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EXPERIMENTAL ARTICLES

Heterologous Expression of *Bacillus intermedius* Gene of Glutamyl Endopeptidase in *Bacillus subtilis* Strains Defective in Regulatory Proteins

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Abstract—Expression of the gene of glutamyl endopeptidase from *Bacillus intermedius* (*gseBi*) cloned on the plasmid pV has been studied in *Bacillus subtilis* recombinant strains with mutations of the regulatory proteins involved in sporogenesis and spore germination. It has been established that inactivation of the regulatory protein Spo0A involved in sporulation initiation resulted in a decrease in the expression of the *gseBi* gene by 65% on average. A mutation in the gene of the sensor histidine kinase *kinA* had no effect on the biosynthesis of the enzyme. Inactivation of Ger proteins regulating bacterial spore germination resulted in a 1.5–5-fold decrease in glutamyl endopeptidase activity. It has been concluded that expression of the *B. intermedius* glutamyl endopeptidase gene from plasmid pV in recombinant cells of *B. subtilis* is under impaired control by the regulatory system of Spo0F/Spo0A phosphorelay, which participates in sporulation initiation. The regulatory Ger proteins responsible for spore germination also affect expression of the gene of this enzyme.

Key words: Bacillus intermedius, glutamyl endopeptidase, recombinant strain, gene expression, sporulation.

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Glutamyl endopeptidases are enzymes with narrow substrate specificity towards the peptide bond formed by glutamic and aspartic amino acids [1]. More than 100 proteins belonging to the class of glutamyl endopeptidases have been characterized. These enzymes have been isolated from pathogenic microorganisms and from actinomycetes, streptomycetes, and bacilli [1–3]. Glutamyl endopeptidases have been found in animals and plants [4, 5]. However, the function of these specific enzymes, which have been revealed in all groups of living organisms, is still not completely clear.

Glutamyl endopeptidase was isolated from the culture liquid of the spore-forming bacterium *Bacillus intermedius* [6, 7]; the conditions of the biosynthesis of this enzyme were elucidated in [8, 9], and the gene encoding this protein was cloned and sequenced in [10]. The production of this enzyme commences during the phase of growth retardation; the maximal level of endopeptidase accumulation occurs in the late stationary growth phase when the cells produce various adaptive responses to nutrient deficiency and, in particular, the process of spore formation is initiated. The induction of sporulation in bacilli is a serial transfer of phosphate from several sensor histidine kinases to the regulatory protein Spo0A, which triggers transcription of the genes encoding different spore-specific proteins. At present, five histidine kinases have been described, which are involved in this process and autophosphorylate in response to as-yet unidentified signals [11]. The transfer of a phosphate group to the Spo0A protein also involves two phosphotransferases: Spo0F and Spo0B [12]. The main regulatory proteins in the process of spore germination are Ger proteins. Mutations in the genes encoding these proteins result in impaired or arrested spore germination and transformation into vegetative cells [13, 14].

The goal of this work was to study the expression of the gene encoding glutamyl endopeptidase from *B. intermedius* in strains with mutations in the regulatory proteins of the Spo0A phosphorelay system, which are involved in sporulation initiation, and in Ger proteins participating in spore germination.

MATERIALS AND METHODS

The multicopy plasmid pV constructed on the basis of vector pCB22 [15] was used in the work. The plasmid carries a 6.2-kb *Bacillus intermedius* DNA fragment including the gene of glutamyl endopeptidase

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Bacterial strains provided by Bacillus Genetic Stock Center (Ohio State University, USA)

Strain	Description
B. subtilis 168	*trpC2*
B. subtilis JH642	*pheA1 trpC2*
B. subtilis 9V	*spo0A9 trpC2*
B. subtilis JH648	*pheA1 spo0B136 trpC2*
B. subtilis JH649	*pheA1 spo0F221 trpC2*
B. subtilis R15-13	*spo0A12abrB23pheA1trpC2*
B. subtilis 4744	*gerA11thr5trpC2*
B. subtilis 4593	*gerD19trpC2*
B. subtilis 4751	*gerE36leu2*
B. subtilis MB170	*kinA82*

with its own promoter and the adjacent regions of *B. intermedius* chromosomal DNA. Strain *B. subtilis* AJ73, deficient in extracellular proteinases, was used as a recipient of plasmid DNA. Bacterial strains defective in the *spo-*, *kin-* and *ger* genes were obtained from the collection of *Bacillus* Genetic Stock Center (BGSC); they are listed in the Table. Transformation was performed as described in [16].

