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EXPERIMENTAL ARTICLES

Bacillus pumilus Strains with Inactivated Genes of Extracellular Serine Proteinases

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Abstract—Two *Bacillus pumilus* strains with inactivated genes of extracellular serine proteinases (subtilisin-like proteinase and glutamyl endopeptidase) were obtained. Inactivation of the *gseBp* and *aprBp* genes resulted in an increase in cell size, changed colony shape, and more rapid cell lysis that started during the growth retardation phase. Protease-deficient stains partially changed the ability to decompose carbohydrates (sugars), reduced resistance to variations in temperature of cultivation, and did not respond to the fluctuations of phosphate concentration in the medium. Proteinases gene disruption resulted in alteration of hydrolases secretion level by these bacteria.

Keywords: *Bacillus pumilus*, serine proteases, gene inactivation, homologous recombination

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Bacilli are attractive objects for gene expression of heterologous proteins in microbial biotechnology. Genomes of many *Bacillus* members have been sequenced, and their physiology and biochemistry is well-studied [1–3]. The advantage of bacilli is their ability to secrete proteins into the culture liquid, allowing their effective isolation [4, 5]. However, the application of bacilli in biotechnology has a number of limitations determined by (1) the presence of extracellular proteases in the growth medium, (2) instability of the plasmids, and (3) incorrect folding of recombinant proteins. The presence of extracellular proteolytic enzymes is the main factor hampering the expression of recombinant proteins. Removal of extracellular proteases genes from *B. subtilis* DNA substantially increases the yield of gene expression product [6, 7]. Hence, the creation of protease-deficient strains is considered to be a promising technology for production of heterologous proteins [8]. It was shown that a minimal set of 270 genes is sufficient for *B. subtilis* cultivation in a rich medium [9]. Thus, reduction of the inessential genome part does not lead to suppression of bacilli viability.

B. pumilus isolated from soil samples (Tatarstan republic) [10] are producers of extracellular serine proteinases: subtilisin-like proteinase and glutamyl endopeptidase [11, 12]. It was interesting to obtain *B. pumilus* cells with the inactivated genes of subtilisin-like proteinase (*aprBp*) and glutamyl endopeptidase (*gseBp*) to investigate the physiological functions of these enzymes and their potential involvement in the extracellular proteins processing.

The goal of this research was to obtain and characterize *B. pumilus* strains with knockout genes of extracellular serine proteinases.

MATERIALS AND METHODS

The objects of research were *B. pumilus* 3-19 strains with inactivated extracellular serine proteinase genes: subtilisin-like proteinase *aprBp* (AN AY754946.2) and glutamyl endopeptidase *gseBp* (AN Y15136.1). The bacteria were grown in the Luria–Bertani (LB) medium in 250-mL flasks with a 1 : 7 medium to flask volume ratio in a thermostatic shaker (Inkubations-Schüttelschrank BS4, Braun, Germany) at 200 rpm at 37°C. The agarized LB medium additionally contained 2% agar. Antibiotics were added to the medium at final concentrations (μg/mL): streptomycin, 10 (*B. pumilus* 3-19); ampicillin, 20 (*E. coli*); erythromycin, 20 (*B. pumilus* MK10/2A-5). The 18-h overnight culture diluted to the optical density value of 0.1 (OD_{590}) was used for inoculation of bacillary strains. The biomass augmentation was measured nephelometrically on a photoelectric colorimeter FEK-56 PM with light filter 9 at 590 nm in a 1-cm cuvette. The cells were stained according to Peshkov protocol for counting free spores [13]. The spores quantity expressed as a percentage relative to the total number of vegetative and sporulating cells (100%) counted in a phase contrast microscope at a 1600× magnification in 5 visual fields.

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Table 1. Oligonucleotides used in the work

Primer	Sequence (5'–3')*	Description
Sub left up	GAAGTCAAACAAACGGTGTCC	5'-end of the left flanking *aprBp* gene region
Sub left low	GAATACCAACATGACGAATCCCCCACGTTGTAATCAAGGACC	3'-end of the left flanking *aprBp* gene region
Sub right up	GGGCACTCTAATAACTACCAAAATTGGTGTTCTTGGGGTCG	5'-end of the right flanking *aprBp* gene region
Sub right low	AGAGAGGGATCCGAGAGGCAGGGGTGACGTCTTTTC	3'-end of the right flanking *aprBp* gene region
Em up	GGGATTCGTCATGTTGGTATTC	5'-end of the *erm* gene
Em low	ATTGGTAGTTATTAGAGTGCCC	3'-end of the *erm* gene
Glu pd	GACAAT*GGATCC*GTAAAAGATGTGTGGAG	*Bam*HI, 5'-end of the *gseBp* gene
Glu stop	ATAAGG*AAGCTT*CAGTGATCCAGCCTCTTC	*Hin*dIII, 3'-end of the *gseBp* gene
Em 1	CTACCAGTGACTAATCTTATG	5'-end of the *erm* gene
Em 2	TACCCTTTAGTAACGTGTAAC	3'-end of the *erm* gene

Note: Restriction sites are in italic; the regions complementary to the *erm* gene are underlined.

