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EXPERIMENTAL ARTICLES

Biosynthesis of the Subtilisin-like Serine Proteinase of *Bacillus intermedius* under Salt Stress Conditions

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Abstract—The biosynthesis of the subtilisin-like serine proteinase of *Bacillus intermedius* 3-19 by the recombinant strain *Bacillus subtilis* AJ73(pCS9) was found to be enhanced under salt stress conditions (growth in a medium containing 1 M NaCl and 0.25 M sodium citrate). In a recombinant strain of *B. subtilis* deficient in the regulatory proteins DegS and DegU, which control the synthesis of degradative enzymes, the expression of the proteinase gene was inhibited. In contrast, in the strain *B. subtilis degU32*(Hy), which provides for the overproduction of proteins positively regulated by the DegS–DegU system, the biosynthesis of the subtilisin-like proteinase of *B. intermedius* 3-19 increased by 6–10 fold. These data suggest that the DegS–DegU system is involved in the positive regulation of the expression of the subtilisin-like *B. intermedius* proteinase gene in recombinant *B. subtilis* strains.

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Key words: *Bacillus*, subtilisin-like serine proteinase, recombinant strain, salt stress, two-component system.

In natural environments, microorganisms are constantly exposed to stressful conditions, such as heat, cold, and salt shock, deficiency of nutrients and microelements, etc. To tolerate and counteract the impact of these stresses, microorganisms have evolved various adaptive mechanisms based on the signal transduction pathway, which represents a two-component system consisting of a sensory protein (histidine kinase) and a regulatory protein. The phosphorylated form of the latter protein activates the transcription of specific genes [1]. Nutrient deficiency may activate various two-component signal transduction systems. For example, the PhoP–PhoR system responds to a deficiency of inorganic phosphate in the medium by activating the biosynthesis of enzymes of phosphorus metabolism [2]. The DegS–DegU system controls the biosynthesis of degradative enzymes. This system is related to the ComP–ComA system responsible for cell competence [3, 4]. The activation of these regulatory systems results in the synthesis and secretion of various extracellular hydrolases, antibiotics, and peptides. The synthesis of degradative enzymes in *Bacillus subtilis* considerably depends on the medium salinity. For example, salt stress enhances the production of extracellular levan sucrase in *B. subtilis* by nine times [5]. Dartois et al. [6] showed that the *WapA* gene encodes a protein associated with the cell wall, which is synthesized by bacteria during their growth at low salt concentrations. The presence of 0.7 M sodium succinate in the medium inhibits the biosynthesis of WapA due to suppression of the expression of the gene responsible for the formation of the phosphorylated form of the regulatory protein DegU.

The bacterium *Bacillus intermedius* secretes into the medium various proteinases, which are dominated (more than 80% of the total extracellular proteinases of this bacterium) by the subtilisin-like serine proteinase encoded by the *aprBi* gene [7]. This proteinase appears in the medium in the growth retardation phase and reaches its maximum content in the late stationary phase, when mature endospores are released into the medium [7]. Analysis of particular proteinases isolated from the culture liquid of *B. intermedius* grown for 24 and 48 h showed that their *N*-terminal sequences are identical [8, 9]. The gene that encodes these proteinases was cloned and sequenced in its regulatory and coding regions (AN AY754946) [10].

The aim of this work was to study the effect of salt stress on the expression of the *aprBi* gene in a recombinant *B. subtilis* strain deficient in extracellular proteinases and to investigate the regulatory mechanism of proteinase biosynthesis under salt stress conditions.

MATERIALS AND METHODS

The streptomycin-resistant strain *Bacillus intermedius* 3-19 was obtained from the Culture Collection at

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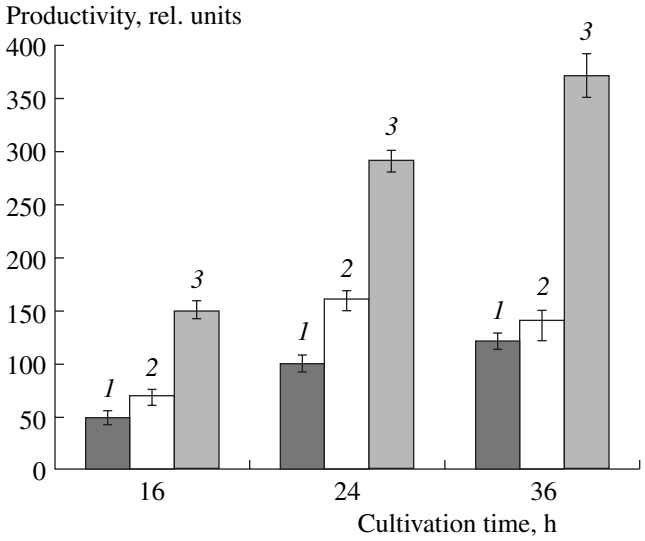

