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EXPERIMENTAL ARTICLES

Effect of the Regulation System of Metabolic Nitrogen Exchange on Biosynthesis of Serine Proteinases from *Bacillus intermedius*

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Abstract—The regulatory link between biosynthesis of *Bacillus intermedius* subtilisin-like serine proteinase and nitrogen metabolism in *B. intermedius* cells was determined. The level of the enzyme biosynthesis by the recombinant strain of *Bacillus subtilis* in the medium containing ammonium ions was three- to fivefold less than that in the medium with poorly utilized sodium nitrate. Accumulation of glutamyl endopeptidase in a culture liquid of this microorganism did not depend on the source of nitrogen present in the medium. During cultivation in the rich medium, the productivity of subtilisin-like proteinase in the recombinant *B. subtilis* strain carrying a mutation in the NrgB sensor protein was demonstrated to increase threefold compared to that of the control strain. In the minimal culture medium, mutation in the *nrgB* gene abolished the effect of a nitrogen source on the level of the subtilisin-like proteinase gene expression. At the same time, this mutation did not affect glutamyl endopeptidase biosynthesis. Thus, expression of the gene coding for subtilisin-like proteinase from *B.intermedius* is suggested to be positively regulated by the regulatory system of nitrogen metabolism.

Key words: Bacillus intermedius, subtilisin-like proteinase, glutamyl endopeptidase, nitrogen catabolite repression.

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Proteinases are the enzymes of strategic importance for microorganisms. In bacterial cells they are involved in cell differentiation, in a set of regulatory processes, and carry out the trophic function as well. The big class of proteolytic enzymes is the class of serine proteinases [1]. Most of the extracellular proteinases are sensitive to the carbon catabolite repression. In the presence of an easily metabolized carbon source (glucose), the CcpA regulatory protein suppresses expression of the genes coding for the proteins which provide utilization of alternative carbon sources such as citrate [2, 3]. On the other hand, co-regulation between carbon catabolite repression and nitrogen catabolite repression is established: the genes involved in utilization of amino acids were shown to be controlled by both systems [4], and nitrogen deficiency may partially switch off the catabolite repression caused by glucose [5]. The preferable nitrogen sources for bacteria are ammonium salts and glutamine. The absence of these substances in the culture medium leads to an activation of the genes coding for the enzymes responsible for extraction of nitrogen from various organic and inorganic compounds (nitrate, nitrite, urea, purines and pyrimidines, amino acids, etc.) In this case ammonium ions formed on the cell membrane by the nitrate-reductase complex are directed into the cell by the NrgA transport protein [6]. NrgA acts in complex with the NrgB protein, which exhibits close homology to PII sensor proteins. Probably, NrgB provides the cell with the information concerning accessibility of nitrogen [6, 7].

Bacterium *Bacillus intermedius* 3-19 effectively secretes into the medium proteolytic enzymes such as subtilisin-like proteinase and glutamyl endopeptidase. The enzymes have been previously isolated in homogenous form, and their properties have been thoroughly studied [8, 9]. Mechanisms of the enzymes biosynthesis were described [10, 11], the genes coding for proteinases were cloned and sequenced [12, 13]. Biosynthesis of the enzymes was shown to be regulated by catabolite repression [10, 11, 13]. In the optimal peptone medium, ammonium ions stimulate glutamyl endopeptidase synthesis [14] while suppressing accumulation of subtilisin-like proteinase in the culture liquid [9]. Here, we report the study of the effect of a nitrogen source on the expression of the genes for AprBi and GseBi proteinases when bacteria are grown in synthetic culture medium without organic nitrogen.

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The objective of this work was to elucidate the impact of nitrogen catabolite repression, as well as the NrgA and NrgB proteins on the expression of the genes of *B. intermedius* serine proteinases.