The procedures for obtaining recombinant plasmid DNA were performed in *E. coli* cells as described previously [14]. *E. coli* transformants were grown on the plates with agarized medium containing 5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside (40 mg/mL) and isopropyl-β-S-1-thiogalactopyranoside (32 mg/mL). After 24-h incubation of the transformants, white bacterial colonies were selected for PCR screening by the combination of oligonucleotide primers. The primers were constructed using the Oligo Calc software package (http://www.basic.northwestern.edu/biotools/oligocalc.html) and were based on the *aprBp*, *gseBp*, and *erm* gene sequences (Table 1). The primer synthesis and sequencing was carried out by Synthol Company (Moscow). Amplification was performed in a Tertsik thermocycler (DNA Technology, Russian Federation). Restriction and ligation were carried out by the methods described previously [14]. The enzymes from SibEnzyme Ltd. (Moscow) were used in the work. DNA was purified with a Fermentas kit (Lithuania). DNA transformation in *B. pumilus* 3-19 cells was performed as described [15]. The plasmids used in the work are listed in Table 2.

B. pumilus 3-19 strain with the inactivated *aprBp* gene was obtained by three-stage PCR method (Fig. 1). (1) Flanking regions of the *aprBp* gene and the erythromycin resistance gene (*erm*) were amplified from plasmid pSC9 using primers Sub left up/Sub left low, Sub right up/Sub right low, and Em up/Em low, respectively (Table 1). (2) Derived PCR products were used to obtain double constructions: the left flanking region *aprBp* + *erm* (LE) and the right flanking region *arpBp* + *erm* (ER). These constructions were cloned into pGEM-T Easy vector by ligation at the sticky T-ends of the plasmid and A-ends of the amplificates, followed by the formation of the plasmids pGEMTE-LE and pGEMTE-ER. (3) These plasmids were used as a template for triple construction synthesis. Final PCR product (Sub left up/Sub right low) with the expected size (~3 kb) was used for transformation into *B. pumilus* 3-19 cells. As a result of homologous recombination, the antibiotic resistance *erm* gene replaced the *aprBp* protease gene. The colonies from LA medium with erythromycin were collected and tested on the medium containing 2% milk agar. Proteolytic activity was detected by appearance of a clarification area on milk agar (the zone of the cleavage of milk proteins). As a result of selection, two independent clones were chosen to determine the specific activity (of the subtilisin-like protease). Strains with the lost proteolytic activity were selected.

The glutamyl endopeptidase *gseBp* gene was inactivated by the technique of open reading frame disruption (Fig. 2). The *gseBp* gene was amplified from plasmid p58.21 using the Glu pd/Glu stop primers. The PCR product was cloned in vector pUC19 at the sites *Bam*HI and *Hin*dIII with pUCG plasmid generation. Since the glutamyl endopeptidase gene carries the unique restriction site *Eco*RV, the pUCG plasmid was restricted at this site for cloning the erythromycin resistance gene. The *erm* gene was amplified from plasmid pCB22 using primers Em 1/Em 2. T4-poly-

Table 2. Plasmids used in the work

Plasmid	Genotype*	Oligonucleotides used	References
pCS9	*bla*, *erm*, *rep19035*, 6.0-kb genomic DNA fragment of *B. pumilus* 3-19 (P_{aprBp}-*aprBp*)	Sub left up/Sub left low; Sub right up/Sub right low; Em up/Em low	S.V. Kostrov, IMG RAS, Moscow
pGEM-T Easy	T7 RNA polymerase initiation site, P_{T7}, SP6 RNA polymerase initiation site, P_{SP6}, *lacZ*, lac-operon, *bla*, the region of phage f1	–	Promega, United States
pGEMTE-LE	Derivative of pGEM-T Easy (left *aprBp-erm*)	Sub left up/Em low	Obtained in this work
pGEMTE-ER	Derivative of pGEM-T Easy (*erm-aprBp* right)	Em up/Sub right low	Obtained in this work
p58.21	2.5-kb genomic DNA fragment of *B. pumilus* 3-19 (P_{gseBp}-*gseBp*), *rep* pCB22, *cat*	Glu pd/Glu stop	S.V. Kostrov, IMG RAS, Moscow
pUC19	*lacZ*a, *rep* pMB1, *bla*	–	From the fund of the laboratory
pCB22	*bla*, *erm*, *rep19035*	Em 1/Em 2	S.V. Kostrov, IMG RAS, Moscow
pUCG	Derivative of pUC19 (*gseBp*)	–	Obtained in this work
pUCGE	Derivative of pUCG and pCB22 (*gseBp' erm 'gseBp*)	–	Obtained in this work