Fig. 1. The effect of high salt concentrations in the cultivation medium on the biosynthesis of the subtilisin-like serine proteinase of *B. intermedius* 3-19: (*1*) medium without added salts, (*2*) medium supplemented with 1 M NaCl, (*3*) medium supplemented with 0.25 M sodium citrate.

the Kazan State University. Plasmid pCS9 carries the erythromycin resistance gene and the complete gene of the subtilisin-like serine proteinase of *B. intermedius* 3-19 within a 6.0-kb region of its chromosome [10]. The proteinase-free recipient strain *Bacillus subtilis* AJ73, whose chromosome contains no genes of extracellular proteinases, was kindly provided by Yu. Yomantas from the Institute of Genetics and Selection of Industrial Microorganisms. The other strains used in this study were obtained from Jan Maarten van Dijjl from the University of Groningen, The Netherlands. These strains included *B. subtilis* 8G5 *degSdegU* (like 8G5 Δ*degS*Δ*degU*; *Km*^{r}) deficient in the *degS* and *degU* genes of the DegS–DegU system; *B. subtilis* 8G5 *degU32*(Hy) (like 8G5 *degU32*(Hy), *Km*^{r}) with a mutation in the *degU* gene leading to the Hy phenotype; and *B. subtilis* 8G5 (*trpC2*, *tyr, his, nic, ura, rib, met, ade*), which is auxotrophic with respect to tryptophan, tyrosine, histidine, nicotinamide, uracyl, riboflavin, methionine, and adenine and lacks the signal peptidase SipP genes but contains the functionally active regulatory proteins DegS and DegU. The last strain was used as a control in experiments on the strains with mutations in the respective genes.

B. intermedius 3-19 and the recombinant *B. subtilis* strains were cultivated in LB broth [11]. Salt stress was produced by adding 1 M NaCl or 0.25 M sodium citrate to this medium. Antibiotics were added to the following final concentrations (μ/ml): erythromycin, 20; streptomycin, 100; and kanamycin, 10. The inoculum (15-h-old culture grown in LB broth supplemented with an antibiotic) was added in an amount of 1 vol %. Cultivations were carried out at 30°C in 100-ml Erlenmeyer flasks one-seventh full of the growth medium on a shaker (200 rpm). Growth was monitored by measuring culture turbidity at 590 nm using a KFK-2 photoelectrocolorimeter.

Proteolytic activity was determined with the chromogenic substrate Z-Ala-Ala-Leu-pNa [12]. One unit of activity was defined as the amount of enzyme that hydrolyzed 1 nmol substrate per 1 min.

The specific activity of the enzyme (the biosynthetic productivity of the culture) was determined as the ratio of the proteolytic activity of the culture liquid to the biomass and expressed in relative units.

B. subtilis cells were transformed with plasmid DNA as described by Chang and Cohen [13].

Gene sequences were analyzed by using the known proteinase gene sequences (http://www.ncbi.nlm.nih.gov) with the aid of BLAST software available at http://www.ncbi.nlm.nih.gov/blast [14].

The results were statistically processed using Student's *t*-test statistics for significance level $P \leq 0.05$. Calculations were carried out with the aid of SPSS 12.0 software. The results were considered to be confident when the standard deviation σ was equal to or less than 10%.

RESULTS AND DISCUSSION

Measurements of the amount of subtilisin-like serine proteinase in the culture liquid of *B. intermedius* 3-19 grown in various media (Fig. 1) showed that the addition of 1 M NaCl and 0.25 M sodium citrate enhanced the biosynthesis of this proteinase by 1.5 and 3 times, respectively, as compared to the control (proteinase synthesis in LB broth without added salts). It should be noted that the addition of sodium citrate inhibited bacterial growth by 1.5–2 times.