MATERIALS AND METHODS

Bacterial strains and plasmids. Plasmid pCS9 containing the complete gene for subtilisine-like *B. intermedius* 3-19 proteinase within a 6.0-kb chromosome region from this microorganism and the gene conferring resistance to erythromycin was used in the study [13]. The gene coding for glutamyl endopeptidase was cloned on a pV plasmid carrying a 6.3-kb DNA region from *B. intermedius* 3-19's chromosomal DNA and the gene conferring resistance to erythromycin [12]. Extracellular protease-deficient strain *B. subtilis* BG20-36 was kindly supplied by Prof. Ferrari (Genencor International, California, United States) and was used as the plasmid host for expression studies. Strains *B. subtilis* 168 (*trpC2*), *B. subtilis* GP254 (*trpC2, nrgA*, Cm^r), and *B. subtilis* GP253 (*trpC2*, *nrgB*, Cm^r) were kindly provided by Prof. Schtulke, University of Göttingen, Germany. Proteolytic activity in the culture liquid of the control and mutant plasmid-free strains against synthetic substrates Z-Ala-Ala-*p*Na and Z-Glu-*p*Na was 3–5% from the activity of recombinant strains carrying pCS9 and pV plasmids, respectively.

Cutlture conditions. *B. subtilis* recombinant strains were cultivated in a Luria–Bertani (LB) medium [15] containing (%): tryptone, 1.0; yeast extract, 0.5; and sodium chloride, 0.5.

The minimal synthetic medium SMM [16] contained the following (g/l): K_2HPO_4, 14; KH_2PO_4, 6; $MgSO_4 \cdot 7H_2O$, 0.2; and sodium citrate, 0.2. This medium was supplemented with 20 mM sodium nitrate or 20 mM ammonium chloride as a nitrogen source. As a carbon source, 0.5% of glucose or 0.6% of sodium citrate were added. The medium was supplemented with erythromycin at 20 μg/ml, or chloramphenicol at 10 μg/ml. The cultures were inoculated from overnight cultures (4% vol/vol) grown on SMM medium supplemented with sodium citrate, sodium nitrate, and with an antibiotic. The native and recombinant cells were routinely grown at 37°C in 100-ml flasks with a culture volume of 14 ml on a rotary shaker at 200 rpm. The growth of bacterial cultures was monitored at a wavelength of 590 nm (OD_{590}) using a FEK-56M photocolorimeter. The biomass content was expressed as units of light absorbation in a 1-cm cuvette.

Proteinase assay. Determination of the proteinase activity was carried out as described earlier [17] using synthetic oligopeptides Z-Ala-Ala-Leu-*p*NA and Z-Glu-*p*NA as substrates. The activity unit was defined as the amount of the enzyme that hydrolyzed 1 nm of a substrate per min under specific conditions.

Productivity of the cells (specific activity) was calculated as the ratio of proteinase activity to the value of biomass and was expressed in conventional units.

DNA isolation was performed as described by Sambrook et al. [15]. Transformation of *B. subtilis* competent cells with plasmid DNA was carried out using the standard technique [16].

Statistical analysis of the data was carried out using the SPSS 12.0 software package. Standard deviation σ $\sigma \le 10\%$ was taken as a difference which was not significant. *P* values of ≤ 0.05 were considered statistically significant.

RESULTS AND DISCUSSION

Bacteria *B. intermedius* secrete into the medium two serine proteinases: subtilisin-like proteinase AprBi and glutamyl endopeptidase GseBi. Since amino acids, the products of proteolytic protein hydrolysis, can be utilized by bacteria both as the carbon and nitrogen source, we wished to examine whether the system of nitrogen catabolite repression could affect biosynthesis of proteinases. The preferable nitrogen sources for these bacteria are ammonium and glutamine; therefore, if these compounds are present in the culture medium, the expression of the genes coding for the enzymes involved in the degradation of alternative nitrogen sources could be suppressed [18]. *B. subtilis* BG20-36 pCS9 and *B. subtilis* BG20-36 pV recombinant strains were grown in synthetic SMM culture medium supplemented with 0.5% glucose as the carbon source, and with chloride ammonium (experimental medium) or sodium nitrate (control medium) as the sole nitrogen source, and expression of the genes coding for AprBi and GseBi proteinases was examined (Fig. 1).

When bacteria were grown in liquid medium containing sodium nitrate, an insignificant prolongation of the lag phase was recorded. As a result, bacterial culture reached the stationary phase 6 hours later (Figs. 1a, 1b). In the presence of ammonium ions, the decrease in expression of subtilisin-like proteinase was observed. Sodium nitrate, present in the medium in 20-mM concentration (control medium), caused an eight- to tenfold increase in subtilisin-like proteinase activity compared to the activity of the enzyme in the medium with ammonium chloride (Fig. 1c). Bacterial growth did not differ significantly (Fig. 1a). These data suggest the regulatory effect of nitrogen catabolite repression on the expression of the *B. intermedius* AprBi proteinase gene. On the contrary, if bacteria were cultivated in the medium containing ammonium chloride (a preferred nitrogen source), expression of the glutamyl endopeptidase gene increased twofold (Fig 1d). Probably, glutamyl endopeptidase biosynthesis does not depend on the availability of reduced nitrogen to the microbial cell.