Note: Resistance cassettes: *erm*, erythromycin; *bla*, ampicillin; *cat*, chloramphenicol.

nucleotide kinase was used for phosphorylation of the 5'-end of the amplificate (*erm*). Phosphorylated *erm* genes were cloned in linearized pUCG plasmid by the *Eco*RV restriction site. The obtained pUCGE plasmid was used for transformation into *B. pumilus* 3-19. The colonies from erythromycin-containing LA medium were selected and tested on the specific proteolytic activity (of glutamyl endopeptidase).

The bacteria were monitored under a MicrosMC 300 microscope, Austria, at different magnifications (40×/0.65; 100×/1.25 with immersion oil).

Recombinant strains were analyzed for the ability to utilize sugars using Hiss media. The media contained peptone, indicator, and the respective carbohydrate (g/L): glucose, 16; lactose, 20; mannitol, 20; or sucrose, 15. The culture was stab-inoculated into test tubes with 5 mL of the medium. Culture growth was controlled by discoloration of the nutrient medium.

Total proteolytic activity was determined by azocasein (Sigma, United States) cleavage as described in the work [16]. The enzyme quantity hydrolyzing 1 μg of substrate per 1 min under experimental conditions was taken as an activity unit. The subtilisin-like protease and glutamyl endopeptidase activities were assayed by the cleavage of the chromogenic synthetic substrates Z-Ala-Ala-Leu-pNA and Z-Glu-pNA, respectively, by the methods described previously [17]. The enzyme quantity hydrolyzing 1 μg of substrate per 1 min under experimental conditions was taken as an activity unit. RNase activity was assayed by the accumulation of acid-soluble products of RNA hydrolysis [18]. The amount of RNase causing an increase in optical density by 1 optical unit per 1 h of incubation per 1 mL of the enzyme solution (OD/(mL h)) was taken as an RNase activity unit. Phosphomonoesterase activity [18] was assayed by the effect of the enzyme on *p*-nitrophenylphosphate (p-NPP) (Serva, Germany). The amount of the enzyme causing an increase in optical density by 1 optical unit at 410 nm per 1 mL of the enzyme solution after 1 h of incubation was taken as a unit of phosphatase activity.

Statistical analysis of experimental data was performed in Microsoft Excel by calculating the standard deviation (σ). The results were considered reliable at $\sigma \leq 10\%$. The reliability of differences obtained was calculated by the Student's criterion with $P \leq 0.05$ taken as a reliable level of significance.

RESULTS AND DISCUSSION

The approach used for disruptions in the *B. pumilus* serine proteinase genes (*aprBp* and *gseBp*) was based on homologous recombination between the exogenous and genomic DNA regions (Figs. 1 and 2). Mutants were selected on the plates with erythromycin