Bacilli are known to be active producers of various proteolytic enzymes. Thus, *B. intermedius* synthesizes two serine proteinases and one neutral proteinase [7]. To estimate the effect of salt stress on the subtilisin-like serine proteinase, we transformed the strain *B. subtilis* AJ73 deficient in its own extracellular proteinases with plasmid pCS9 carrying the subtilisin-like serine proteinase gene of *B. intermedius* 3-19. The addition of 1 M NaCl did not affect the growth of the recombinant strain *B. subtilis* AJ73(pCS9) (Fig. 2a) but increased the level of proteolytic activity in the culture liquid by two times. This level remained higher than in the control for 36 h of growth (Figs. 2b, 2c). In the medium with 0.25 M sodium citrate, bacterial growth was slower than in the control (Fig. 2a) probably because of the inhibitory action of citrate on the bacterium [19].

The addition of citrate extended the exponential and retardation growth phases, due to which enzyme production remained high for as long as 48 h of growth (Figs. 2b, 2c). The productivity of enzyme biosynthesis was 15 times as great as in the control. During growth in the saline medium, spores began to appear after 40 h of cultivation, whereas in the salt-free medium, they

appeared as soon as after 28 h of cultivation. This observation suggests that the addition of salts to the medium extends the period of vegetative bacterial growth. The first peak of proteolytic activity, which was observed in the salt-free medium after 24 h of growth (Fig. 2b), was delayed for 4 h in the presence of 1 M NaCl and for 16 h in the presence of 0.25 M sodium citrate. These data provide evidence that salt stress enhances the production of subtilisin-like serine proteinase in the recombinant *B. subtilis* strain.

According to the data available in the literature, bacilli respond to salt stress by increasing the synthesis of proteins controlled by the DegS–DegU system, which is activated by high concentrations of sodium and potassium ions [5, 6]. With this in mind, we hypothesized that this system is also involved in the regulation of the biosynthesis of the subtilisin-like serine proteinase of *B. intermedius* 3-19.

The potential binding sites of the regulatory DegU protein were sought in the promoter region of the *aprBi* gene with the known sequence [10]. In the regulatory regions of the target genes, DegU interacts with the respective consensus sequence AGAA-$N_{(11–13)}$-TTCAG (DegU box) [6]. In the 5'-nontranscribable region of the *aprBi* gene, located 460 bp from the potential initiation site of translation, we identified eight DNA regions with a high degree of homology (70–80%) to the DegU box (Fig. 3). These regions are located in the regulatory region of the proteinase gene as direct tandem repeats. It should be noted that Jan et al. [16] also identified binding sites for the DegU protein in the promoter region of the *aprE* gene, which encodes the *B. subtilis* subtilisin. A comparative analysis of the *aprBi* gene promoter with the gene promoters of subtilisin-like proteinases in various bacilli (*B. thuringiensis* subsp. *kurstaki*, *B. pumilus* TYO-67, *Bacillus* sp., and *B. licheneformis* x91261) showed that the regulatory regions of these genes also contain regions homologous to the DegU boxes, which represent direct tandem repeats (Fig. 4). Such a disposition of the regulatory sites must enhance the frequency of transcription initiation in response to activation of the signal transduction pathways [17]. Therefore, the observation of DNA regions homologous to the DegU boxes implies the involvement of the regulatory DegS–DegU system in the regulation of the expression of the *aprBi* gene.

The biosynthesis of the *B. intermedius* proteinase was studied in the *B. subtilis* strains with mutations in the genes encoding the proteins of the regulatory DegS–DegU system. In *B. subtilis* 8G5 Δ*degS*Δ*degU* with deletions in the *degS* and *degU* genes transformed with the *B. intermedius* subtilisin-like serine proteinase gene *aprBi*, the productivity of proteinase synthesis during growth in LB broth was 60% lower than in the case of the control strain *B. subtilis* 8G5(pCS9) with the functional regulatory DegS–DegU system (Fig. 5a). These data indicate that the efficient expression of the *aprBi* gene requires a functionally active regulatory DegS–DegU system.

