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EXPERIMENTAL ARTICLES

The Biosynthesis of New Secretory High-Molecular-Weight Ribonucleases in *Bacillus intermedius* and *Bacillus subtilis*

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Abstract—The investigation of new secretory ribonucleases, the *Bacillus intermedius* binase II expressed in the recombinant *B. subtilis* strain 3922 and the native RNase Bsn of *B. subtilis*, showed that they are synthesized in the growth retardation phase, when inorganic phosphate is exhausted in the medium. The biosynthesis of these ribonucleases was found to be suppressed by the presence of inorganic phosphate in the medium and activated by small amounts of the transcriptional inhibitor actinomycin D. The cultivation media of the producing strains were optimized for the maximum production of the enzymes.

Key words: *Bacillus intermedius*, *B. subtilis*, binase II, RNase Bsn, biosynthesis, phosphate starvation, actinomycin D.

It is well documented that bacilli synthesize two types of secretory ribonucleases, low-molecular-weight guanyl-specific cycling ribonucleases composed of about 110 nucleotides and high-molecular-weight ribonucleases composed of about 240 nucleotides. Ribonucleases of the first type (EC 3.1.27.1) have been isolated and characterized in *Bacillus amyloliquefaciens*, *B. intermedius*, *B. pumilus*, *B. thuringiensis*, *B. circulans*, *B. coagulans*, and other bacillar species. These enzymes are 85–100% identical in primary structure, stable in a wide range of pH (3–10), have a pH optimum at 8.5, and require no metal ions for activity. They cleave RNA with the formation of 2',3'-phosphodiesters, which are spontaneously hydrolyzed to nucleoside-3'-phosphates [1–3]. The genes encoding these enzymes have been sequenced and their nucleotide sequences have been deposited in the GenBank. Guanyl-specific ribonucleases, except for the RNase of *B. amyloliquefaciens* (barnase), are synthesized under conditions of inorganic phosphate deficiency and regulated similarly to the PHO regulon genes of *B. subtilis* [4–6].

Only two RNases of the second type have so far been described: RNase Bsn from *B. subtilis* [7] and binase II from *B. intermedius* [8, 9]. The former enzyme, encoded by the *bsn* gene, is activated by Mg^{2+} ions and cleaves RNA randomly to oligonucleoside-5'-phosphates [7]. It is shown that the *B. subtilis* genome does not contain genes homologous to those of guanyl-specific RNases. The biosynthesis of RNase Bsn has not yet been studied. As for binase II, it is the minor RNase of *B. intermedius*, the major being guanyl-specific binase I. Binases I and II are encoded by the *birA* and *birB* genes, respectively. The second binase was discovered when a fragment of the *B. intermedius* chromosome was cloned in *B. subtilis*. Binase II is 72% homologous to the RNase Bsn of *B. subtilis* in primary structure, the *birB* and *bsn* genes being 65% homologous. Binase II has a molecular mass of 30 kDa, is activated by Mg^{2+} ions, and is almost completely inhibited by EDTA. The optimum pH of binase II is 8.5, as in the case of RNase Bsn from *B. subtilis* [9, 10]. The biosynthesis of binase II has also not yet been investigated.

This work was aimed at studying the biosynthesis of the secretory binase II of *B. intermedius* in the recombinant *B. subtilis* strain 3922 (pJF28) and the native RNase Bsn of *B. subtilis* 3922.

MATERIALS AND METHODS

Bacterial strain and plasmid. The *Bacillus subtilis* strain 3922 (*hisB2 trpC2 leuB*) was obtained from the collection of microorganisms at the Laboratory of Enzyme Synthesis and Bioengineering at Kazan State University. This strain was used as the producer of RNase Bsn and as a host for the cloned gene of binase II from *B. intermedius*. The gene was cloned using plasmid pJF28, which bears the erythromycin resistance gene and a 4.9-kbp fragment of the *B. intermedius* chromosome with the *birB* gene [8].