Expression of *aprBi* and *gseBi* in *B. subtilis* BG20-36 recombinant cells grown in the medium containing

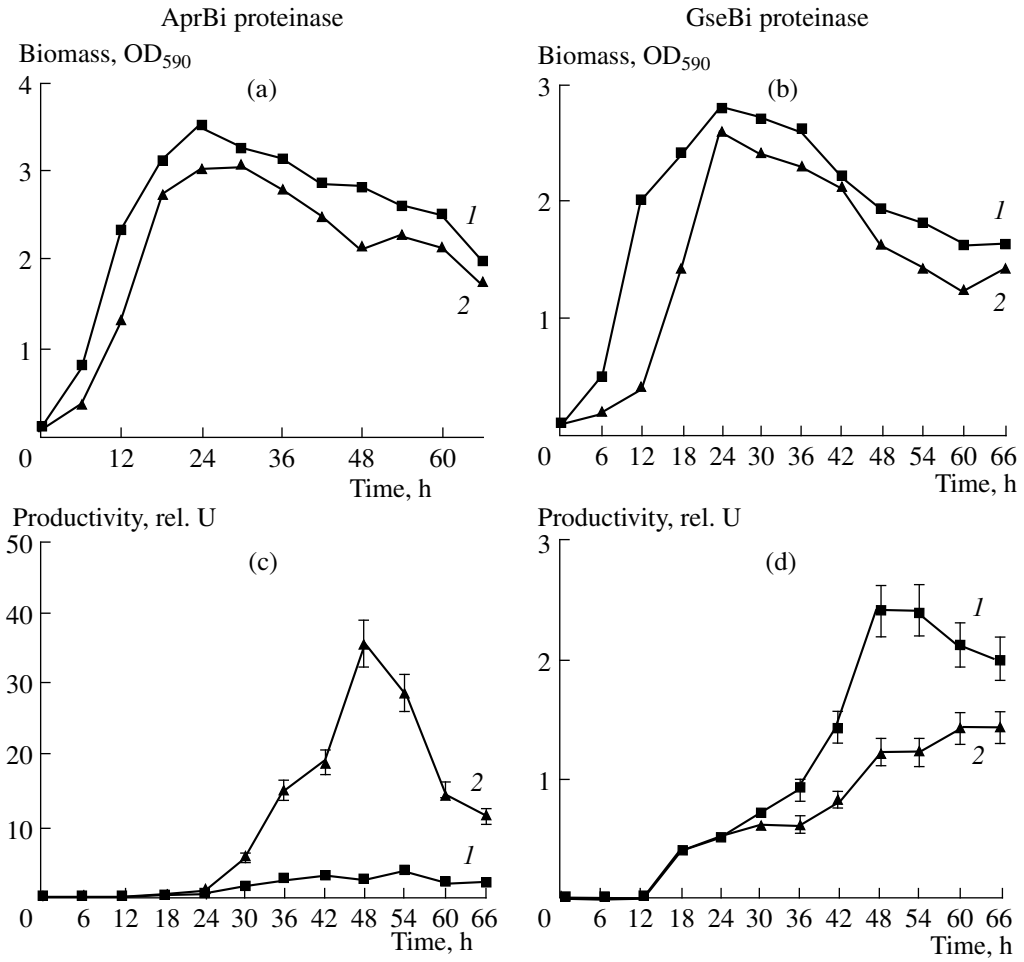

Fig. 1. Effect of the nitrogen source on growth (a, b) and expression of the genes for subtilisin-like serine proteinase AprBi and glutamyl endopeptidase GseBi from *B. intermedius* (c, d) in *B. subtilis* BG20-36 recombinant strains in the minimal culture medium supplemented with 0.5% of glucose; 20 mM NH_4Cl (*1*); 20 mM $NaNO_3$ (control medium) (*2*).