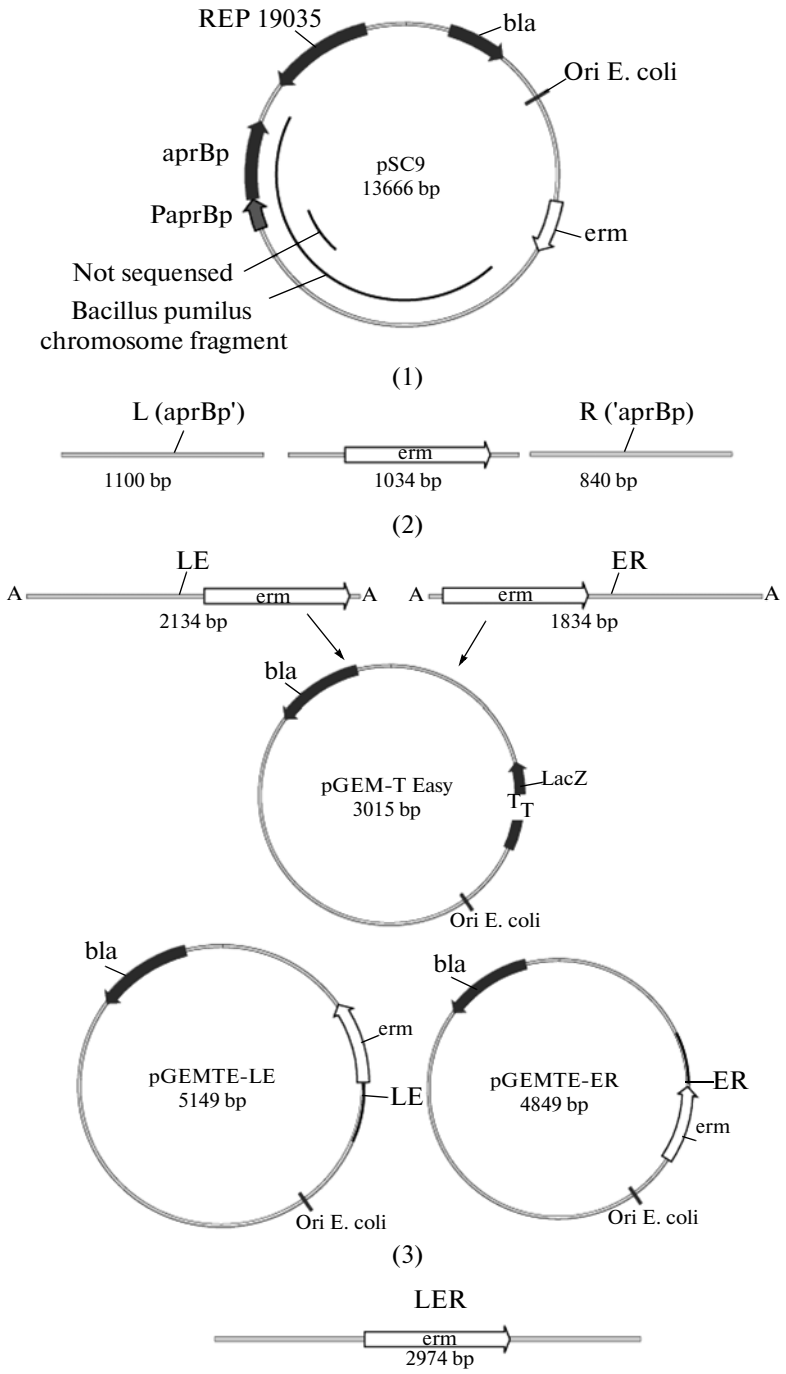

Fig. 1. The scheme of obtaining a triple recombination construction for the *aprBp* gene inactivation in the genome of *B. pumilus* 3-19. (1) The left (L) and right (R) flanking regions of the *aprBp* gene and the erythromycin resistance gene (*erm*) were amplified by PCR from plasmid pCS9. (2) The amplificates of single constructions were used as a template for obtaining double constructions: the left flanking region *aprBp* + *erm* (LE) and the right flanking region *aprBp* + *erm* (ER). The derived double constructions (LE and RE) were cloned in the plasmid pGEM-T Easy with the formation of plasmids pGEMTE-LE and pGEMTE-ER. (3) Both plasmids were used as a template for obtaining a triple recombination construction (LER).

after the transformation of *B. pumilus* 3-19 cells by obtained constructions (see Materials and Methods). Then, separate colonies from antibiotic-containing media were tested on milk agar to observe decreased zones of casein hydrolysis around the colonies. Clarification zones were observed only for the initial strain *B. pumilus* 3-19. In the recombinant strains, the proteolysis zones were absent, indicating disruption of secreted proteolytic enzymes' genes. As a result, the recombinant strains *B. pumilus* MK10 (*aprBp*::*erm*)

