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EXPERIMENTAL ARTICLES

Peculiarities of the Biosynthesis of *Bacillus intermedius* Glutamyl Endopeptidase in Recombinant *Bacillus subtilis* Cells during the Stationary Growth Phase

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Abstract—We studied the biosynthesis of *Bacillus intermedius* glutamyl endopeptidase in the recombinant *Bacillus subtilis* strain AJ73 Δ58.21 during the stationary growth phase. We optimized the composition of the culture medium to favor effective enzyme production during the stationary growth phase and found that the nutritional requirements for glutamyl endopeptidase synthesis were different in the stationary phase and the growth retardation phase. Proteinase accumulation was activated by complex organic substrates (casein and gelatin). During the final stages of the culture growth, the enzyme production was stimulated by Ca^{2+}, Mn^{2+}, and Co^{2+} and inhibited by Zn^{2+}, Fe^{2+}, and Cu^{2+}. The synthesis of glutamyl endopeptidase in the late stationary phase was not inhibited by glucose, unlike that in the trophophase during proliferation. We conclude that the regulatory mechanisms of proteinase synthesis during vegetative growth and sporulation are different.

Key words: glutamyl endopeptidase, regulation of biosynthesis, recombinant strain, growth conditions, sporulation.

Bacteria of the genus *Bacillus* are capable of rapid reorganization of biosyntheses in response to a changing environment. Unfavorable conditions induce in bacilli mechanisms of motility, competence, antibiotic production, and sporulation (transition to anabiosis). During this period, bacilli secrete a number of hydrolytic enzymes, including proteinases, which take part in cell differentiation and selective proteolysis related to metabolic reorganization. According to some data, proteinases play a leading role in sporulation [1]. During the stages of spore maturation and sporangium autolysis, proteinases take part in nonspecific lysis of proteins that form surface structures of the mother cell, which facilitates spore release.

Among the enzymes secreted by *B. intermedius,* we identified glutamyl endopeptidase, a proteinase with a narrow substrate specificity. The enzyme was isolated from culture liquid [2] and the mechanisms regulating its biosynthesis were determined [3–6]. The glutamyl endopeptidase gene of *B. intermedius* was cloned and its expression in *B. subtilis* cells was studied. Two recombinant plasmids were constructed, pV and Δ58.21. These plasmids differ in the size of the *B. intermedius* chromosomal DNA fragment [7] (Fig. 1). Plasmid pV carries a 6.2-kb fragment of *B. intermedius* DNA, which includes the glutamyl endopeptidase gene and adjacent sites of the *B. intermedius* chromosomal DNA. Plasmid Δ58.21 carries a 2.6-kb insertion and contains only the glutamyl endopeptidase gene. We studied the regularities of proteinase excretion by the recombinant strains *B. subtilis* pV and *B. subtilis* Δ58.21 [8–10] and found that the conditions of glutamyl endopeptidase biosynthesis were different for

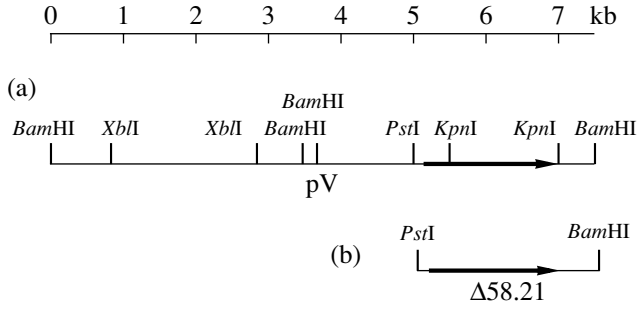

Fig. 1. Restriction map of the *B. intermedius* DNA fragment containing the gene of glutamyl endopeptidase localized in plasmids (a) pV and (b) Δ58.21 [7].

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these two strains in the growth retardation phase. Taking this into consideration, it is of interest to compare the conditions of glutamyl endopeptidase biosynthesis in the stationary growth phase.