B. subtilis cells were cultured in 100-ml flasks, with a 1 : 5 ratio of medium to flask volume, on a shaker (200 rpm), at 30°C. *B. subtilis* strains defective in the *spo-*, *kin-* and *ger* genes were cultured in the Luria–Bertani medium [17]. Sterile Na_2HPO_4 solution was introduced immediately before inoculation to the final concentration of 0.3 g/l. The medium was sterilized at 1 atm. Erythromycin in a final concentration of 20 μg/ml was added to the medium, since plasmid pV carries the gene of resistance to this antibiotic. The 18-h culture was used as an inoculum (1% vol/vol).

Biomass increase was measured nephelometrically at 590 nm.

Proteolytic activity was determined by the method described previously [6], with *para*-nitroanilide of carbobenzoxy-L-glutamic acid (Z-Glu-pNA) as a substrate. The enzyme quantity hydrolyzing 1 nmol of substrate in 1 min under experimental conditions was taken as the unit of proteolytic activity.

Culture productivity with respect to glutamyl endopeptidase synthesis (specific activity) was determined as a ratio of the proteolytic activity in the culture liquid to the biomass and expressed in conventional units (CU).

Rabbit antiserum against glutamyl endopeptidase was obtained by three sequential immunizations of rabbits by pure enzyme solutions (1 mg of protein). The antiserum was purified by affinity chromatography using *B. intermedius* glutamyl endopeptidase immobilized on CNBr-activated Sepharose 4B (Amersham Biosciences).

Immunodiffusion (reaction by Ouchterlony) was carried out using rabbit antibodies to glutamyl endopeptidase. Enzyme preparations (20 μl; average protein content 1 mg/ml) were introduced into the wells. The slides with 1% agar (Difco) prepared in 0.85% NaCl solution were kept for 24 h in a moist chamber at room temperature.

For immunological analysis, glutamyl endopeptidase was isolated from the culture liquid of the native and recombinant strains and purified in a Mono S column using the FPLC chromatography system (Pharmacia) by the method described in [6].

For gene information analysis, the sequence of the *B. intermedius* glutamyl endopeptidase gene (EMBL AN Y15136) was used in the work. The sequence was easily accessible at the NCBI server (http://www.ncbi.nlm.nih.gov). The potential –10 and –35 regions for the recognition by the sigma A factor of transcriptional RNA polymerase were identified using the Softberry BPROM server (http://www.softberry.com). The sequence was analyzed with the Vector NTI V.8 software package.

Mathematical processing of the data obtained was performed in the Microsoft Excel software environment. The standard deviation (σ) was calculated; the results were considered reliable at $\sigma \leq 10\%$. Student's criterion was used to determine the reliability of the obtained differences, with $P \leq 0.05$ as the reliable level of significance.

RESULTS AND DISCUSSION

Expression of *B. intermedius* glutamyl endopeptidase gene (*gseBi)* on plasmid pV in *B. subtilis* recombinant cells starts in the phase of growth retardation and continues through the stationary phase of bacterial growth (Fig. 1). The maximal accumulation of the enzyme was recorded after 22, 38, and 78 hours of growth. The study of sporulation dynamics showed that the quantity of free spores reached 25% after 48 h of growth, 50% after 78 h, and did not change afterwards.