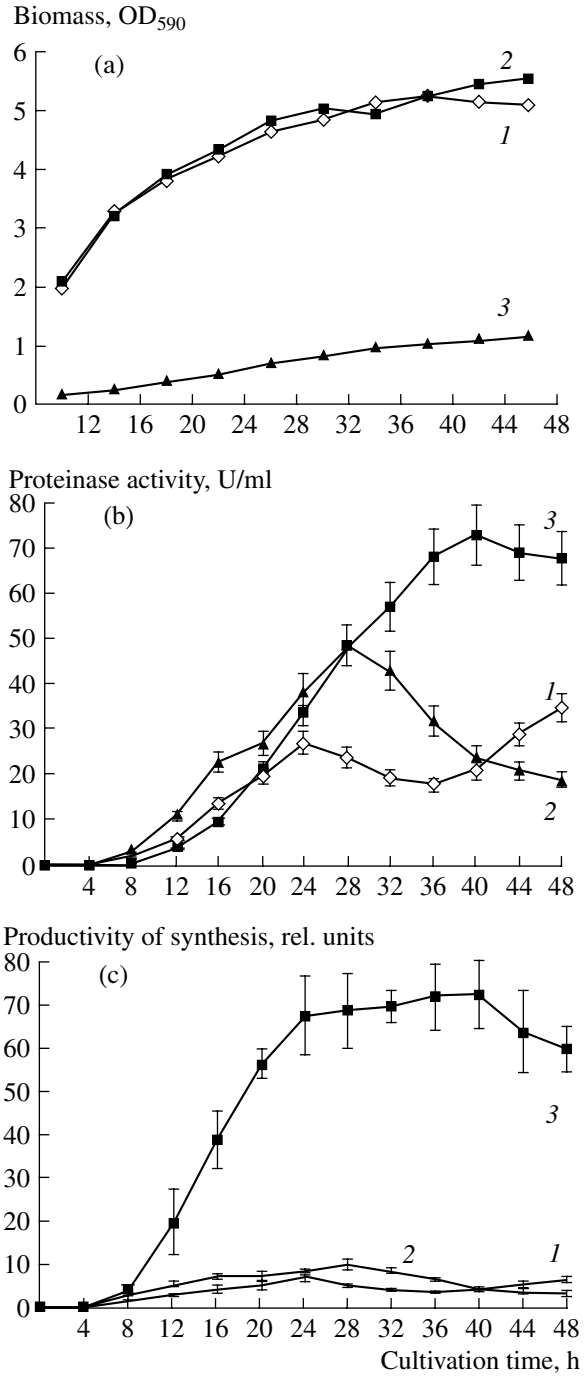

Fig. 2. The effect of high salt concentrations in the cultivation medium on the biosynthesis of the subtilisin-like serine proteinase of *B. intermedius* 3-19 in the recombinant strain *B. subtilis* AJ73(pCS9): (a) biomass, (b) enzyme activity, (c) productivity of enzyme synthesis, (*1*) medium without added salts, (*2*) medium supplemented with 1 M NaCl, (*3*) medium supplemented with 0.25 M sodium citrate.

We also studied the expression of the cloned *aprBi* gene in the mutant strain *B. subtilis* 8G5 *degU32*(Hy)

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1   GAATGGAAGGTCCTTGATTACAACGTGGTCAGCCATTTACTC
                                    78%
43  CATCCTCCCCTTTTTAAAGAACCTGTTATTGTAACAGGTTNT

85  TTTTNAATGCCAAAACCAAAAAATAATATTTTTTTATATCGA
                        67%
127 AATTCGAAATAGATGCTAGACGTTTCTACCTATTTTAAGGCT

169 TTTCGGGTATCGAATATTTGTCCGAAAATGGATCATAAGAAA
          78%                       67%
211 AAAAGCACACTTCCTTTTTTAATAGATAACCGCTGAAACAGCA
                    67%
253 GAACAAACATATTTTCCCAACGTTTCCAAGTGACTTAATTCC
                                  78%
295 CCAATTTTCGCTAGGACTTTCACAAAAATTCGGGTCTACTCT
                                          67%
337 TATTTGCCTACTTCCCTTAAACTGAATATACAGAATAATCAA
                                  78%
379 ACGAATCATTCTTATAGACTACGAATGATTATTCTGAAATAA

421 GAAAAAAGGGATGTGGATTGTGCGTGAAAAAGAAAAATGTGA
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Fig. 3. Promoter region of the subtilisin-like serine proteinase of *B. intermedius* 3-19 with potential binding sites for the regulatory protein DegU (A(G/A)AA-$N_{(11–13)}$-TTCAG). Interaction sites are underlined, canonical sequences are highlighted. The initiating codon GTG is given in italics and underlined.