sodium citrate instead of glucose was analyzed (Fig. 2). Maximal growth of bacterial cells on this medium was found to be two times lower than that recorded on the medium containing 0.5% of glucose, and did not depend on the nitrogen source. During cultivation on the minimal culture medium, the carbon source is probably the limiting factor. The level of subtilisin-like proteinase biosynthesis in the control medium was 3–4 times higher than that in the medium containing ammonium chloride (Fig. 2c). Thus, in the *B. subtilis* BG20-36 recombinant strain, *aprBi* expression could be regulated by nitrogen catabolite repression since proteinase biosynthesis decreased significantly in the presence of the preferable nitrogen source (ammonium). The suppressing effect of ammonium on enzyme synthesis was observed mainly at the early stationary phase of growth (24–36 h of growth) and was much less pronounced at the late stationary phase (48–60 h) (Fig. 2a). Influence of the regulatory systems involved in the vegetative growth (including the system of metabolic nitrogen exchange regulation) on *aprBi* expression is probably minimized during this period of growth. It should be noted that if bacteria were grown in a minimal culture medium with nitrate, an addition of 0.5% glucose had a slight inhibitory effect on formation of subtilisin-like proteinase causing a 30–40% decrease in the enzyme production (Figs. 1c, 2c). In culture medium containing ammonium as the source of nitrogen and in the presence of glucose, the productivity was 4–5 times lower (Figs. 1c, 2c). In LB medium after the addition of glucose the level of the enzyme production decreased tenfold [13]. This effect probably can be attributed to starvation of bacteria during their growth in the minimal culture medium. This suggestion is in accord with the results reported earlier for *Bacillus licheniformis*, indicating that nitrogen deficiency in the medium completely abolished the effect of lucose catabolite repression on proteinase biosynthesis [5].

If a recombinant strain was cultivated in minimal culture medium supplemented with sodium citrate, its growth and the level of glutamyl endopeptidase pro-