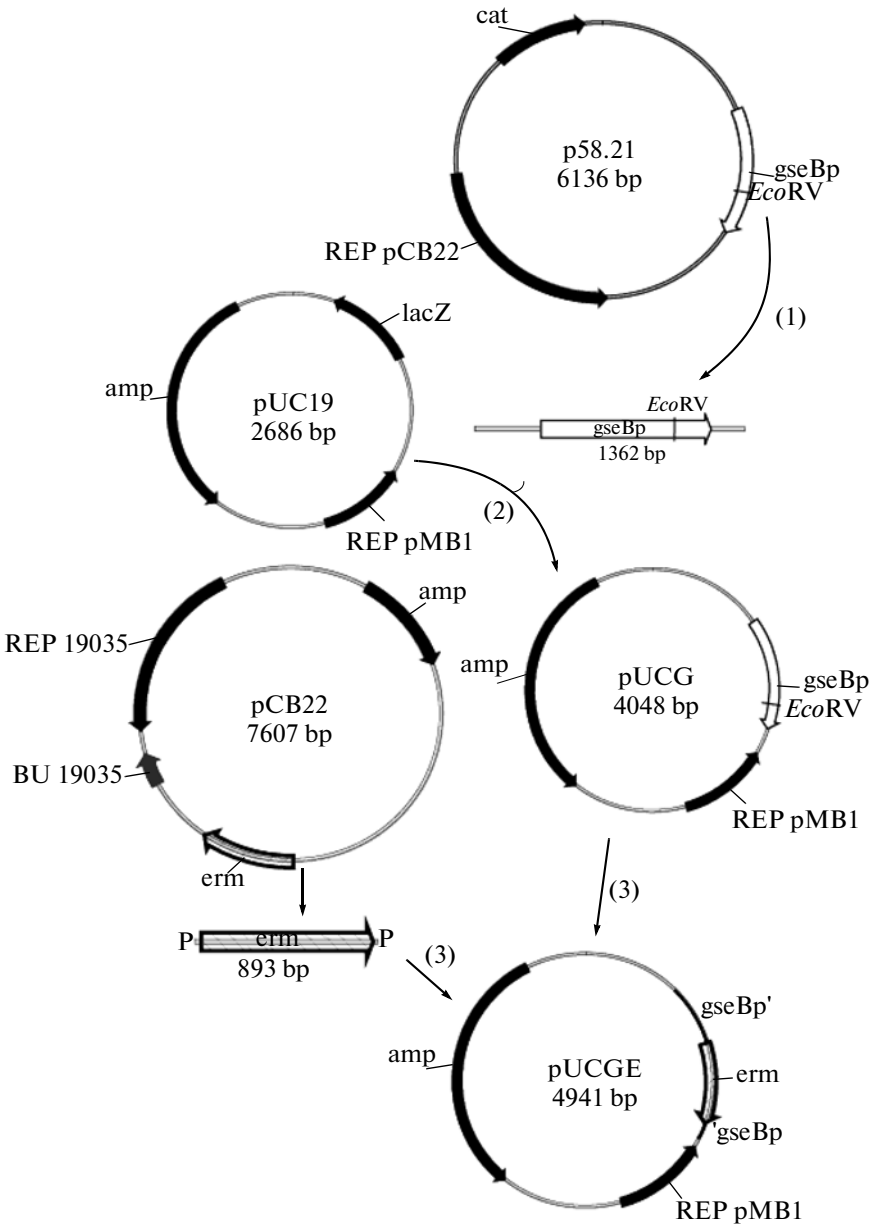

Fig. 2. Obtaining the plasmid pUCGE carrying a recombination construction for the *gseBp* gene inactivation in the genome of *B. pumilus* 3-19. (1) The protease gene (*gseBp*) was amplified by PCR from the plasmid p58.21. (2) The amplification product was cloned in the vector pUC19 with the formation of plasmid pUCG. (3) After restriction of plasmid pUCG at the *Eco*RV site, the amplificate of the *erm* gene carrying phosphate groups and obtained from plasmid pCB22 was integrated into plasmid pUCG. As a result, the pUCGE plasmid was obtained, where the *gseBp* gene sequence was disturbed by the erythromycin resistance gene (*erm*).

and *B. pumilus* 2A-5 (*gseBp*::*erm*) were selected. Both strains demonstrated the absence of specific proteolytic activity on the respective substrates.

Microscopy of recombinant strains showed that their cells were larger compared to the wild type strain (Fig. 3): their length was about 14 μm versus 8.0 μm for the wild type strain. The colonies of protease-free strains during the growth on agarized medium were characterized by rough surface and uneven edges, compared to the smoother, even edges and slimy consistency of the initial strain.