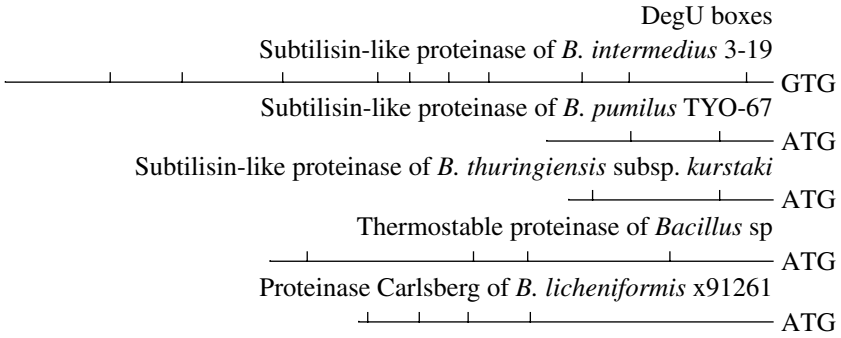

Fig. 4. Schematic disposition of interaction sites for the regulatory DegU protein in the promoter sequences of the subtilisin-like serine proteinase of various bacilli.

with an enhanced production of the phosphorylated form of the regulatory DegU protein [3] (this mutant is distinguished by the overproduction of the proteins whose synthesis is regulated by the DegS–DegU system). The transformation of this strain with plasmid pCS9 carrying the *aprBi* gene resulted in an increase in the proteinase productivity in LB broth by 6–10 times as compared to the control strain *B. subtilis* 8G5(pCS9) (Fig. 5b). According to the data of Msadek et al. [3] and Dartois et al. [6], the level of degradative enzymes regulated by the DegS–DegU system (such as levan sucrase, amylase, and glucanase) with the *degU32*(Hy) mutation can increase tenfold, whereas in a strain deficient in the DegU protein, gene expression can decrease to as low as 3–6%. These results allowed us to infer that the phosphorylated form of the regulatory DegU protein enhances the expression of the *aprBi* gene and, hence, that the synthesis of the subtilisin-like proteinase of *B. intermedius* 3-19 in the recombinant *B. subtilis* strain is controlled by the regulatory DegS–DegU system.

To conclude, the data obtained in the present study show that the two-component signal transduction DegS–DegU system is a positive regulator of the expression of the subtilisin-like serine proteinase gene of *B. intermedius.* This proteinase may be involved in the adaptive processes activated during transition to the stationary growth phase. Earlier, we showed that high

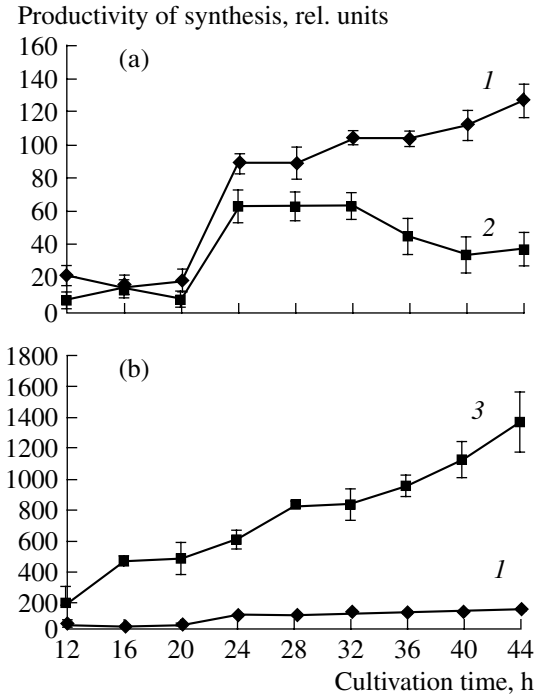

Fig. 5. Biosynthesis of the subtilisin-like serine proteinase of *B. intermedius* 3-19 in LB broth by the recombinant *B. subtilis* strains with mutations in the genes encoding the regulatory DegS and DegU proteins: (*1*) *B. subtilis* 8G5(pCS9) (control), (*2*) *B. subtilis* 8G5 Δ*degS*Δ*degU* (pCS9), (*3*) *B. subtilis* 8G5 *degU32*(Hy) (pCS9).

medium salinities stimulate the synthesis of another serine proteinase, glutamyl endopeptidase, of *B. intermedius* [18]. At high salt concentrations, bacterial cells accumulate various low-molecular-weight osmoprotectants (such as glutathione, glutamine, glutamate, and proline [19, 20]), which are not synthesized by the cells but are transported from the medium [21, 22]. An intense degradation of various carbon-containing substrates, including proteins, is necessary to maintain a required intracellular pool of the mentioned low-molecular-weight substances. It is tempting to suggest that the enhanced level of biosynthesis of the extracellular proteinase, which is involved in the nonspecific cleavage of proteins to oligopeptides and amino acids, serves as a defense mechanism against salt stress.

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