Microbiology, Vol. 71, No. 4, 2002, pp. 420–424. Translated from Mikrobiologiya, Vol. 71, No. 4, 2002, pp. 494–499.

EXPERIMENTAL ARTICLES

Hydrolytic Enzymes and Sporulation in *Bacillus intermedius*

M. R. Sharipova*, [1], N. P. Balaban*, L. A. Gabdrakhmanova*, M. A. Shilova*, Yu. M. Kadyrova, G. N. Rudenskaya, and I. B. Leshchinskaya***

**Kazan State University, ul. Kremlevskaya 18, Kazan, 420008 Russia*

***Moscow State University, Vorob'evy gory, Moscow, 119899 Russia*

Received May 28, 2001; in final form, February 19, 2002

Abstract—The investigation of the activity of extracellular hydrolytic enzymes and sporulation in the bacterium *Bacillus intermedius* 3-19 showed that the activity of ribonuclease is maximal in the glucose-containing growth medium, in which sporulation is suppressed. At the sporulation stages II–IV, the synthesis of phosphatase was not regulated by the factors that influence this synthesis in the phase of growth retardation. Caseinolytic activity exhibited two peaks. The first peak was observed when thiol-dependent proteinase began accumulating in the medium. The second peak corresponded to the late stages of sporulation, i.e., the stages of spore maturation and the autolysis of sporangium. The regulatory relationship between proteinase synthesis and sporulation and the possible role of extracellular phosphatases and proteinases in the sporulation are discussed.

Key words: alkaline phosphatase, ribonuclease, thiol-dependent proteinase, glutamyl endopeptidase, sporulation, *Bacillus intermedius*.

Sporulation in bacilli and some other prokaryotes provides for their survival under extreme conditions. The mechanism controlling the transition of a bacillar culture from the stage of vegetative growth to the stage of sporulation is a fundamental biological problem. Bacilli initiate sporulation over a short time period after the onset of replication, modifying their metabolism in accordance with acquired information on the surrounding conditions. Morphological and metabolic changes are controlled by a special program of differential gene expression [1, 2]. Some aspects of sporulation are known, while others, including the role of hydrolytic enzymes in this process, remain unclear.

The aim of this work was to study the relationship between sporulation and the accumulation of extracellular hydrolytic enzymes in the bacterium *Bacillus intermedius* 3-19.

MATERIALS AND METHODS

The strain *Bacillus intermedius* 3-19 (Str 500) used in this study is a streptomycin-resistant mutant of the wild-type strain *B. intermedius* 7P from the Culture Collection of Kazan State University.

The strain was grown in a medium containing (%) peptone, 2; $CaCl_2 \cdot 2H_2O$, 0.01; $MgSO_4 \cdot 7H_2O$, 0.03; NaCl, 0.3; and $MnSO_4$, 0.01 (pH 8.5). The medium was sterilized at 1 atm. $CaCl_2$, $CoCl_2$, Na_2HPO_4, glucose, gelatin, and lactate solutions were sterilized separately at 0.5 atm. and added aseptically immediately before inoculation to give the following final concentrations: 5 mM $CaCl_2$, 2 mM $CoCl_2$, 0.01% Na_2HPO_4, 1% glucose, 1% gelatin, and 0.3 and 3% lactate. Streptomycin sulfate was also added to the medium before inoculation in an amount of 500 μg/ml. The strain was cultivated in Erlenmeyer flasks one-fifth full of the growth medium on a temperature-controlled shaker (200 rpm; 30°C) from Braun (Germany). The medium was inoculated with a 48-h culture grown in the presence of streptomycin. The inoculum size was 1 vol %. Streptomycin was added to the growth medium at a concentration of 0.5 mg/ml. Growth was monitored by measuring culture turbidity at 590 nm using a KFK-2 photoelectrocolorimeter.

To reveal endospores, cells were Gram stained as described by Gusev and Mineeva [3]. Cells with endospores were enumerated using a Carl Zeiss phase-contrast microscope (Jena, Germany), at a magnification of 1600×. The total number of vegetative and sporulating cells was determined in no fewer than 5 microscope fields, and the relative number of the latter cells was expressed as a percentage of the total number of cells. Sporulation phases were determined as described by Schlegel [4].

The activity of RNase was estimated by measuring the amount of the products of RNA hydrolysis soluble in 4% $HClO_4$. The products were assayed with 12% uranyl acetate [5]. One unit (U) of RNase activity was defined as the amount of enzyme that increases the

[1]Corresponding author. E-mail: margarita.sharipova@ksu.ru

0026-2617/02/7104-0420$27.00

extinction E_{260} of the reaction mixture by one optical density unit per 1 hour.