Culture media. The starting nutrient medium for the synthesis of guanyl-specific ribonucleases in recombinant *B. subtilis* strains contained (%) peptone, 2; glucose, 1; $CaCl_2$, 0.01; $MgSO_4 \cdot 7H_2O$, 0.03, $MnSO_4$, 0.01; and NaCl, 0.3 [11]. Media that provided for the maximum synthesis of the *B. intermedius* binase

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Table 1. The effect of inorganic phosphate on the growth and biosynthesis of RNase Bsn and binase II in the *B. subtilis* strains 3922 and 3922 (pJF28)

P_i, μg/ml	Biomass				RNase				Specific activity			
	B. subtilis 3922		*B. subtilis* 3922, pJF28		*B. subtilis* 3922 (Bsn)		*B. subtilis* 3922, pJF28(binase II)		*B. subtilis* 3922		*B. subtilis* 3922, pJF28	
	OD units	%	OD units	%	E/ml	%	E/ml	%	OD units	%	OD units	%
Control	4.2	100.0	3.0	100.0	190	100	5990	100	79	100	1996.7	100
50	4.9	116.7	4.5	150.0	91	47.9	2550	42.6	18.6	23.5	566.7	28.4
100	5.6	133.3	5.2	173.3	25	13.2	1212	20.2	4.5	5.7	233.1	11.7
150	6.0	142.9	5.4	180.0	10	5.3	1005	16.8	1.7	2.2	186.1	9.3
200	6.5	154.8	5.6	186.7	10	5.3	910	15.2	1.6	2.0	162.5	8.1
250	6.8	161.9	5.7	190.0	10	5.3	865	14.4	1.5	1.9	151.8	7.6

Note: The control medium contained only P_i that is present as impurities in the medium ingredients.

II in the recombinant *B. subtilis* strain 3922 (pJF28) and the native RNase Bsn of *B. subtilis* 3922 were elaborated in this work. The media were sterilized at 1 atm for 20 min. The pH of the media were preliminarily adjusted to 8.5 with 40% NaOH. Glucose solutions were prepared and sterilized separately. To study the effect of inorganic phosphate (P_i) on bacterial growth and RNase synthesis, the necessary volume of a sterile solution of Na_2HPO_4 was added to the culture medium together with the inoculum to avoid the precipitation of calcium phosphates during the sterilization of the medium. The recombinant *B. subtilis* strains bearing plasmid pJF28 were grown in the presence of 10 μg/ml erythromycin.

Cultivation conditions. The bacterial strains were grown either in test tubes or in 100-ml flasks containing culture medium in a medium-to-flask volume ratio equal to 1 : 7.5. The flasks were inoculated with cultures grown in optimized media. The inoculum was added in an amount of 1%. The inoculated flasks were incubated at 30°C on a shaker (220 rpm).

In experiments with short-lived cultures, bacterial cells were grown in the optimized medium with 100 μg/ml P_i to the stationary phase, collected by centrifugation, washed twice with sterile physiological saline solution (0.75% NaCl), and resuspended in the tested media to a cell density corresponding to that in the stationary growth phase.

To determine biomass and enzymatic activities, cell samples were withdrawn at 1-h intervals.

P_i was added before inoculation in the form of Na_2HPO_4 salt to a concentration of 50–100 μg P/ml.

Biomass determination. Biomass was determined from the culture turbidity measured at 590 nm using an FEK-56M photometer. One unit of biomass was defined as its amount corresponding to one optical density unit in a 1-cm-pathlength cuvette. The mean specific growth rate μ (h^{-1}) over the time period ($t_1 - t_0$) was calculated by the formula [12] $\mu_{mean} = (\ln m_1 - \ln m_0)/(t_1 - t_0)$, where m_0 and m_1 are the biomass values at times t_0 and t_1, respectively.

Cell transformation. *B. subtilis* cells were transformed by the method described in [13].

RNase assay. The activity of ribonucleases was determined from the amount of acid-soluble products of RNA hydrolysis, using a modification of the method of Anfinsen *et al.* [14]. The reaction mixture was supplemented with 1 mM $MgSO_4$. One unit of activity (E) was defined as the amount of enzyme causing an increase in absorbance at 260 nm (A_{260}) equal to 1.0 OD unit per 1 h at 37°C in a 1-ml volume. The productivity of RNase synthesis was estimated as the ratio of the RNase activity of the culture liquid to the culture biomass. The mean specific rate of RNase synthesis (ε), measured in h^{-1}, was determined analogously to the mean specific growth rate. In estimating the activity of binase II in the recombinant *B. subtilis* strain, the activity of the native RNase (Bsn) of this strain was neglected, as it was more than 25 times lower than that of binase II.