The aim of the present work was to study the conditions of biosynthesis of *B. intermedius* 3-19 glutamyl endopeptidase in the recombinant strain *B. subtilis* Δ58.21 in the late growth stages.

MATERIALS AND METHODS

Bacillus subtilis strain AJ73 (*amy*E4, *npr*512, *apr*73) with deleted genes of extracellular proteinases was used as a recipient of plasmid DNA. The strain was kindly provided by Yu. Jomantas (State Research Institute of Genetics and Selection of Industrial Microorganisms). We used the multicopy plasmid Δ58.21, which carries the complete *B. intermedius* glutamyl endopeptidase gene [7] and differs from plasmid pV in the smaller size of the DNA insertion at the 5' regulation site. *B. subtilis* cells were transformed with plasmid DNA as described by Glover [11].

The basal cultivation medium contained (%) peptone, 2.4; yeast extract, 1.0; gelatin, 1.0; $CaCl_2 \cdot 2H_2O$, 0.02; $MgSO_4 \cdot 7H_2O$, 0.03; NaCl, 0.3; $MnSO_4$, 0.01; and Na_2HPO_4, 0.03; pH 8.5 [10]. The medium was supplemented with 20 μg/ml chloramphenicol since plasmid Δ58.21 carries a chloramphenicol resistance gene. The medium was sterilized at 1 atm. The solutions of inorganic phosphate (Na_2HPO_4), NH_4Cl, $C_6H_6O_7(NH_4)_2$, and metal salts were sterilized separately at 1 atm and added to the medium immediately before inoculation. The solutions of casein and gelatin were sterilized separately at 0.5 atm and also added to the medium immediately before inoculation. Peptone, yeast extract, gelatin, and Hammarsten casein were obtained from a Tbilisi plant, Difco Laboratories, Sigma, and Serva, respectively.

Incubation was performed in 100-ml flasks containing 20 ml of medium on a shaker (200 rpm) at 30°C. The flasks were inoculated with an 18-h culture in an amount of 1 vol %.

Biomass was evaluated nephelometrically at 590 nm.

Proteolytic activity was determined as described previously [2] with carbobenzoxy-L-glutamic acid *p*-nitroanilide (Z-Glu-pNA). One unit of proteolytic activity was defined as the quantity of enzyme hydrolyzing 1 μmol of substrate per minute under the conditions of the experiment. The result obtained was multiplied by 1000.

The efficiency of glutamyl endopeptidase synthesis (culture productivity) was defined as the ratio of proteolytic activity of the enzyme in culture liquid to the biomass and was expressed in arbitrary units.

The data were statistically analyzed [12]. The results of multifactorial experiments were processed with the STATGRAPHICS PC software, operating with regression equations, degrees of model reliability, and graphic display of response surfaces.

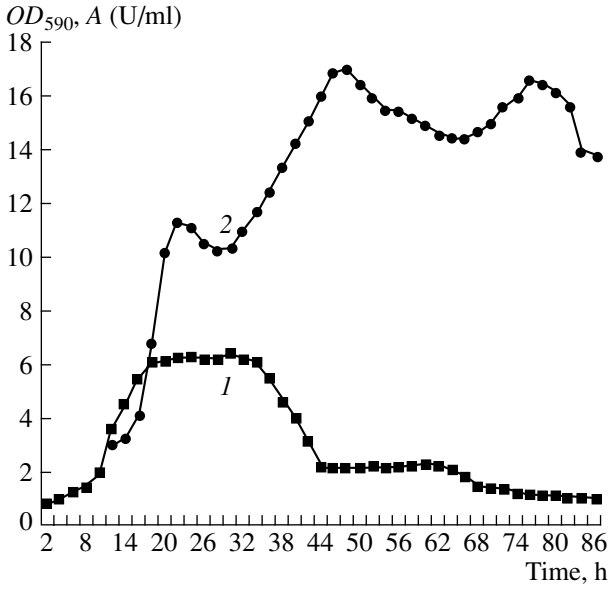

Fig. 2. Dynamics of (*1*) biomass growth and (*2*) glutamyl endopeptidase activity in a culture of the recombinant *B. subtilis* strain AJ73.