According to the literature data, serine proteinases are involved in the processes of cell growth and differentiation [19]. Monitoring the growth of bacteria in liquid medium revealed that exponential growth and biomass accumulation were the same for all three strains. The strains simultaneously entered the stationary phase. However, the stationary phase of the initial strain 3-19 was longer than the stationary phase

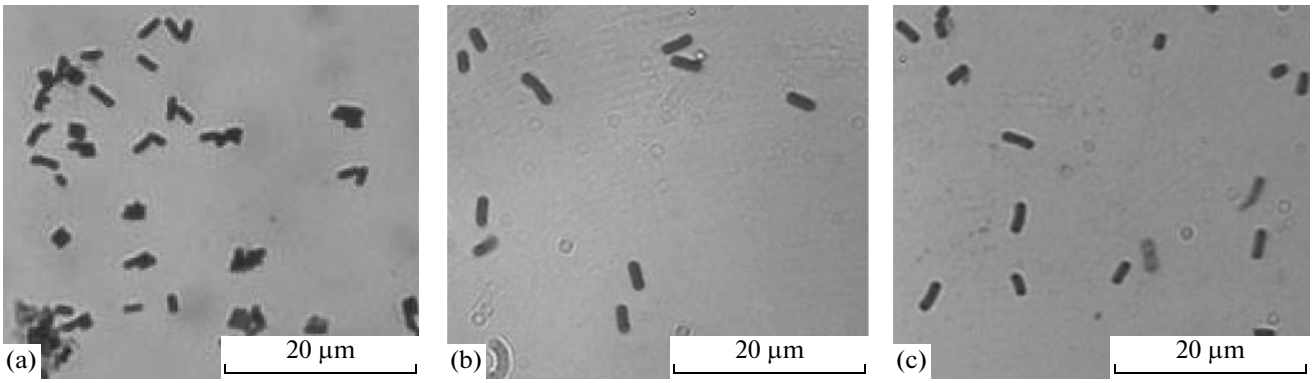

Fig. 3. Cells of the initial 3-19 (a) and modified MK10 (b) and 2A-5 (c) strains of *B. pumilus* under identical cultivation conditions. Microscopy with immersion; 100× immersion system (MicrosMC 300, Austria).

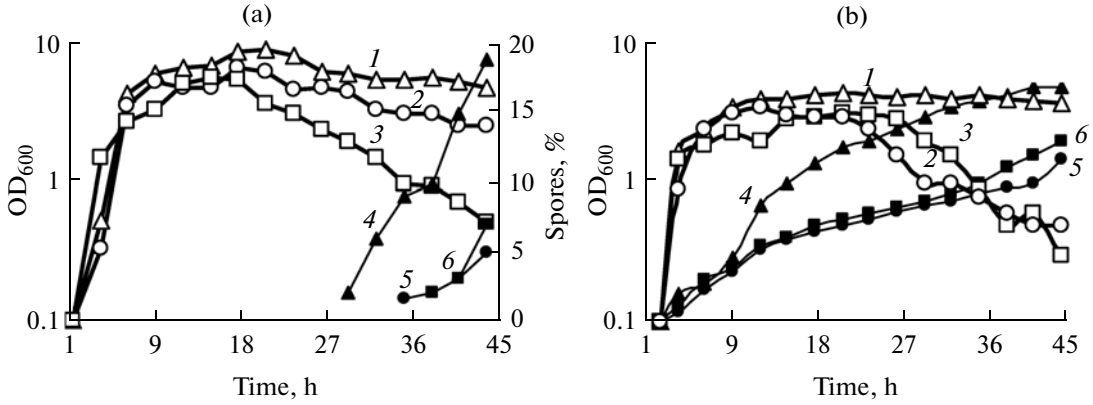

Fig. 4. Strains growth and sporulation in liquid LB medium. (a) Cell growth/sporulation: *B. pumilus* 3-19 (*1/4*); the *aprBp::erm* MK10 gene knockout (*2/5*), the *gseBp::erm* 2A-5 gene knockout (*3/6*). (b) Cell growth at 42°C/15°C: *B. pumilus* 3-19 (*1/4*); the *aprBp::erm* MK10 gene knockout (*2/5*), the *gseBp::erm* 2A-5 gene knockout (*3/6*).

of recombinant strains MK-10 and 2A-5 (Fig. 4a). It was noted that autolysis began earlier in the strain 2A-5 with the glutamyl endopeptidase gene knockout. Release of the free spores in the wild type strain began from hour 30, and by hour 45 their abundance reached 20%. In both mutant strains, spore formation was impaired: free spores appeared by 36 h, and by 45 h their abundance was ~5% (Fig. 4a). Thus, disruption of extracellular subtilisin-like proteinase and glutamyl endopeptidase genes influenced bacterial sporulation, the stationary growth phase, and autolysis.

The growth of recombinant (2A-5, MK-10) and wild type (3-19) strains at low (15°C) and high (42°C) cultivation temperatures was studied. At a high temperature (42°C), bacterial growth did not change substantially, although all strains entered the stationary phase earlier; at the same time, unlike strain 3-19, autolysis of the recombinant strains was observed after hour 20 of growth (Fig. 4b). At a low temperature (15°C), the wild type strain 3-19 showed the priority of growth: its biomass under these conditions accumulated similar to the control (Fig. 4a, 4b). Both mutant strains were retarded in growth rate and were characterized by the lower level of biomass accumulation.