Glutamyl endopeptidase corresponding to 48 and 78 h of growth was purified from the culture liquid of the recombinant strain *B. subtilis* pV and identified by the method of immunodiffusion using rabbit antiserum against native glutamyl endopeptidase from *B. intermedius*. After the enzyme preparations isolated from the recombinant strain had been introduced into the wells, precipitates were formed against the hole containing the antiserum to glutamyl endopeptidase isolated from the initial strain. These data confirmed the identity of the enzymes secreted by the initial (*B. intermedius*) and recombinant (*B. subtilis*) strains, as well as identity of the enzymes from the early and late stationary phases.

Biosynthesis of various proteolytic enzymes is associated with the change of the physiological status of the cells in response to stresses [18]. For potential assessment of the mechanisms underlying the regulation of

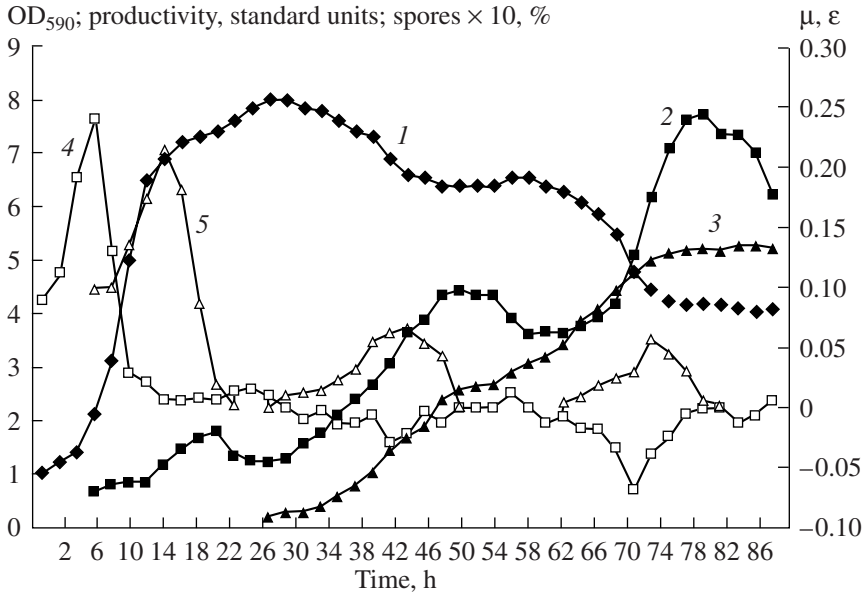

Fig. 1. The dynamics of culture growth, productivity of *B. intermedius* glutamyl endopeptidase, and spore formation by the recombinant strain *B. subtilis* AJ73pV: OD_{590}, optical units (*1*); productivity of *B. intermedius* glutamyl endopeptidase, conventional units (*2*); spore formation (*3*); specific growth rate, μ (*4*); specific rate of enzyme accumulation, ε (*5*).

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   1 cagctgccaccttcatctgaagtaccgatgatcatgatcggaccgggtacaggaatcgct
  61 ccatttagagggtttgttcaggcaagagaagtgtggcagaaggaaggaaanccactaggc
 121 gaagctcacctgtattttggctgtcgtcaccctctTGAAGATgatctgtatttgaggaa
 181 atgcagcttgcagcgcaaaaaggagttgttcacatccaccgggcttattctcgtcacaaa
 241 gagcaaaaagtatatgtccagcattgttgaaagaagacggcggcatgctgatcaagtta
 301 ctgaccaaggtgggtatctttacgtgtgcggggacggaaaagttatggcaccagacgta
 361 gaggctacgcttatcgacctctatcaaaacgagaaacattgctcgaaggaaacagctgaa
 421 aattggctgacaacccttgcaaatgacaatagatatgtaaaagatgtgtggagctgaaaa
 481 attaggaagaccgccaattaggcggtttttctatttcatagagagagatcaatagaatg
 541 aaggttggaaagatacaaaacacctaatttaaaaatgaaatattttgtaaaaaataagaa
                 −35                    −10          +1        SD
 601 tattctctcatttactccaatatgaaacaatcgtatgattttgatataggacataaagg