RESULTS AND DISCUSSION

Figure 2 shows the dynamics of culture growth and glutamyl endopeptidase accumulation in the culture liquid of the recombinant *B. subtilis* strain expressing *B. intermedius* glutamyl endopeptidase gene from plasmid Δ58.21. The glutamyl endopeptidase activity had two peaks, at the 48th and 78th hours of cultivation, which exceeded the level of the enzyme activity in the growth retardation phase (the 20th hour) by 1.5 times. Similar results were obtained earlier for the recombinant *B. subtilis* strain AJ73 pV [9]. We defined glutamyl endopeptidase produced during the growth retardation phase and the late stationary phase as early and late enzyme, respectively.

As the synthesis of extracellular enzymes is substantially determined by the composition of a culture medium, we studied the influence of different components of the medium on glutamyl endopeptidase accumulation in the culture liquid of the recombinant *B. subtilis* strain AJ73 Δ58.21 in the late stationary phase of growth.

In bifactorial experiments, we determined optimal concentrations of peptone and inorganic phosphate for the synthesis of the late glutamyl endopeptidase by the strain Δ58.21. These concentrations were varied at three levels. The defined levels of nutrients, biomass values (optical density, the means of triplicate experiments), glutamyl endopeptidase activity, and productivity of proteinase synthesis are presented in Table 1.

The enzyme accumulation in culture liquid as a function of concentrations of peptone and inorganic phosphate is presented in Fig. 3 in the form of a response surface and isolines of glutamyl endopepti-

Table 1. Optimization of the nutrient medium for the biosynthesis of glutamyl endopeptidase by the recombinant *B. subtilis* strain

Factor levels				48th hour of cultivation			78th hour of cultivation		
Peptone		Inorganic phosphate		Biomass, OD units	Activity, U/ml	Productivity, %	Biomass, OD units	Activity, U/ml	Productivity, %
*X*1	g/l	*X*2	g/l						
0	5	–	0.1	9	31.08	3.453	4.8	23.68	4.93
+	9	+	0.3	4.8	28.416	5.92	2	22.2	11.1
–	1	0	0.2	7.44	34.632	4.655	3.4	27.824	8.18
+	9	0	0.2	5.46	26.64	4.879	2.22	19.832	8.93
0	5	+	0.3	6.75	31.672	4.692	2.8	24.568	8.77
+	9	–	0.1	8.1	25.16	3.106	3.26	18.648	5.72
–	1	+	0.3	7.5	33.744	4.499	3.3	26.64	8.07
0	5	0	0.2	7.8	32.56	4.174	3.44	27.528	8
–	1	–	0.1	9.15	24.568	2.685	3.66	19.832	5.418

dase activity. In the picture, a zone of an optimum is clearly visible. The maximal enzyme production at the 48th hour of cultivation was observed at 4 g/l peptone and 0.28 g/l inorganic phosphate, whereas at the 78th hour it was observed at 4 g/l peptone and 0.26 g/l inorganic phosphate. Thus, the recombinant strain needed a six times lower peptone concentration for the enzyme synthesis in the late growth phases than in the growth retardation phase. This can partly be explained by reduction of nutrient demands due to transition of some part of the cells to anabiosis. In addition, the optimal peptone concentration for the late enzyme production for *B. subtilis* AJ73 Δ58.21 was five times less than that for the initial *B. intermedius* strain [6] and the recombinant *B. subtilis* strain AJ73 pV [9] (Table 2). This may be due to differences in the synthesis regulation determined by the size of the *B. intermedius* DNA fragment.