Caseinolytic activity was determined using a 2% solution of casein in 0.1 M Tris–HCl buffer (pH 8.5) as described by Kaverzneva [6]. One unit of caseinolytic activity was defined as the amount of enzyme that liberates 1 μmol tyrosine per 1 min.

Thiol-dependent serine proteinase was assayed with the chromogenic peptide substrate Z-Ala-Ala-Leu-pNA [7]. One unit of proteinase activity was defined as the amount of enzyme that hydrolyzes 1 nmol of the substrate per 1 min.

Glutamyl endopeptidase was assayed with *N*-carbobenzoxy-L-glutamic acid *p*-nitroanilide (Z-Glu-pNA) [7]. One unit of endopeptidase activity was defined as the amount of enzyme that hydrolyzes 1 nmol of the substrate per 1 min.

Phosphomonoesterase activity was determined with *p*-nitrophenylphosphate (*p*-NPP) using a calibration curve plotted as the extinction E_{410} versus the concentration of *p*-NPP [5]. One unit (U) of phosphatase activity was defined as the amount of enzyme releasing 1 μmol of *p*-nitrophenol per 1 min.

The specific activity of the enzymes was expressed as the ratio of the enzymatic activity of 1 ml of the culture liquid to the cell concentration.

The results were statistically processed using Student's *t*-test statistics for significance level P 0.05. Calculations were carried out with the aid of the Microsoft Excel program and were considered to be confident when the standard deviation σ was equal to or less than 15%.

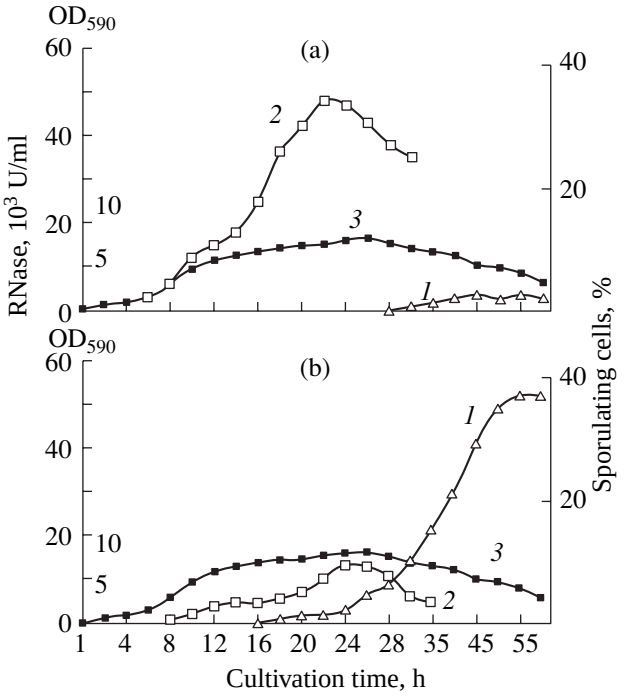

Fig. 1. Growth, sporulation, and RNase activity in the *B. intermedius* culture grown in the medium (a) with 1% glucose and (b) without glucose: (*1*) sporulating cells; (*2*) RNase; and (*3*) biomass. The total number of vegetative and sporulating cells was taken as 100%. For all data points, σ < 12%.

RESULTS AND DISCUSSION

When *B. intermedius* was grown in the medium without glucose, sporulation began after 28 h of growth (Fig. 1b). In the medium with 1% glucose, the activity of extracellular RNase was 3.5 times higher than in the glucose-free medium, and sporulation was suppressed (Fig. 1a).

It is known that the regulatory region of the RNase gene of *B. intermedius* contains promoter sequences recognizable by the vegetative σ^A factor and the regulatory proteins of the PHO regulon [8], although RNase synthesis and sporulation are likely to be regulated separately. According to our data, however, these two processes are related reciprocally. If RNase performs a catabolic function during the post exponential growth phases, this may be associated with phosphorus metabolism.