Analysis of inorganic phosphate. P_i in the culture liquid was determined as described elsewhere [10].

Reagents. The cultivation media were prepared using chemical and analytical grade salts from Reakhim (Russia), peptone with a P_i content not exceeding 1.5 mg/g dry weight (a product of a meat processing plant in Semipalatinsk), erythromycin purchased from the OAO Biokhimik (Saransk, Russia), actinomycin D from CALBIOCHEM (United States), and chloramphenicol from Fluka (Switzerland). RNases were assayed using high-molecular-weight yeast RNA purchased from the Research and Technology Institute of Biologically Active Substances (Novosibirsk, Russia).

Data processing. The results were statistically processed using the BIOPT software [15]. The cultivation media were optimized for the maximum RNase production in terms of the experimental factorial design B_2 [16].

Table 2. The effect of peptone and glucose on the growth and biosynthesis of RNase Bsn in the *B. subtilis* strain 3922 according to the data of experiments designed in terms of the factorial experimental design B_2

Experiment number	Factor level				Biomass, OD_{590}	RNase	
	peptone		glucose			E/(ml h)	specific activity, arb. units
	X_1	%	X_2	%			
1	+	5.0	+	2.0	5.6	475.0	84.8
2	–	1.0	+	2.0	2.7	152.5	56.5
3	+	5.0	–	0	4.1	262.5	64.1
4	–	1.0	–	0	2.4	13.75	5.9
5	+	5.0	0	1.0	5.7*	487.5*	84.9
6	–	1.0	0	1.0	2.9	212.5	73.6
7	0	3.0	+	2.0	4.3	355.0	88.5*
8	0	3.0	–	0	3.4	150.0	44.1

Regression equations: $Y = f(x_i)$,

$$Y_{\text{biomass}} = 4.50 + 1.24X_1 + 0.45X_2 - 0.18X_1^2 - 0.63X_2^2 + 0.30X_1X_2, \quad (1)$$

$$Y_{\text{RNase}} = 377 + 141X_1 + 93X_2 - 27X_1^2 - 124X_2^2, \quad (2)$$

$$Y_{\text{specific activity}} = 93 + 16X_1 + 19X_2 - 14X_1^2 - 26X_2^2 - 8X_1X_2. \quad (3)$$

* The asterisks mark the maximum values of response factors, which were taken as 1.0 when constructing the response factor isolines presented in Fig. 1.

Table 3. The effect of peptone and glucose on growth and binase II biosynthesis in the recombinant *B. subtilis* strain 3922 (pJF28) according to the data of experiments designed in terms of the factorial experimental design B_2

Experiment number	Factor level				Biomass, OD_{590}	RNase	
	peptone		glucose			E/(ml h)	specific activity, arb. units
	X_1	%	X_2	%			
1	+	6.0	+	2.0	7.3	9477.5	1298.5
2	–	2.0	+	2.0	2.3	7930.0	3461.0
3	+	6.0	–	0	4.4	7967.5	1589.0
4	–	2.0	–	0	2.45	4890.0	1980.5
5	+	6.0	0	1.0	6.0*	10000.0*	1667.0
6	–	2.0	0	1.0	2.4	8952.5	3676.5*
7	0	4.0	+	2.0	4.6	9925.0	2165.5
8	0	4.0	–	0	3.85	7517.5	1953.0

Regression equations: $Y = f(x_i)$,

$$Y_{\text{biomass}} = 4.31 + 1.75X_1 + 0.58X_2 - 0.011X_1^2 - 0.087X_2^2 + 0.76X_1X_2, \quad (4)$$

$$Y_{\text{RNase}} = 10631 + 945X_1 - 1155X_2 - 1155X_1^2 - 1910X_2^2 - 385X_1X_2, \quad (5)$$

$$Y_{\text{specific activity}} = 2648 - 760X_1 + 233X_2 + 23X_1^2 - 589X_2^2 - 442X_1X_2. \quad (6)$$

* The asterisks mark the maximum values of response factors, which were taken as 1.0 when constructing the response factor isolines presented in Fig. 1.