It is known that the rate of biosynthesis of an enzyme with narrow specificity sharply increases in the presence of the corresponding substrate or its derivative [13]. Figure 4 presents the effect of complex organic substrates (casein and gelatin) on the production of late glutamyl endopeptidase by the recombinant *B. subtilis* strain AJ73 at the final growth stages. Addition of 2%

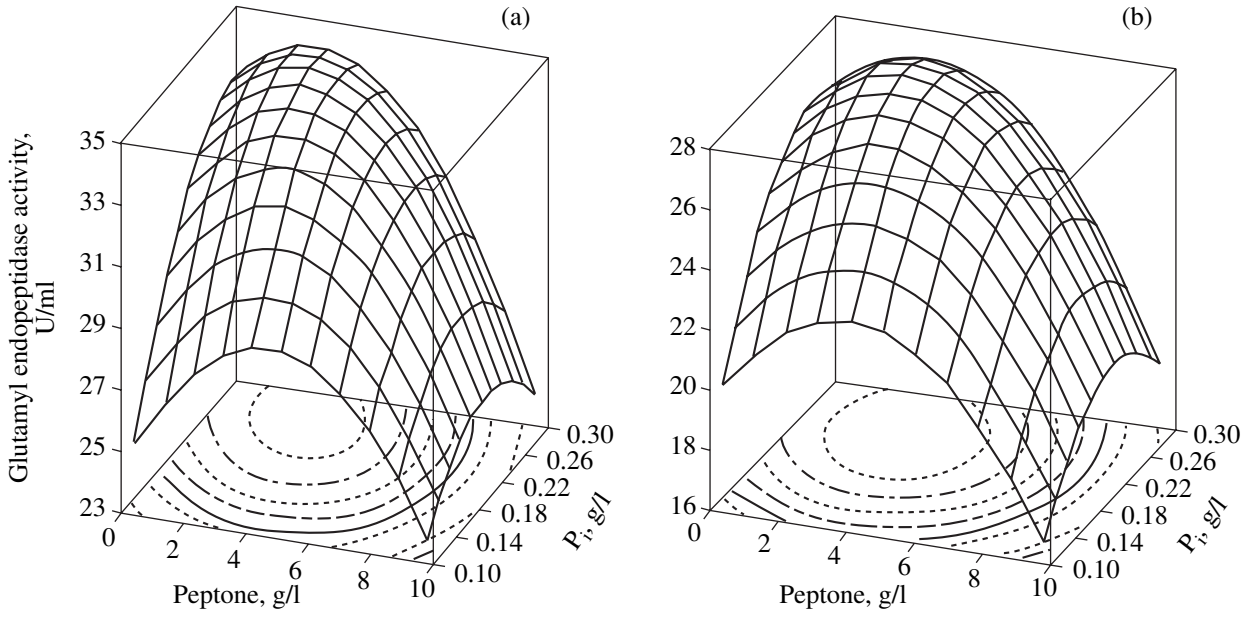

Fig. 3. Response surface and isolines of glutamyl endopeptidase activity obtained in bifactorial experiments with the recombinant *B. subtilis* strain AJ73 after (a) 48 and (b) 78 h of cultivation.

Table 2. Influence of the components of the nutrient medium on glutamyl endopeptidase accumulation in the culture liquids of *B. intermedius* and the recombinant *B. subtilis* strains