The growth of recombinant strains did not vary in the media with different concentrations of phosphorous compounds (data not shown). Inactivation of the subtilisin-like proteinase and glutamyl endopeptidase genes is probably not associated with bacterial resistance to phosphate starvation.

Thus, inactivation of the extracellular proteinase genes in bacilli resulted in increased cell size and changes in the colony shape and acceleration of cell lysis.

Investigation of *B. pumilis* strains (MK10, 2A-5, and 3-19) ability to utilize sugars in the indicator media showed that, under identical cultivation conditions, the strains with inactivated extracellular proteinase genes decomposed glucose and sucrose with less intensity and did not decompose mannitol and lactose, unlike the initial culture. Thus, the strains' ability to metabolize carbohydrates changed somewhat as a result of inactivation of the subtilisin-like proteinase and glutamyl endopeptidase genes.

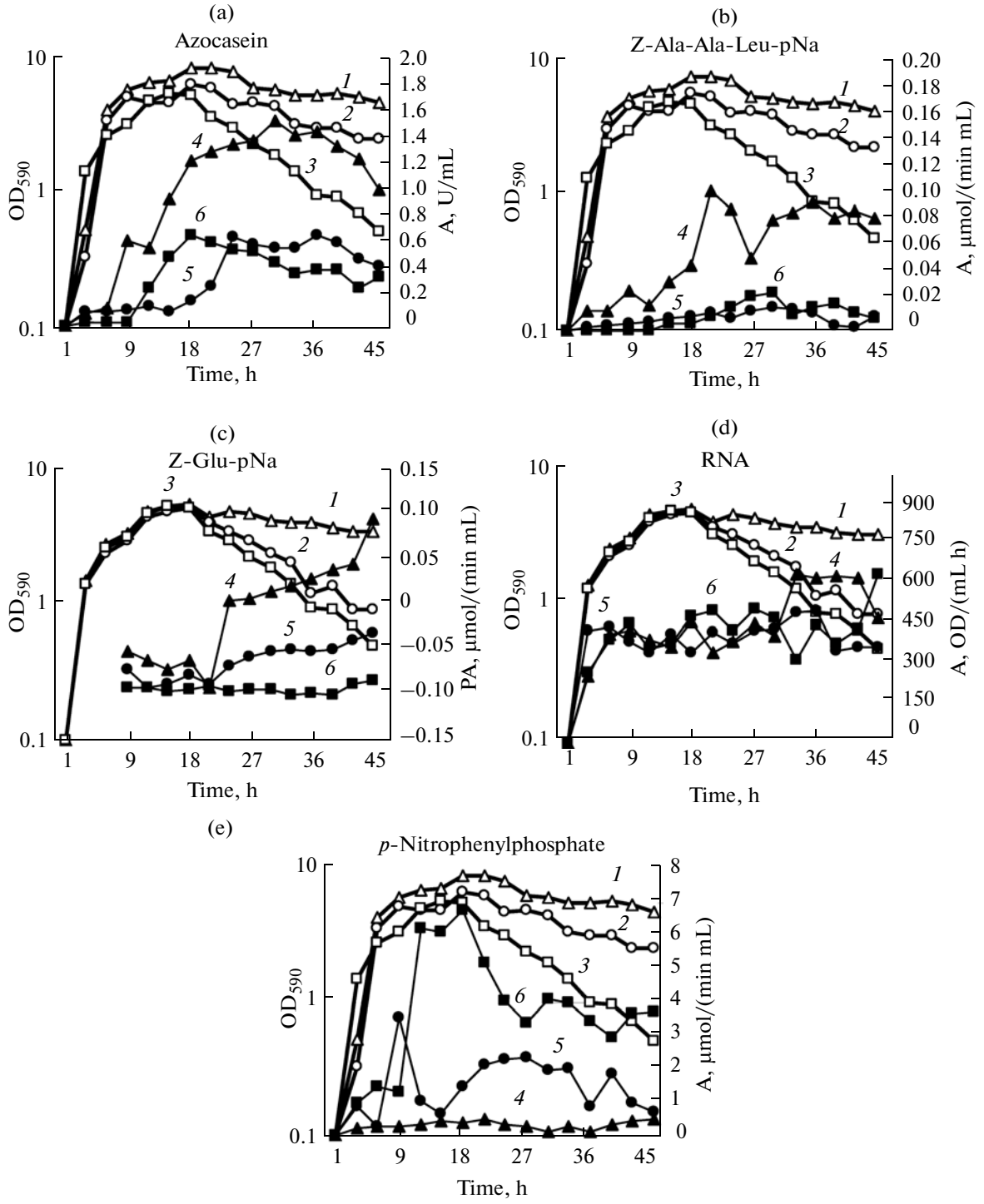

Fig. 5. Strains growth and accumulation of enzyme activities on different substrates. Cell growth: *B. pumilus* 3-19 (*1*); the *aprBp::erm* MK10 gene knockout (*2*), the *gseBp::erm* 2A-5 gene knockout (*3*). Enzyme activities: *B. pumilus* 3-19 (*4*); the *aprBp::erm* MK10 gene knockout (*5*), the *gseBp::erm* 2A-5 gene knockout (*6*).

It was most important to detect the changes in the ability to secrete extracellular hydrolases in the protease-deficient strains: MK10 and 2A-5. According to the data of *B. subtilis* genome sequencing, the bacilli can excrete into the medium the complex of extracellular proteases comprising nine enzymes [9]. Previously we have shown that the subtilisin-like proteinase activity was predominant in the proteolytic pool of *B. pumilus* (over 80%), with less than 10% accounted for the glutamyl specific endopeptidase under the con-

ditions of synthesis induction [11, 12]. Disruption of both serine proteinases' genes resulted in decreased level of recombinant strains proteolytic activity. The activity of azocasein hydrolysis decreased in 3.5 times for both strains; in the strain MK10 (*aprBp*$^-$), azocasein hydrolysis activity appeared in the medium only after 21 h of growth (Fig. 5a).

Activities of hydrolases in the culture liquid against specific substrates (Z-Ala-Ala-Leu-pNA for subtilisin-like proteinase and on Z-Glu-pNA for glutamyl endopeptidase) were changed during the growth of knockout strains. In the recombinant strains, the activity was absent on the substrates corresponding to knockout. At the same time, the activity against Z-Ala-Ala-Leu-pNA was almost absent in both mutant strains (Fig. 5b), whereas the activity against Z-Glu-pNA was not