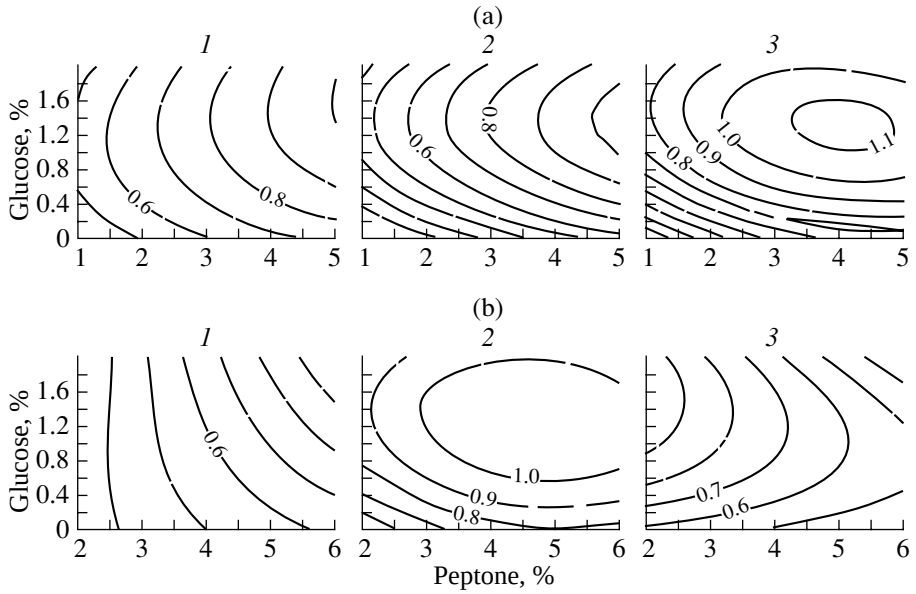

Fig. 1. The isolines of (*1*) the biomass and the (*2*) total and (*3*) specific activities of (a) RNase Bsn and (b) binase II computed from the data of experiments designed in terms of the factorial experimental design B_2. The maximum values of response factors, taken as 1.0, can be found in Tables 2 and 3.

Experiments were performed at least in triplicate. The results were computed for a 95% confidence level.

RESULTS AND DISCUSSION

Optimization of cultivation media for the maximum production of RNases. It is known that the activity of native binase II in *B. intermedius* is masked by the activity of binase I, which has the same pH optimum as binase II. For this reason, the biosynthesis of binase II was studied in the recombinant *B. subtilis* strain 3922 transformed with plasmid pJF28 bearing the binase II gene.

As was shown earlier, the expression of genes coding for secretory guanyl-specific bacillar ribonucleases is activated under conditions of inorganic phosphate deficiency, whereas an excess of P_i in the medium suppresses the synthesis of secretory ribonucleases at least in the bacillar species *B. intermedius, B. pumilus, B. thuringiensis*, and *B. circulans*. At the same time, the biosynthesis of barnase does not depend on the concentration of P_i in the medium [4, 6, 17]. Taking into account these data, we investigated the effect of the P_i concentration in the medium on the production of RNase Bsn and binase II (Table 1). As can be seen from this table, increasing the concentration of P_i in the medium led to an increase in the cell biomass and to a decrease in the total and specific activities of RNase Bsn and binase II. For this reason, P_i was not included into the composition of the starting nutrient media for the optimization of ribonuclease production.

Optimization experiments were designed in terms of two variable factors, the concentration of P_i-deficient peptone and the concentration of glucose, which were varied at three levels. The results of these experiments, including computer-derived regression equations, are presented in Tables 2 and 3. The isolines of the biomass and the total and specific activities of RNase Bsn and binase II are depicted in Fig. 1. As is evident from the positive sign of constants at the terms X_1 and X_2 in equations (1) and (4), both factors are necessary for active growth of the producing strains. The concentration of RNase Bsn in the culture liquid of *B. subtilis* 3922 will be at a maximum at concentrations of peptone and glucose equal to 6.0 and 1.4%, respectively. For the maximum concentration of binase II in the medium, the concentrations of peptone and glucose, calculated from the regression equations, must be 4.8 and 1.3%, respectively. The cell biomass was found to be proportional to the concentration of peptone in the medium, so that the specific activity of ribonucleases per unit biomass decreased with increasing peptone concentration. The maximum intensity of RNase Bsn biosynthesis was calculated to be at concentrations of peptone and glucose equal to 4.0 and 1.3%, respectively (Fig. 1a), whereas binase II is synthesized at a maximum rate in medium with 2.0% peptone and 1.4% glucose (Fig. 1b).