Microorganism	Enzyme		Components of nutrient medium														
			Glucose, 1%	Inorganic phosphate, g/l	Peptone, g/l	Protein substrates, %		Ammonium ions, mM		Bivalent cations, mM							
						Casein	Gelatin	Chloride	Citrate	Ca	Co	Zn	Mg	Mn	Fe	Cu	
B. intermedius 3-19	Glutamyl endopeptidase 1 (18th hour of growth) [3, 4]		Inhibition	0.2	20	No influence (0.5–2)		4	–	5	Stimulation of biosynthesis but inhibition of culture growth (1–10)	Biosynthesis inhibition (1–10)	2	Biosynthesis inhibition (1–10)	Biosynthesis inhibition (1–10)	Biosynthesis inhibition (1–10)	
	Glutamyl endopeptidase 2 (40th hour of growth) [6]		–	0.3	19	–	–	Biosynthesis inhibition (2–8)		–			–				
B. subtilis AJ73 pV	Early enzyme (22nd hour of growth) [8]		Inhibition	0.36	17.5	0.5	1	2	–	10			2				
	Late enzyme [9]	48th hour of growth	No influence	0.18	16	0.5	1	4	5	5			1				
		78th hour or growth		0.2	22	0.5	1	4	5	5			1				
B. subtilis AJ73 Δ58.21	Early enzyme (20th hour of growth) [10]		Inhibition	0.3	24	1	1	No influence (1–5)	–	2			1				
	Late enzyme [9]	48th hour of growth	No influence	0.28	4	2	2	Biosynthesis inhibition (1–6)		2			Biosynthesis inhibition (2–10)	1			
		78th hour or growth		0.26	4	2	2	1	No influence (1–6)	2				1			

Note: "–" means "no data."

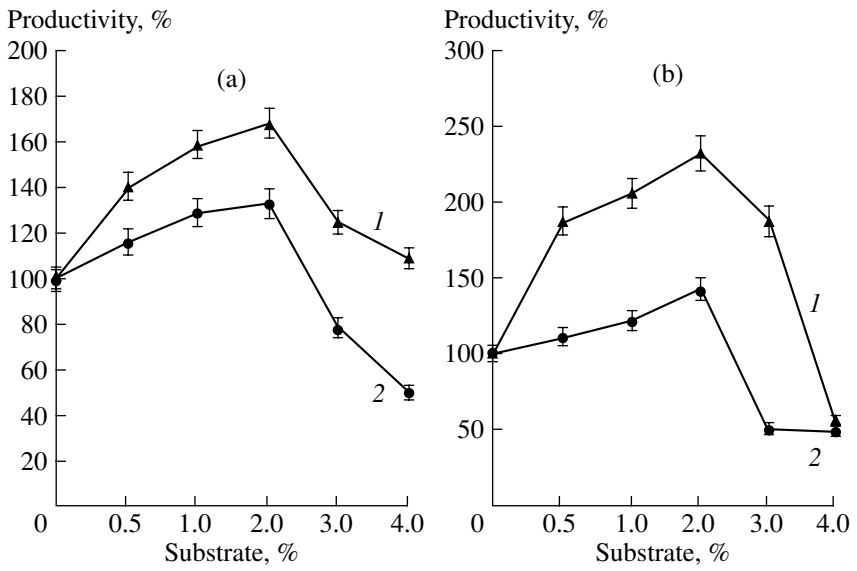

Fig. 4. Effect of (*1*) casein and (*2*) gelatin on the production of glutamyl endopeptidase by the recombinant *B. subtilis* strain AJ73 as determined after (a) 48 and (b) 78 h of cultivation. Productivity in medium without protein substrates was taken as 100%.

casein to the medium containing no organic substrates resulted in a 170% increase of glutamyl endopeptidase productivity by the 48th hour of incubation and a 230% increase by the 78th hour of incubation. Addition of 2% gelatin resulted in 130 and 140% increases by the 48th and the 78th hours of incubation, respectively. Thus, complex organic substrates stimulated glutamyl endopeptidase synthesis in the recombinant *B. subtilis* strain AJ73 Δ58.21 in the late growth phases, as in the growth retardation phase [10]. Similar data were obtained earlier for *B. subtilis* strain pV [8, 9]. However, the rate of production of glutamyl endopeptidase by the initial *B. intermedius* strain was not influenced by these substrates [3] (Table 2). This can result from the differences in the synthesis regulation in plasmidless and recombinant strains. It is known that recombinant plasmids can modify the levels of certain metabolites and affect the processes of cell metabolism regulation [14].

