RNase Bci (*B. circulans* BCF 247, AN Z29626)

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       A                       T    T  A                                                                     –10
GTTGGTTCATGCCGGGGTTTATTTTTCGCTGGATCAAAAGTACTATTTTTAAATTCTATCTATTCCTTTCTTTCGATGCTGATACAATAAAAAGG
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Fig. 1. Promoters of the genes of ribonucleases of *B. intermedius* and *B. circulans.* The mRNA synthesis initiation sites are underlined; supposed sequences recognized by the regulatory protein TnrA (5'-TGTNAN$_7$TNACA-3') are underlined; supposed PucR boxes (5'-WWWCNTTGGTTAA-3') are enclosed. Promoter regions corresponding to the *pho* boxes (5'-TTA(T/C)ACA-3') are in bold.

The goal of this work was to determine the molecular mechanisms of regulation of the synthesis of secreted ribonucleases of *Bacillus intermedius* and *Bacillus circulans* under nitrogen starvation.

MATERIALS AND METHODS

The strains used in the work were as follows: *B. subtilis* 168 trpC2 (*Bacillus* Genetic Stock Center), strains defective in the genes of ammonium transport system: *B. subtilis* GP253 (*trpC2 amyE::(nrgB–lacZ aphA3) ΔnrgB::cat pGP183GP250 Cm^r*), *B. subtilis* GP254 (*trpC2 amyE::(nrgA–lacZ aphA3) ΔnrgA::cat pGP184GP250 Cm^r*) [7], and a strain defective in the gene of the regulatory protein TnrA: *B. subtilis* FA6 (*trpC2 amyE::(ykoL600'–lacZ cat) tnrA::Tn917 Em^r*) [9].

Recombinant strains producing the binase ribonuclease from *B. intermedius* and RNase Bci from *B. circulans* were obtained using plasmids pMZ55 [4] and pMZ59 [3] carrying the genes of respective RNases and the gene of kanamycin resistance.

The bacteria were cultivated at 30°C in 100-ml flasks (medium to flask volume ratio 1 : 7) on laboratory shakers at 200 rpm. Experimental flasks were inoculated with one volume percent of the inoculum.

The following media were used for cultivation: phosphate-free synthetic BPS medium (g/l: glucose, 5.0; ammonium sulfate, 2.0; yeast extract, 0.5; potassium chloride, 5.0; sodium chloride, 1.0; sodium citrate, 1.0; magnesium sulfate, 0.2; Tris, 2.0; pH 8.0), BPS-1 (BPS modification with sodium nitrate instead of ammonium sulfate), BPS-2 (BPS modification: pH 5.7); and BPS-3 (BPS modification without ammonium sulfate). Kanamycin was added to the medium in the concentration of 10 μg/ml.

Culture growth was controlled by measuring the optical density of culture liquid at 590 nm (OD_{590}).

B. subtilis cells were transformed by plasmid DNA according to the standard method [15].

Ribunoclease activity in the culture liquid was determined by the modified Anfinsen's method by the quantity of acid-soluble products of hydrolysis of a model substrate: high-polymer yeast RNA [16]. Specific ribonuclease activity, which is an index of culture productivity with respect to enzyme synthesis, was calculated as a ratio of total enzyme activity to the biomass value.

The results were statistically analyzed with the Excel 2003 software package. The root-mean-square deviation (σ) was calculated. The results were considered reliable at $\sigma \leq 10\%$. The reliability of the obtained differences was calculated using the Student's criterion, taking $P \leq 0.05$ as a reliable level of significance.

RESULTS AND DISCUSSION

Comparative analysis of the structure of promoters of the genes of secreted ribonucleases of *B. intermedius* and *B. circulans*. Previously, the experiments with mutant genes of guanyl-specific RNases have shown that their expression is determined by a promoter [17]. In the present work, comparative analysis of the structure of promoters of the genes of guanyl-specific ribonucleases from *B. intermedius* (binase) and *B. circulans* (RNase Bci) was carried out in order to reveal the nucleotide sequences determining the regulatory mechanisms of expression of the these genes (Fig. 1).

The promoter of the phosphate-regulated binase gene was shown to contain, apart from PHO boxes [4], the sequences homologous to the recognition sites of the *B. subtilis* regulatory protein TnrA. This sequence is a promoter region that contains two conservative sites separated by seven nucleotides and has the appearance of TGTNAN$_7$TNACA. The expression of gene products of the degradation enzymes and permeases involved in nitrogen metabolism is regulated by complex global regulating systems, each of them functioning under different nutritional conditions. The TnrA protein is known to regulate gene expression under nitrogen-limited growth [8].