The dynamics of growth and ribonuclease synthesis in *B. subtilis* strains. The dynamics of growth and the synthesis of native binase II and RNase Bsn in the parent *B. subtilis* strain 3922 and the recombinant strain 3922 (pJF28) are shown in Fig. 2. The synthesis of the ribonucleases began in the phase of active growth and reached a maximum intensity in the seventh and

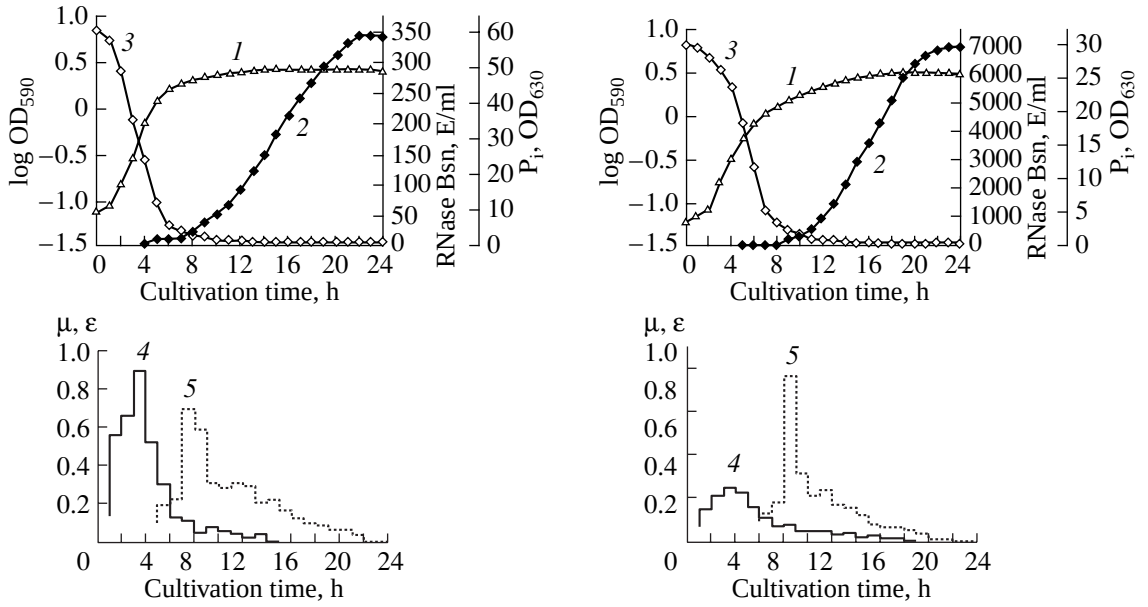

Fig. 2. The dynamics of the (*1*) biomass, (*2*) RNase activity in the culture liquid, and (*3*) P_i uptake and the calculated mean values of (*4*) the specific growth rate (μ, h^{-1}) and (*5*) the specific rate of RNase synthesis (ε, h^{-1}) in (a) *B. subtilis* 3922 and (b) *B. subtilis* 3922 (pJF28).