It is known that proteinase synthesis and secretion are strongly influenced by the presence of an additional nitrogen source in the form of an inorganic or an organic salt. Figure 5 presents the effect of ammonium salts (NH_4Cl and $C_6H_6O_7(NH_4)_2$) on the synthesis of late glutamyl endopeptidase by the recombinant *B. subtilis* strain AJ73 Δ58.21. Addition of ammonium citrate to a final concentration of 1–6 mM at the 48th hour of incubation resulted in an insignificant decrease in productivity (5–10%), whereas the inhibitory effect of ammonium chloride was more pronounced (up to 30%). On the contrary, addition of 1 mM ammonium chloride at the 78th hour of incubation resulted in a 45% increase in the enzyme production in comparison with the control, whereas addition of ammonium citrate did not cause a significant impact.

Earlier, we showed a repressive effect of ammonium ions on the production of the late glutamyl endopeptidase in *B. intermedius* cells [6]. However, the enzyme secretion increased in the presence of both organic (peptone) and inorganic (ammonium ions) sources of nitrogen [3]. It was shown that ammonium salts significantly stimulated the synthesis of early [8] and late [9] glutamyl endopeptidase by the recombinant *B. subtilis* strain pV but did not have any impact on early glutamyl endopeptidase secretion by the recombinant *B. subtilis* strain Δ58.21 [10] (Table 2). The difference in response of glutamyl endopeptidase secretion to nitrogen supplementation at different growth stages may be the consequence of modulation of mechanisms of glutamyl endopeptidase gene expression at different stages of the bacillar development cycle.

It is known that the presence of Ca^{2+} is obligatory for stabilization of glutamyl endopeptidase molecules [2, 15]. Earlier, we showed that Co^{2+} supplementation also resulted in an increase in glutamyl endopeptidase secretion [3].

We studied the effect of a number of bivalent cations on the production of late glutamyl endopeptidase by the recombinant *B. subtilis* strain AJ73 Δ58.21 (Fig. 6). We found that Ca^{2+} stimulated glutamyl endopeptidase production. In the presence of 2 mM Ca^{2+}, culture productivity increased by 180% at the 48th hour of incubation and by 166% at the 78th hour of incubation in comparison with the control (basic medium). Addition of 1% Mg^{2+} did not influence the glutamyl endopeptidase level, whereas a further increase of the Mg^{2+} concentration to 10 mM caused a 15% drop in productivity. The optimal Mn^{2+} concentration was found to be 1 mM (increases in activity by 135 and 156% at the 48th and the 78th hours of incubation, respectively). Addition of

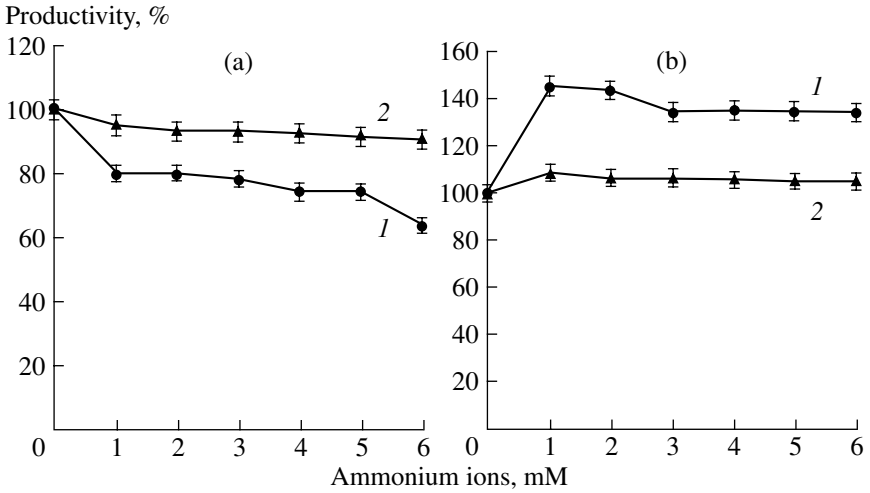

Fig. 5. Effect of (*1*) ammonium chloride and (*2*) ammonium citrate on the production of glutamyl endopeptidase by the recombinant *B. subtilis* strain AJ73 as determined after (a) 48 h and (b) 78 h of cultivation. Productivity in medium without ammonium ions was taken as 100%.