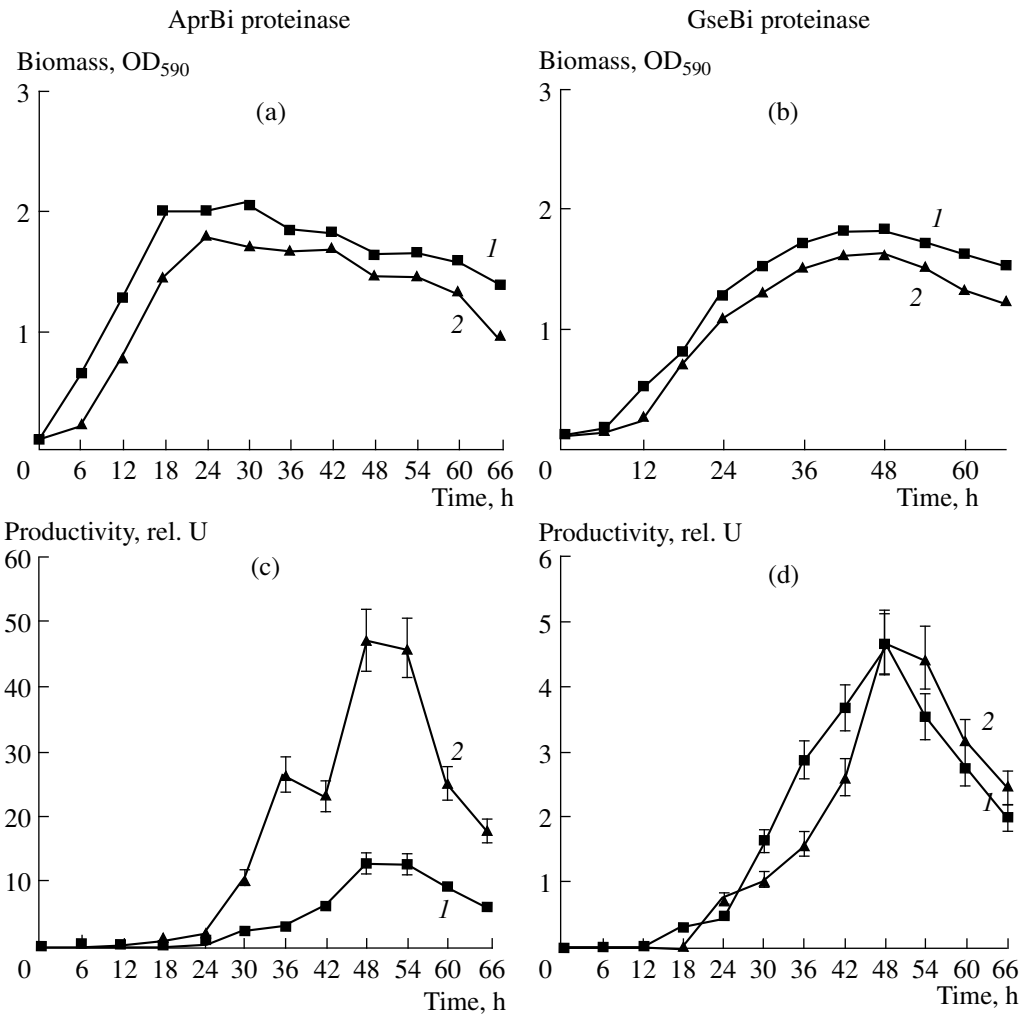

Fig. 2. Effect of the nitrogen source on growth (a, b) and expression of the genes for subtilisin-like serine proteinase AprBi and glutamyl endopeptidase GseBi from *B. intermedius* (c, d) in *B. subtilis* BG20-36 recombinant strains in the minimal culture medium supplemented with 0.6% of sodium citrate; 20 mM NH_4Cl (*1*); 20 mM $NaNO_3$ (control medium) (*2*).

duction did not depend on the nitrogen source (Figs. 2b, 2d) in contrast with LB medium [14] and with the medium containing glucose (Fig. 1d). Therefore, the data obtained in this study lead to the conclusion that *gseBi* expression is not under control of nitrogen catabolite repression.

In *B. subtilis* cells the transport of ammonium ions into the cell was shown to be mediated by the membrane-bound NrgA protein associated with the NrgB regulatory protein [6, 7]. NrgA deficient *B. subtilis* strain GP254 (*trpC2, nrgA*, Cm^r), NrgB deficient *B. subtilis* GP253 (*trpC2, nrgB*, Cm^r), and the control strain *B. subtilis* 168 (*trpC2*) were transformed with plasmid DNA pCS9 and pV as described in the Materials and Methods.

The study of the growth dynamics and subtilisin-like proteinase's gene expression of the regulatory mutants in the LB medium was carried out. The absence of functionally active NrgA was demonstrated not to affect *B. intermedius* proteinase formation in the rich LB medium (Fig. 3c). At the same time, mutation in the *nrgB* gene led to a two- to threefold enhanced level of the enzyme biosynthesis. For the AprBi proteinase, the effect was detected at the early stationary phase (28–30 h of cultivation). Expression of the gene for *B. intermedius* glutamyl endopeptidase under the same conditions did not differ from the control (Fig. 3d).

In order to support the assumption that NrgB may be involved in regulation of the AprBi proteinase biosynthesis, we examined the dynamics of enzyme synthesis in a synthetic minimal medium containing sodium citrate as the nitrogen source. In the experimental medium ammonium chloride was used as the source of nitrogen, and sodium nitrate was used in the control medium. In the NrgA-deficient strain expression of the gene for proteinase was found to be controlled by nitrogen