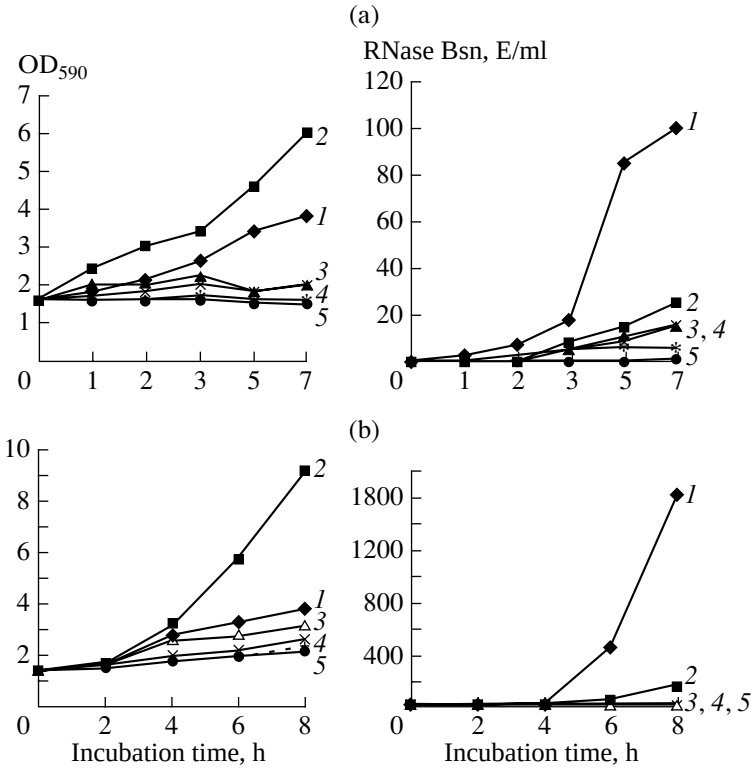

Fig. 3. The effect of chloramphenicol and inorganic phosphate on growth and RNase biosynthesis in (a) *B. subtilis* 3922 and (b) *B. subtilis* 3922 (pJF28): (*1*) control medium; (*2*) medium supplemented with 100 μg/ml P_i; (*3–5*) medium without added P_i but supplemented with 25, 50, and 100 μg/ml chloramphenicol, respectively.

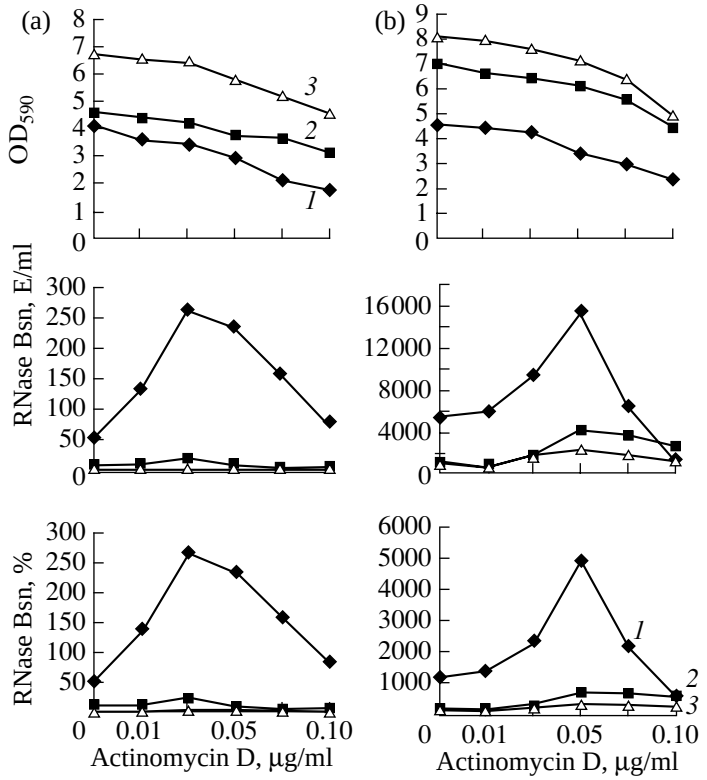

Fig. 4. The effect of actinomycin D and inorganic phosphate on the biosynthesis of (a) RNase Bsn in *B. subtilis* 3922 and (b) binase II in *B. subtilis* 3922 (pJF28): (*1*) control medium; (*2*, *3*) medium supplemented with P_i at concentrations of 50 and 100 μg/ml, respectively.

eighth hours of cultivation, when the specific growth rate of the strains was diminishing and the residual concentration of P_i in the medium had decreased to 3–4 μg/ml. The maximum activities of RNase Bsn (about 340 E/ml) and binase II (about 6800 E/ml) were observed between 20 and 22 h and 23 and 24 h of cultivation, respectively, when the producing strains were in the stationary growth phase.