No recognition sites of regulatory protein TnrA were found in the promoter of the RNase Bci gene. However, this fact does not eliminate the possibility of the involvement of TnrA in the regulation of the RNase Bci gene expression. This regulatory protein may influence the expression of the RNase Bci gene indirectly,

by means of other regulatory proteins. Previously it was shown that the promoter region of the urease-encoding operon *ureABC* did not contain a TnrA site, but TnrA regulation of the *ureABC* operon is accomplished through transcription regulator PucR [12]. PucR is known to regulate the expression of genes involved in purine catabolism. Induction of the *pucR* gene expression under nitrogen deficiency is directed by the global protein of nitrogen regulation, TnrA [13]. Probably, the regulation of the RNase Bci gene expression is similar to TnrA regulation of the *ureABC* operon, because the RNase Bci gene promoter contains a site homologous to the *PucR* box (Fig. 1). The system of nitrogen regulation of bacilli and, in particular, the TnrA factor reacts to a signal of yet unknown nature. One of these signals is supposedly generated as a result of the functioning of proteins of the ammonium ion transport system: NrgA and NrgB [7].

Expression of the genes of secreted ribonucleases from *B. intermedius* and *B. circulans* by *B. subtilis* strains under nitrogen starvation. The nitrogen sources preferable for bacteria are ammonium and glutamine. In the environment, ammonium is present in two forms: gaseous ammonia NH_3 and ammonium ion NH_4^+. At low concentrations or at decreased pH, the equilibrium between gaseous ammonia, which can enter the cells by diffusion, and ammonium ions is shifted towards ammonium. Under these conditions, there is a need of ammonium transporter NrgA associated with the NrgB protein. It is believed that NrgB can transmit information about ammonium availability to the regulation factors of other signal systems and thus regulate their activity [7].

Recombinant *B. subtilis* strains carrying plasmids pMZ55 and pMZ59 with the binase and RNase Bci genes, respectively, were obtained to study the patterns of biosynthesis of *B. intermedius* and *B. circulans* ribonucleases under nitrogen starvation. Strains with mutations in the *nrgA* (*B. subtilis* GP254) and *nrgB* (*B. subtilis* GP253) genes were used in addition to *B. subtilis* strains with the complete ammonium transport system.

The expression of the binase and RNase Bci genes was studied during the cultivation of recombinant strains on BPS medium containing ammonium sulfate as the major nitrogen source. The BPS medium was modified to reduce nitrogen availability: in BPS-1, pH was reduced to 5.7; in BPS-2, ammonium sulfate was replaced by sodium nitrate; and in the third variant, ammonium sulfate was lacking in the cultivation medium (BPS-3).

The decrease in pH and substitution of sodium nitrate for ammonium sulfate had no significant effect on the growth of all tested strains of *B. subtilis.* At the same time, the specific activity of secreted ribonucleases, binase and RNase Bci, exceeded the specific activity for the strains grown on BPS medium by 10–30% and 15–36%, respectively (Fig. 2). The amount of biomass of recombinant strains on BPS-3 was much lower than on other media due to the absence of ammonium sulfate. Specific ribonuclease activity of the cultures grown on BPS-3 was twice as high as those grown on BPS.

Biomass accumulation by recombinant strains *B. subtilis* GP254 (*ΔnrgA*) pMZ55, *B. subtilis* GP254 (*ΔnrgA*) pMZ59, *B. subtilis* GP253 (*ΔnrgB*) pMZ55, and *B. subtilis* GP253 (*ΔnrgB*) pMZ59 was less by 30–50% than in the strains with the complete NrgAB system. This effect may result from lower nitrogen availability to the cultures lacking the NrgAB system. Against the background of a decrease in growth, there was an increase of specific activities of RNase Bci and binase both on BPS (by 40–51% for binase and by 31–36% for RNase Bci) and on modified media (by 56–196% for binase and by 45–180% for RNase Bci) as compared with the control (recombinant strain with the complete NrgAB system grown on BPS). At cultivation on BPS-2 medium, the difference in the levels of biosynthesis was less marked. The specific activity was maximal at cultivation of the mutant strains on BPS-1 (182% for binase and 185% for RNase Bci) and BPS-3 (296% for binase and 280% for RNase Bci).

Thus, nitrogen starvation increases the level of biosynthesis of secreted RNases from *B. intermedius* and *B. circulans* both by unimpaired *B. subtilis* strains carrying plasmids pMZ55 and pMZ59 and by strains with deletions in the proteins of the ammonium transport system, NrgA and NrgB. In other words, the expression of the binase and RNase Bci genes in *B. subtilis* cells is activated in response to the lower availability of nitrogen to bacterial cells.