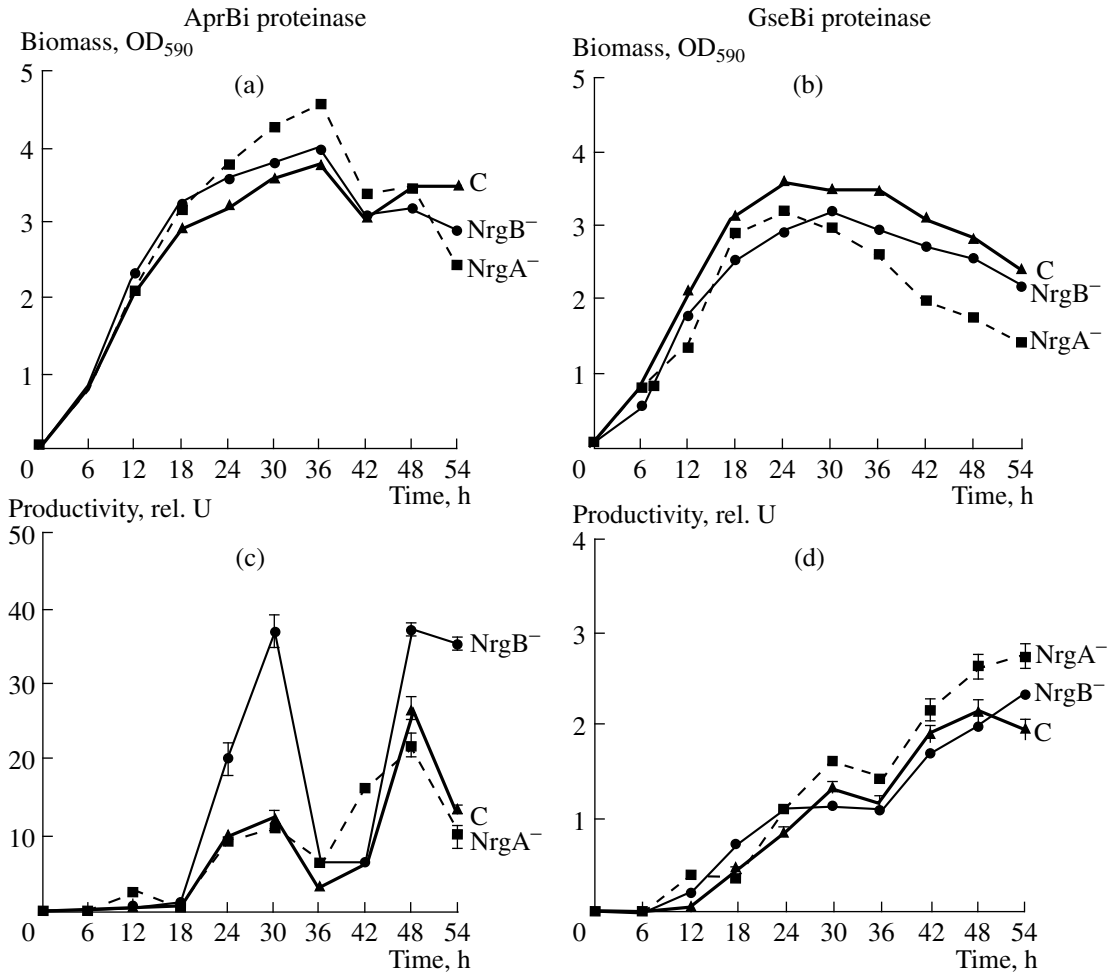

Fig. 3. Expression of the *aprBi* and *gseBi* genes in *B. subtilis* GP254 (*trpC2, nrgA*, Cm^r) and *B. subtilis* GP253 (*trpC2, nrgB*, Cm^r) recombinant strains deficient in NrgA and NrgB proteins, respectively. Bacteria were grown in LB medium. The strain *B. subtilis* 168 (*trpC2*) was used as a control.

catabolite repression (Fig. 4a). The level of the enzyme production was the same as in the control strain carrying the functional NrgA and NrgB proteins (Figs. 2c, 4a). Expression of the *aprBi* gene in the strain carrying the mutation in NrgB did not depend on the type of nitrogen source (Fig. 4c). The level of proteolytic activity in the medium supplemented with nitrate was equal to that in the medium supplemented with ammonium; it was the same as the level of proteinase synthesis by the control strain grown in the medium with ammonium chloride. Based on the data obtained, we suggested that the regulatory function of the NrgB protein could be an activation (instead of suppression) of the gene expression under conditions of nitrogen starvation.

Expression of the glutamyl endopeptidase gene in NrgA- and NrgB-deficient recombinant strains during bacterial growth in the minimal culture medium did not depend on the source of nitrogen (Figs. 4b, 4d). The level of the proteinase gene's expression both in the mutant and in control strains did not depend on the nitrogen source and was equal to that in the control (Figs. 4b, 4d, 2d).

Thus, we have established for the first time the expression of the gene encoding a subtilisin-like proteinase (AprBi proteinase from *B. intermedius*) to be controlled in *B. subtilis* recombinant strains by nitrogen catabolite repression. In the medium containing the preferable nitrogen source (ammonium ions), suppression of proteinase production was detected. This confirms close interaction between the regulation of nitrogen and carbon metabolism in a bacterial cell and the cross-regulation of the genes involved in these processes [4]. However, expression of the gene for glutamyl endopeptidase, another serine proteinase, does not depend on the nitrogen status of the cell but is suppressed by glucose [10, 14]. These results may be due to the fact that glutamyl endopeptidase, while possessing narrow substrate specificity, plays in bacterial cells a regulatory role in addition to its traditional trophic function.