The effect of P_i and chloramphenicol on the biosynthesis of binase II and RNase Bsn. The inhibitory action of P_i present in the medium on ribonuclease activity could be due either to the suppression of enzyme synthesis or to the inhibition of enzyme activity. In attempts to discriminate between these possibilities, we incubated *B. subtilis* 3922 and 3922 (pJF28) cells for 8 h in the P_i-deficient medium with and without the protein synthesis inhibitor chloramphenicol, which was added at concentrations of 25, 50, and 100 μg/ml. Chloramphenicol at high concentrations diminished the *B. subtilis* 3922 biomass by 48–61% and the activity of RNase Bsn in the medium by 75–99%. In this case, the biomass of the recombinant strain *B. subtilis* 3922 (pJF28) decreased by 16–43%, and the activity of binase II fell by 98–99% (Fig. 3). The drastic decrease in ribonuclease activity in the presence of chloramphenicol against the background of its weak effect on the biomass indicates that the rise in ribonuclease activity observed when washed cells grown in the P_i-rich medium were placed in the P_i-deficient medium was due to the de novo synthesis of ribonucleases, and not to activation of already existing enzymes.

The effect of actinomycin D on the synthesis of binase II and RNase Bsn. According to our earlier data, the transcriptional inhibitor actinomycin D stimulates the biosynthesis of the major ribonuclease of *B. intermedius*, binase I, only if this enzyme is actively synthesized under the conditions of inorganic phosphate deficiency [18, 19]. This prompted us to study the effect of actinomycin D on the biosynthesis of the other ribonuclease of *B. intermedius*, binase II, and the native RNase Bsn of *B. subtilis.*

In these experiments, actinomycin D was added to the culture media after 5 and 6 h of cultivation (in the growth retardation phase) at concentrations of 0.01, 0.025, 0.05, 0.075, and 0.1 μg/ml. The 22-h incubation of *B. subtilis* 3922 and 3922 (pJF28) cells with actinomycin D resulted in the suppression of the growth of both strains, the concentration of actinomycin D and the biomass accumulated in its presence being inversely proportional.

The addition of actinomycin D to the P_i-deficient medium at concentrations of 0.025 and 0.05 μg/ml

enhanced the activities of RNase Bsn and binase II in the culture liquid by 4.3 and 2.9 times and augmented the rates of their synthesis by 5.1 and 4.1 times, respectively. However, at higher concentrations of actinomycin D, its stimulating effect on the synthesis of ribonucleases decreased (Fig. 4).

The addition of actinomycin D to the medium containing P_i at concentrations inhibitory to ribonuclease biosynthesis increased the activities of RNase Bsn and binase II by only 41 and 57%, respectively, as compared with the ribonuclease activity observed in the medium with 50 μg/ml P_i. The addition of actinomycin D to the medium containing 100 μg/ml P_i virtually did not influence the ribonuclease activity. It can be suggested that the activating effect of actinomycin D on the synthesis of ribonucleases is not due to the removal of the inhibitory effect of P_i, just as is the case with the alkaline RNase of *B. intermedius* [18].

To conclude, the active biosynthesis of native RNase Bsn in the parent *B. subtilis* strain requires a higher concentration of peptone in the cultivation medium than the active biosynthesis of binase II in the recombinant strain. The nutrient medium optimal for the biosynthesis of binase II by this strain turned out to be almost identical to the medium optimal for the production of guanyl-specific cycling ribonucleases by recombinant *B. subtilis* strains [10]. The active synthesis of the ribonucleases begins in the growth retardation phase, when P_i is nearly completely exhausted in the medium. The biosynthesis of both RNases is subject to negative regulation by one of the metabolic effectors, P_i, and is activated by small doses of actinomycin D. The stimulating effect of this antibiotic is pronounced during the active synthesis of ribonucleases, but is insignificant when ribonuclease synthesis is inhibited by inorganic phosphate. The genes encoding binase II and RNase Bsn have been sequenced and their nucleotide sequences have been deposited in GenBank under the accession numbers X98086 and D01097, respectively. These genes exhibit 65% homology in the nucleotide sequences of their structural parts, but considerably differ in the structure of their promoters and the leader sequences. The investigation of molecular mechanisms involved in the regulation of the expression of the binase II and RNase Bsn genes is in progress in our laboratory.

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