 661 aggaatatg
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Fig. 2. The nucleotide sequence of the promoter region of the *B. intermedius* glutamyl endopeptidase gene. The site of binding with ribosome (Shine–Dalgarno) as well as the –35 and –10 regions for recognition by transcription sigma A factor are underlined. The proposed site of transcription initiation is designated as +1. Potential sites for binding with the regulatory protein Spo0A (TGNCGAA) are marked in bold and underlined; those of binding with the AbrB protein (WAWWTTTWCAAAAAAW) are marked with color.

the expression of the enzyme gene, the analysis of its promoter region is required.

Fig. 2 presents the nucleotide sequence of the *gseBi* gene promoter region, within which we have identified potential sites of regulation with 86% homology to the consensus region for the interaction with the regulatory protein Spo0A (TGNCGAA). The revealed sequences are located as direct tandem repeats, which may increase the frequency of initiation of the proteinase gene transcription under certain conditions. In the promoter region of the *gseBi* gene, we have also found a nucleotide sequence with 81% homology to the con-

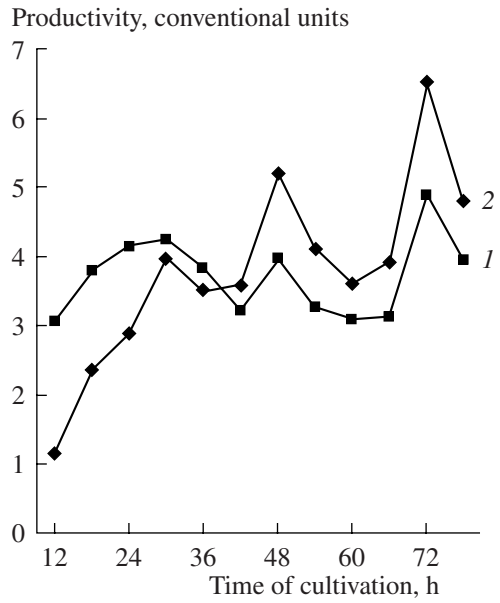

Fig. 3. Expression of the *B. intermedius* glutamyl endopeptidase gene in the strain with mutation in the *kinA* gene (*1*). The strain *B. subtilis* 168 with functionally active regulatory proteins was used as a control (*2*). $\sigma \leq 10\%$.

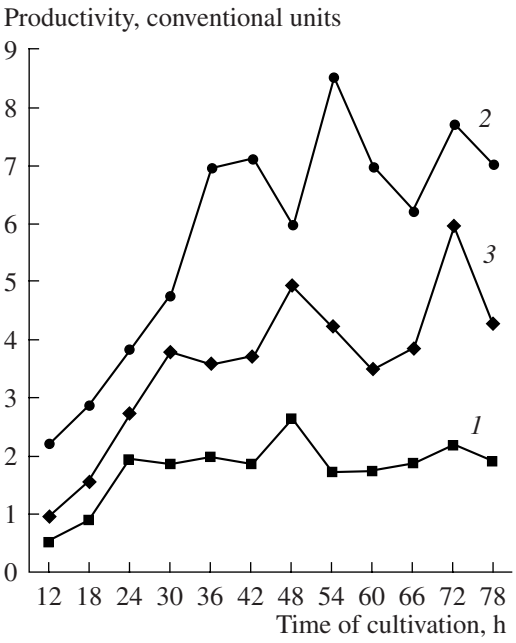

Fig. 4. Expression of the *B. intermedius* glutamyl endopeptidase gene in the strains with mutations in the *spo0A* and *spo0A*/*abrB* genes (*1*). The strain *B. subtilis* 168 with functionally active regulatory proteins was used as a control (*2*). $\sigma \leq 10\%$.

sensus sequence for binding by the ArbB regulatory protein (WAWWTTTWCAAAAAAW) that inhibits Spo0A during vegetative growth [19].