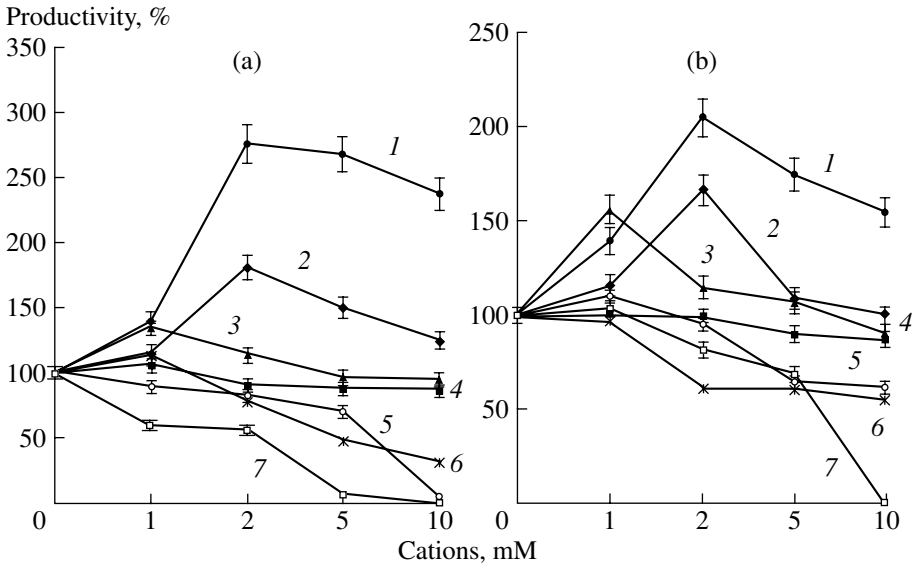

Fig. 6. Effect of the bivalent cations (*1*) Co^{2+}, (*2*) Ca^{2+}, (*3*) Mn^{2+}, (*4*) Mg^{2+}, (*5*) Zn^{2+}, (*6*) Fe^{2+}, and (*7*) Cu^{2+} on the production of glutamyl endopeptidase by the recombinant *B. subtilis* strain AJ73 as determined after (a) 48 and (b) 78 h of cultivation.

1–10 mM Zn^{2+}, Fe^{2+}, and Cu^{2+} resulted in a decrease in glutamyl endopeptidase level in the culture liquid. On the contrary, addition of 2 mM Co^{2+} resulted in an increase in culture productivity by 270% at the 48th hour of incubation and by 200% at the 78th hour of incubation, although culture growth was inhibited. Similar data were obtained earlier for *B. intermedius* [3] and *B. subtilis* strain AJ73 pV [8, 10] (Table 2). The data obtained on the late glutamyl endopeptidase synthesis by the recombinant *B. subtilis* strain AJ73 Δ58.21 are in accordance with the data on the early enzyme production by *B. intermedius* strain 3-19 and the recombinant *B. subtilis* strains pV and Δ58.21 [3, 8, 10].

According to a number of researchers, production of alkaline proteinases in bacilli correlates with the process of sporulation. Both processes are regulated via catabolic repression [1, 5].We studied the effect of glucose added to the culture liquid at different hours of incubation on the production of late glutamyl endopeptidase by the recombinant *B. subtilis* strain AJ73 Δ58.21 during the final growth phase (Fig. 7). Addition of 1% glucose in a trophophase (0–10 h of incubation) resulted in a sharp decrease in the level of late glutamyl endopeptidase. Addition of glucose from the 20th hour of incubation onwards (after the initiation of spore formation) did not cause a decrease in glutamyl endopep-