The role of the TnrA regulatory protein in the regulation of expression of the genes of secreted ribonucleases from *B. intermedius* and *B. circulans* in the cells of *B. subtilis*. The regulatory protein TnrA is activated under limitation of nitrogen availability and provides a high level of expression of some genes.

B. subtilis FA6 defective in the regulatory protein TnrA and the control strain *B. subtilis* 168 were transformed by plasmids pMZ55 and pMZ59 carrying the binase and RNase Bci genes. The recombinant strains were grown on BPS (with ammonium sulfate as the main nitrogen source) and BPS-3 (without ammonium sulfate).

The growth of recombinant strains on BPS-3 medium was lower by 21–33% than on BPS medium (Fig. 3).

Specific ribonuclease activities of the control strain and the strain defective in the regulatory protein TnrA grown on BPS medium were on the same level. On BPS-3 medium, specific activities of the control strain increased by 38% (binase) and 23% (RNase Bci). The specific activity of recombinant strains with plasmids pMZ55 and pMZ59 (lacking the TnrA regulatory protein) on BPS-3 medium substantially decreased (1.7–2.0 times).

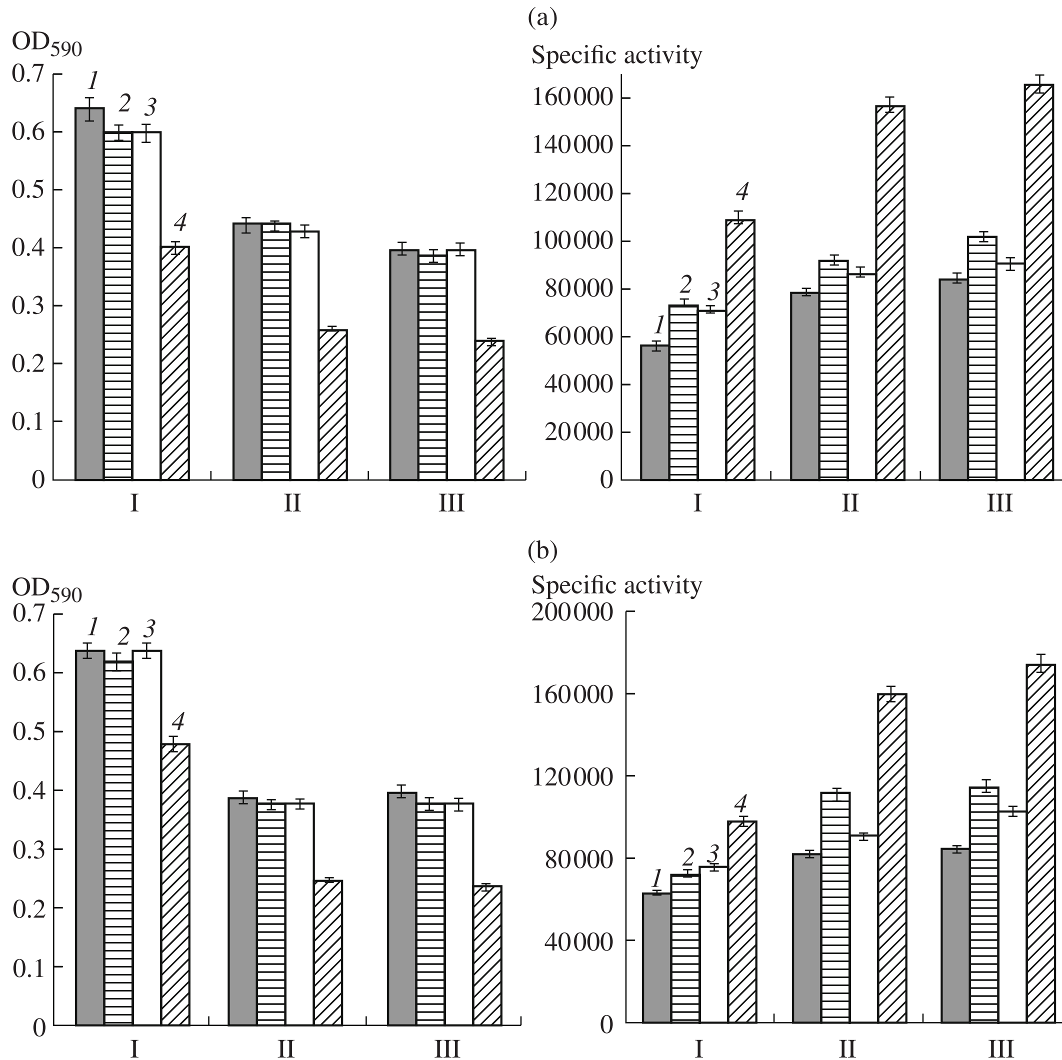

Fig. 2. Expression of the genes of ribonucleases from *B. intermedius* (a) and *B. circulans* (b) in recombinant *B. subtilis* strains under nitrogen starvation: I, *B. subtilis* 168; II, *B. subtilis* GP254 (*Δ nrgA)*; III, *B. subtilis* GP253 (*Δ nrgB*). Cultivation media: BPS (*1*); BPS-1 (*2*); BPS-2 (*3*); BPS-3 (*4*).