The expression of the *gseBi* gene in the *B. subtilis* strains mutant in protein components of the Spo0A phosphorelay system (KinA ⟶ Spo0F ⟶ Spo0B ⟶ Spo0A) has been studied in order to elucidate whether there is a regulatory interrelation between glutamyl endopeptidase synthesis and sporogenesis. *B. subtilis* strains mutant in these regulatory proteins were transformed by the plasmid pV carrying the gene of glutamyl endopeptidase from *B. intermedius*. Expression of the glutamyl endopeptidase gene in recombinant strains defective in the regulatory proteins was compared with expression of the gene in the same strains with the corresponding functionally active proteins.

Proteolytic activity in the strain with a defect in the gene of sensor histidine kinase *kinA* initially exceeded the activity in the control strain and then decreased; after 48 h it was 80% of the control (Fig. 3). The results demonstrate that inactivation of the *kinA* gene insignificantly affects the expression of the glutamyl endopeptidase gene. It is probable that the expression of the *gseBi* gene depends on the effect of other sensor histidine kinases (KinB, KinC, KinD, KinE) involved in the initiation of sporulation [11].

In the recombinant *B. subtilis* strain with inactivated Spo0A regulatory protein, production of the glutamyl endopeptidase from *B. intermedius* decreased by 50% on average (Fig. 4). Thus, the enzyme synthesis may be controlled by the regulatory protein Spo0A but does not fully depend on this transcription factor. Apparently, bacilli cells have a regulatory system which is able to control gene expression under inactivation of the Spo0A regulatory protein.

The *gseBi* gene expression was studied in the suppressor strain *spo0AabsB* carrying an additional mutation in the regulatory protein AbrB, which in vegetative cells suppresses the activity of the Apo0A protein [19]. In the strains with the double mutation, the defect in the *spo0A* gene is compensated for [19]. The glutamyl endopeptidase productivity in this mutant strain not only recovered to the control level but also increased 1.5–2-fold. These findings confirmed that the Spo0A regulatory protein contributes to the positive regulation of gene expression of the glutamyl endopeptidase from plasmid pV.

The effect of *spo0F* and *spo0B* mutations on glutamyl endopeptidase synthesis by recombinant strains of *B. subtilis* was investigated. Inactivation of these regulatory proteins resulted in a decrease in specific enzyme activity in the recombinant strain by 50–70% as compared with the control (Fig. 5). Thus, for the biosynthesis of *B. intermedius* glutamyl endopeptidase, the regulatory proteins Spo0B and Spo0F must be in a functionally active state since they are essential for phosphate transfer from the sensor protein to the response regulator Spo0A and for its maintenance in the active phosphorylated form. We have made

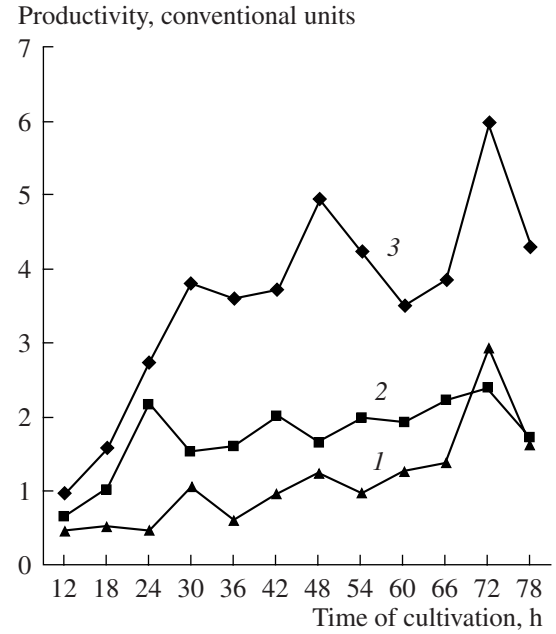

Fig. 5. Expression of the *gseBi* gene in strains with mutations in the *spo0B* and *spo0F* genes (*1*). The strain *B. subtilis* JH642 with functionally active regulatory proteins was used as a control (*2*). $\sigma \leq 10\%$.