With this in mind, in the next set of experiments (Fig. 2a), we studied the effect of 0.1 mM $CoCl_2$, 0.01% Na_2HPO_4, 0.3, and 0.3 and 3% sodium lactate (these substances are known to influence the activity of extracellular phosphatase in the phase of growth retardation) on the dynamics of sporulation and phosphatase activity in the culture liquid of *B. intermedius*. Microscopic observations showed that forespores began forming in the control medium after 24 h of growth, whereas mature spores appeared between the 35th and 40th h of growth (Fig. 2b). Low concentrations of lactate (0.3%) and inorganic phosphate (0.01%) enhanced bacterial growth and increased the number of sporulating cells. Conversely, 0.1 mM Co^{2+}suppressed sporulation. The activity of extracellular phosphatase exhibited two maxima. The first peak of phosphatase activity was observed in the 12th h of growth, i.e., in the phase of growth retardation, before sporulation. At this developmental stage, extracellular phosphatase may provide for the vegetative growth of the culture. The second peak of phosphatase activity, which was 3 times lower than the first peak, corresponded to the sporulation stages II–IV, when various spore structures are synthesized and cells do not reach the state of metabolic dormancy. Of interest is the fact that the regulation of phosphatase synthesis was different in the trophic phase and in the idiophase (Fig. 2b). For instance, inorganic phosphate in the medium considerably decreased the first peak of phosphatase activity but, unlike Co^{2+}, did not influence its second peak. These data allow the suggestion to be made that the phosphatases of *B. intermedius* in these two peaks serve different physiological functions.

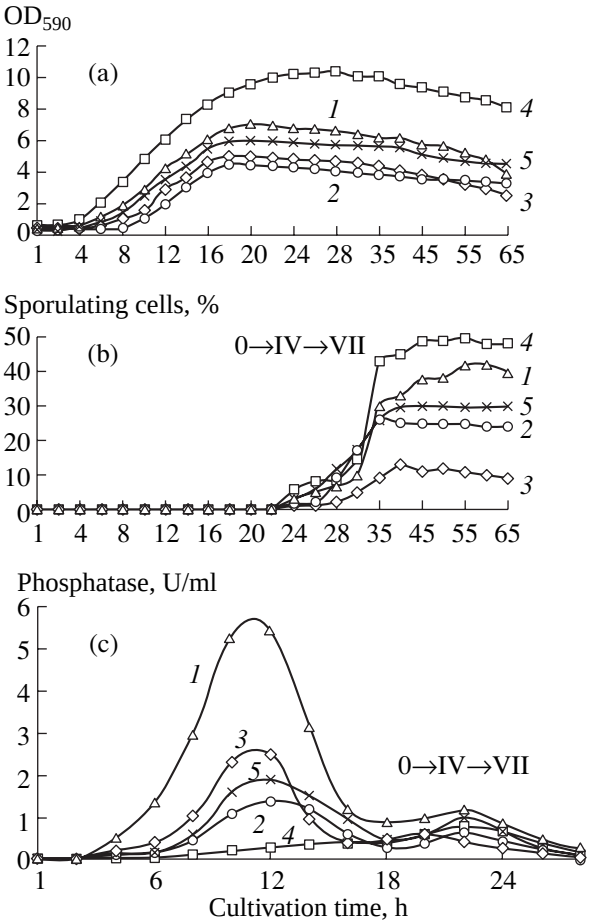

Fig. 2. (a) Growth, (b) sporulation, and (c) the activity of extracellular phosphatase in the *B. intermedius* culture grown (*5*) in the control medium and in the presence of (*1*) 0.3% lactate, (*2*) 3% lactate, (*3*) 0.1 mM $CoCl_2$, and (*4*) 0.01% Na_2HPO_4. The total number of vegetative and sporulating cells was taken as 100%. 0 ⟶ IV ⟶ VII indicate sporulation stages. For all data points, $\sigma < 12\%$.