found for the mutant 2A-5 (*gseBp*$^-$) but was detected, although at a low level, for the mutant MK-10 (*aprBp*$^-$) (Fig. 5c). The functionally active subtilisin-like proteinase probably plays a key role in formation of the minor enzyme glutamyl endopeptidase. Glutamyl endopeptidase, in its turn, influences the formation of the pool of extracellular subtilisin-like proteinase. In general, these data are indicatative of proteases' balance in the total pool of extracellular enzymes and their co-regulation.

Accumulation of other hydrolases in the culture liquid of knockout strains was assayed. The strain *B. pumilus* 3-19 is characterized as a producer of extracellular alkaline ribonuclease [20]. Serine proteinase genes' disruption effect on RNase activity showed that the RNase activity level in the recombinant strains MK10 and 2A-5 was maintained at a level of the strain with the functional *aprBp* and *gseBp* genes (Fig. 5d). The biogenesis of the extracellular ribonuclease is probably independent of proteolytic enzymes.

Previously, the strain *B. pumilus* 3-19 was shown to be capable of secreting alkaline phosphohydrolase during growth in the medium with low phosphate content [21]. The conditions of phosphatase secretion and the factors enhancing the enzyme biosynthesis in the medium were investigated, i.e., the presence of mononucleotides, various carbon sources, and bivalent metal ions [22]. The phosphatase of *B. pumilus* has broad substrate specificity and is able to mobilize inorganic phosphate from natural substrates. Under non-induced conditions, the initial strain 3-19 showed the absence of phosphatase activity at all phases of culture development because the nutrient medium (LB) contains phosphate-rich tryptone. The AprBp protease-deficient strain showed on average a 5-fold increase in accumulation of the enzyme activity in the medium compared to the initial strain. At the same time, the strain with the inactivated *gseBp* gene showed a 10-fold increase in phosphatase activity throughout the cultivation (Fig. 5e). Thus, disturbances in the *gseBp* gene of *B. pumilus* resulted in phosphatase activation, which may be evidence of phosphatase inactivation in the wild type strain in the presence of glutamyl endopeptidase. These data indicate the important physiological function of the specific protease in formation of the extracellular pool of hydrolases of *B. pumilus*.

In total, our results lead to a conclusion that disruption of the serine proteinase genes in bacilli changes the morphology and biochemistry of recombinant strains and influences the formation of the pool of extracellular hydrolases in *B. pumilus*.

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