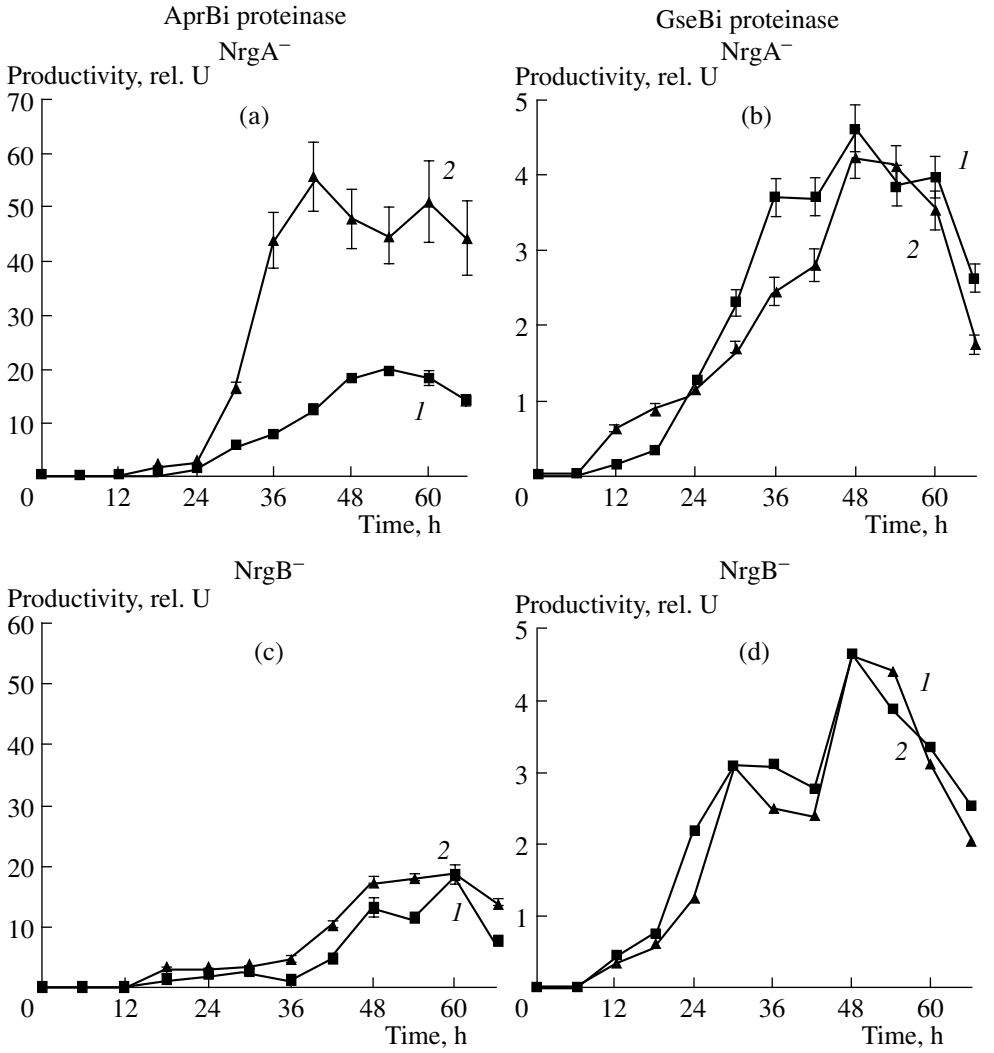

Fig. 4. Expression of the *aprBi* and *gseBi* genes in *B. subtilis* GP254 (*trpC2, nrgA*, Cm^r) and *B. subtilis* GP253 (*trpC2, nrgB*, Cm^r) recombinant strains deficient in NrgA and NrgB proteins, respectively. Bacteria were grown in the minimal culture medium supplemented with 0.6% of sodium citrate; 20 mM NH_4Cl (*1*); 20 mM $NaNO_3$ (control medium) (*2*).

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