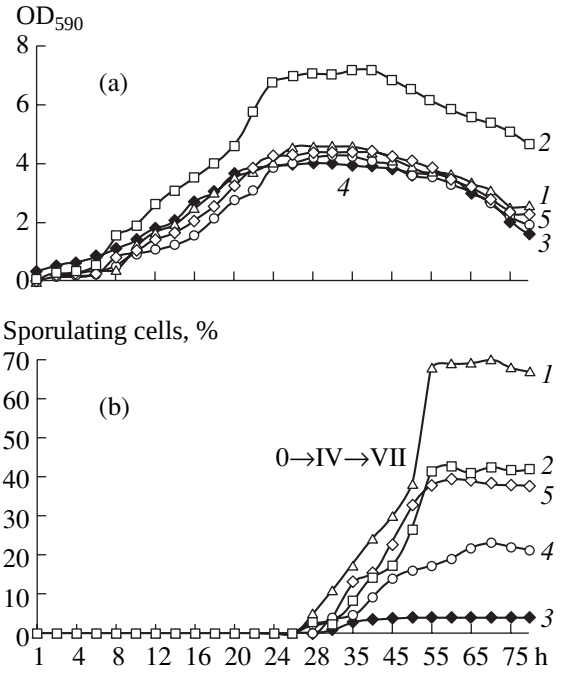

Fig. 3. (a) Growth and (b) sporulation in the *B. intermedius* culture grown (*5*) in the control medium and in the presence of (*1*) 1% gelatin, (*2*) 0.01% Na_2HPO_4, (*3*) 1% glucose, and (*4*) 1% lactate. The total number of vegetative and sporulating cells was taken as 100%. 0⟶IV⟶VII indicate sporulation stages. For all data points, $\sigma < 15\%$.

According to data available in the literature, there are two structural genes of the alkaline phosphatase of *B. subtilis*, *phoA* and *phoB* [9]. These genes are expressed in the postexponential growth phase, when cells are starved of phosphate, and are regulated by proteins of the Pho regulon. During sporulation, the expression of the *phoA* gene is suppressed, while the *phoB* gene is normally expressed. Investigations revealed a tandem of two promoters, P_v and P_s, in the promoter region of the *phoB* gene [10]. The nucleotide sequence of the latter promoter is similar to the conservative sequences recognizable by the spore-specific σ^E factor. The expression of the P_s promoter depends on the *spo*0 and *spo*II genes [11]. The coregulation of the genes of the spore regulon and the Pho regulon suggests that the physiological function of the second-peak phosphatase is related to the processes of cell differentiation. It should also be noted that phosphatases play an important part in the metabolism of polyribonucleotides in the stationary growth phase, when the de novo synthesis of purines and pyrimidines is strongly suppressed [12]. Phosphatases are also involved in the continuous circulation of RNA by bringing about the dephosphorylation of mononucleotides, thereby providing for a constant rate of RNA synthesis.

It is believed that sporulation in bacilli correlates with the synthesis of alkaline proteinases [1]. Our earlier studies showed that *B. intermedius* cells from the early stationary growth phase secrete extracellular proteinases, 75% of which is subtilisin-like thiol-dependent proteinase and 10% of which is glutamyl endopeptidase [13]. Bearing this in mind, we studied the dynamics of extracellular proteolytic activity using three substrates, casein (measures the total proteolytic activity of the culture liquid), Z-Ala-Ala-Leu-pNA (the substrate of thiol-dependent proteinase), and Z-Glu-pNA (the substrate of glutamyl endopeptidase). These experiments were carried out using different growth media (Fig. 4).

In the medium with 1% glucose, the bacterium *B. intermedius* did not produce spores (Fig. 3b) and showed a low level of extracellular proteolytic activity determined with the three substrates used (Fig. 4). This implies that both sporulation and proteinase synthesis are subject to catabolite repression. In the media with 1% gelatin and 0.01% inorganic phosphate, extracellular proteolytic activity assayed with the three substrates

was higher than in the control. In this case, gelatin was found to stimulate spore formation (Fig. 3b). Thiol-dependent proteinase began accumulating after 24 h of growth, i.e., at the onset of sporulation (the 0th sporulation stage) (Fig. 4b). The activity of extracellular thiol-dependent proteinase was at a maximum in the 46th h of growth, i.e., in the late stationary growth phase, which corresponded to the sporulation stages V–VII (the stages of synthesis of spore coats, spore maturation, and sporangium autolysis, respectively). The maximal level of the glutamyl endopeptidase activity was observed in the 18th h of growth, i.e., before the onset of sporulation. However, the background level of this activity (10 U/ml) was observed in the idiophase and at all sporulation stages, including the stage of the release of mature spores (70 h of growth) (Fig. 4c).

The total proteolytic activity, determined with casein as the substrate, also exhibited two peaks in the course of growth. The first peak was observed in the 24th h of growth. The second peak was 4 to 6 times higher and corresponded to the late stages of sporulation (46–50 h of growth) (Fig. 4a). The particular enzymes constituting these peaks of proteolytic activity are unknown. Nevertheless, taking into account the functional and physiological roles of proteinases at the late stages of sporulation in *B. intermedius*, some speculation as to this point can be advanced.

The formation of spore coats requires active synthesis of proteins. This synthesis can be provided with oligopeptides and amino acids through the proteolytic cleavage of extracellular proteins, which are no longer necessary for vegetative cells in the stationary growth phase, when sporulation occurs. There is evidence that proteinases are also involved in at least two other sporulation processes. First, serine proteases may perform the post-translational modification of spore protein precursors. Second, proteinases may cleave the surface structures of sporulating cells, thereby promoting the release of spores into the medium.