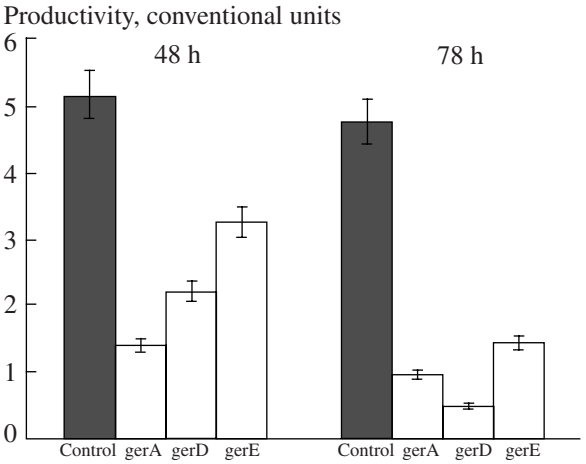

Fig. 6. Expression of the *gseBi* gene in strains with mutations in the *ger* genes. The strain *B. subtilis* 168 was used as a control. Mutations are designated under the corresponding column.

a conclusion that the regulatory system of Spo0A phosphorelay (Spo0F/Spo0A) makes a partial contribution to the positive regulation of the *gseBi* gene expression. Inactivation of the protein components involved in phosphate transfer to a response regulatory protein in this system of signal transduction caused partial suppression of the activity of the glutamyl endopeptidase gene. Thus, participation of this enzyme in spore formation is not apparent.

Germination of a bacterial spore initiates the process of its return to vegetative growth. We have supposed glutamyl endopeptidase to participate in the regulation of this process or some of its stages. The expression of the *gseBi* gene was studied in the cells forming spores unable to germinate. Such strains carried mutations in the genes encoding GerA, GerD, and GerE regulatory proteins involved in the process of spore germination. The maximal inhibitory effect was registered in the strain with the inactivated *gerA* gene (Fig. 6). The expression of *gseBi* in this B. *B. subtilis* strain decreased fourfold on average after 48 h and fivefold after 78 h of growth.

The regulatory protein GerA participates in the regulation of the synthesis of specific receptor proteins necessary for the spores to recognize nutrients (germination factors) [13]. Mutations in the *gerD* gene block the early stages of spore germination up to the stage of its losing heat resistance [13]. The mutations in *gerE* induce changes in the spore envelope ultrastructure [14].

The endopeptidase activity in the strain carrying a mutation in the *gerD* gene was shown to decrease more than twofold after 48 h of growth and tenfold after 78 h of growth. A 1.5-fold decrease in the endopeptidase activity after 48 h and a fourfold decrease after 78 h of growth were observed in the strain with the inactivated *gerE* gene. Thus, the regulatory Ger proteins had a positive effect on the expression of the *gseBi* glutamyl endopeptidase gene, with a more marked effect towards the enzyme synthesized during the late stationary phase.

It has been shown previously that in vegetative cells of *B intermedius* in the phase of growth retardation, the expression of the glutamyl endopeptidase gene is controlled by a DegS/DegU system [20]. Our findings demonstrate that the *gseBi* gene expression in the stationary growth phase is under positive regulation by the main regulatory proteins of the signal-sensor system of the Spo0A phosphorelay, which triggers transcription of the genes of spore-specific proteins and of the proteins responsible for endospore germination. Nevertheless, according to our data, none of the variants corresponding to "switching off" of each of the studied regulation systems shows complete inactivation of the proteinase gene. The level of the *gseBi* gene expression is maintained within 40–80% in the *B. subtilis* regulatory mutants, which may be evidence of crossing pathways of controlling this gene activity. It can be concluded that the expression of the *B. intermedius* glutamyl endopeptidase gene is modulated by different cell regulatory systems and is realized by the type of cross regulation.

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