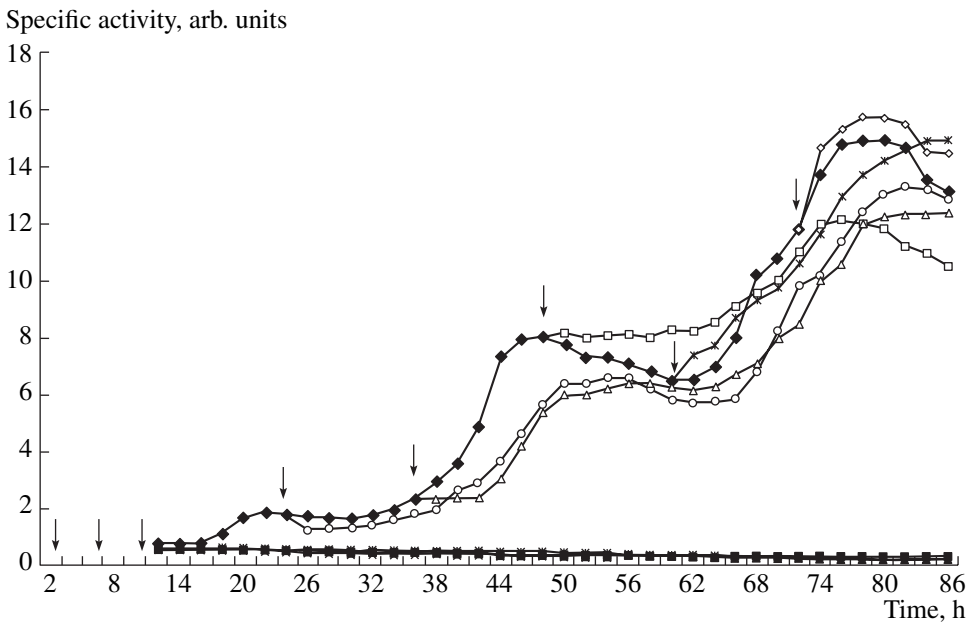

Fig. 7. Effect of glucose added to the culture at different growth stages on the production of glutamyl endopeptidase by the recombinant *B. subtilis* strain AJ73 Δ58.21. The level of glutamyl endopeptidase production in the absence of glucose is set in bold. Arrows indicate moments of glucose addition.

tidase specific activity. Thus, the recombinant strain carrying plasmid Δ58.21 is characterized by different regulatory mechanisms for the expression of the glutamyl endopeptidase gene at the stages of vegetative growth and sporulation.

These data show that cell differentiation in bacteria is accompanied by replacement of mechanisms regulating the expression of late genes. These mechanisms are active during the stationary growth phase, but the ways of their activation are different from those of the vegetative growth phase. Similar data were obtained earlier for *B. subtilis* strain pV [9].

Thus, the study of glutamyl endopeptidase gene expression on plasmids differing in insertion size (2.6 and 6.2 kb) showed that a reduction of the insertion at the 5' untranscribed site (plasmid Δ58.21) resulted in modification of the gene expression (a decrease in peptone demand, changes in nitrogen demand). The data obtained indicate that the gene expression may be influenced by distant regulatory sites within the regulation region.

The results of our work bring us to the conclusion that regulation of *B. intermedius* extracellular proteinase biosynthesis in the recombinant *B. subtilis* strain Δ58.21 during the stationary growth phase complies with general rules for regulation of serine proteinase synthesis. Production of late glutamyl endopeptidase was activated in the presence of one of the complex organic substrates of proteinase, casein or gelatin, and in the presence of bivalent cations (Ca^{2+}, Mn^{2+}, and Co^{2+}). Supplementing the medium with inorganic phosphate and peptone resulted in an increase secretion. Along with this, the mechanisms of regulation of the corresponding genes changed in the late stationary phase, which is determined by the complicated structure of the promoter region of catabolic genes [16]. The results of our study of glutamyl endopeptidase biosynthesis at various stages of culture development in the initial *B. intermedius* strain 3-19 and the recombinant *B. subtilis* strains AJ73 pV and Δ58.21 are summarized in Table 2.

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