Our findings demonstrate that TnrA is involved in the regulation of expression of the binase and RNase Bci genes under nitrogen starvation.

The secreted RNases of spore-forming bacteria not only play a key role in supplying bacterial cells with phosphorus under its deficiency in the environment [6] but also contribute to the utilization of the nitrogen of nucleic acids under nitrogen starvation. The expression of ribonuclease genes encoding the enzymes under study is probably regulated by several successive or concurrent signal systems of gene expression control. By now it has been established that there are at least two regulatory mechanisms responsible for the activation of expression of the genes of extracellular RNases of bacilli: a protein system homologous to the PhoP–PhoR of *B. subtilis* [6] and regulation by a protein similar to the regulatory protein TnrA of *B. subtilis*. There is probably an interrelation between the systems of regulation of cell response to phosphate and nitrogen deficiency, because both stresses are essentially a limitation of the nutrients' availability to the bacterial cell. This assumption is indirectly evidenced by the fact that *B. subtilis* contains the *ykzB-ykoL* operon encoding the genes with an unknown function and controlled by the two global regulation systems: TnrA and PhoP-PhoR [9].

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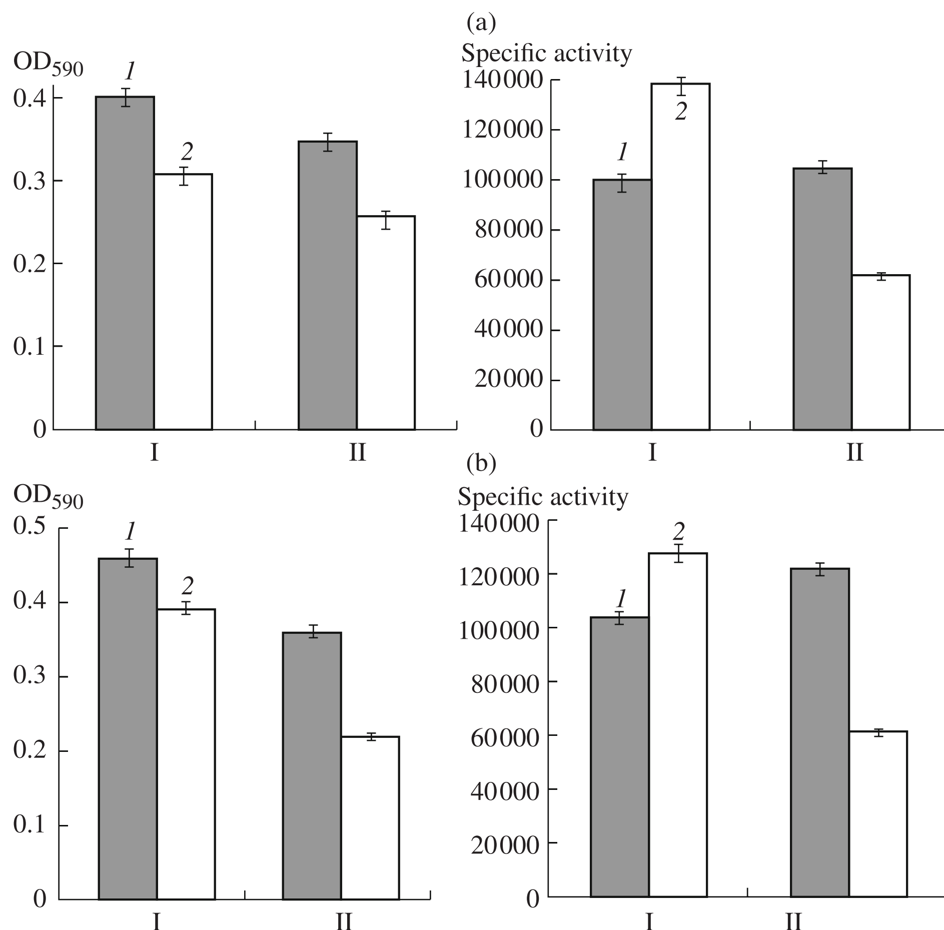

Fig. 3. Expression of the genes of ribonucleases from *B. intermedius* (a) and *B. circulans* (b) in recombinant strains *B. subtilis* 168 (I) and *B. subtilis* JH646 (*tnrA*) (II). Cultivation media: BPS (*1*); BPS-3 (*2*).

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