It is known that sporulation induces changes in the σ factors of transcription, due to which the hitherto nontranscribable genes begin to be transcribed [1, 2]. The key role in the initiation of sporulation is played by the SpoOA protein, whose transcription involves the vegetative (P_v) and sporulation (P_s) promoters [1]. The P_s promoter is subject to catabolite repression and does not function during the stage of vegetative growth [11]. Consequently, catabolite repression is the main regulatory mechanism responsible for the initiation of sporulation and the synthesis of proteolytic enzymes. Our data on the inhibitory action of glucose on both proteinase synthesis (Figs. 4a–4c) and sporulation (Fig. 3b) are in agreement with this inference.

It still remains unknown what signals induce sporulation. The finding that the oligopeptide permease (Opp) mutants of *B. subtilis* are deficient in sporulation [14] suggests that some extracellular peptides are involved either in the regulation of the phosphate flux

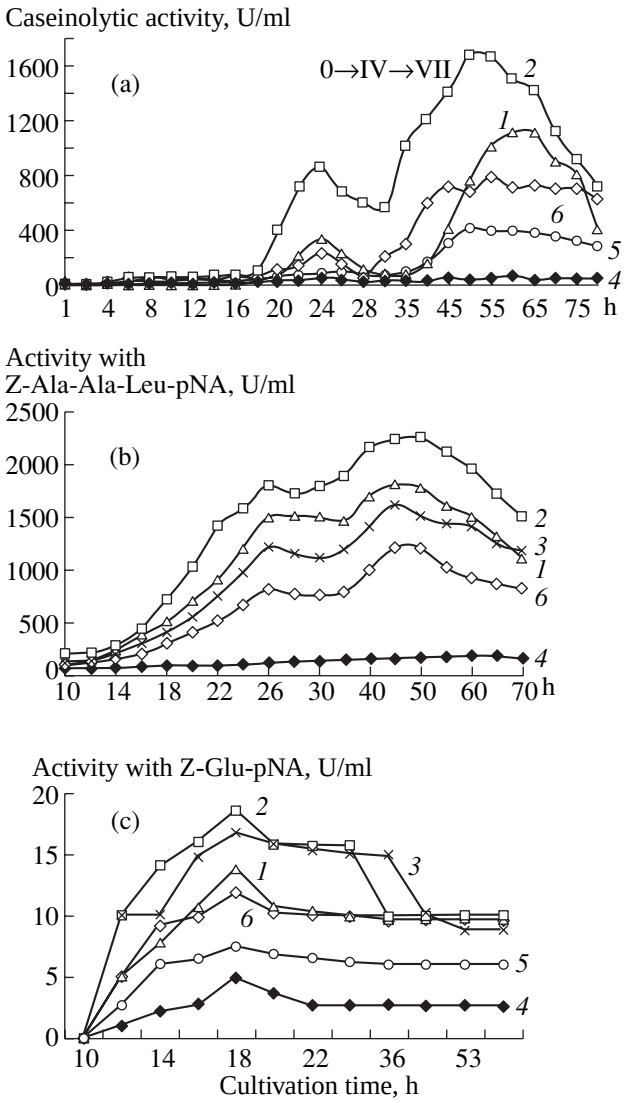

Fig. 4. The activity of extracellular proteinases assayed with (a) casein, (b) Z-Ala-Ala-Leu-pNA, and (c) Z-Glu-pNA during the growth of *B. intermedius* 3-19 in (*6*) the control medium and in the presence of (*1*) 1% gelatin, (*2*) 0.01% Na_2HPO_4, (*3*) 5 mM $CaCl_2$ (b) or 2 mM $CoCl_2$ (c), (*4*) 1% glucose, and (*5*) 1% lactate. 0⟶IV⟶VII indicate sporulation stages. For all data points, σ < 12%.

or in the activation of the spore-specific σ factors responsible for the transcription of late genes. Peptides may be recognized by the Opp system involved in sporulation [14].

Thus, the stationary-phase phosphatases and proteinases of *B. intermedius* may be involved not only in various catabolic processes but also in sporulation.

ACKNOWLEDGMENTS

This work was supported by grants from the Russian Foundation for Basic Research (grant no. 01-04-48037), St. Petersburg University (grant E00-6.0-15),

CRDF (grant Rec 007), and the Academy of Sciences of Tatarstan (grant no. 03-3.10-11/2001(F)).

REFERENCES

1. Errington, J., *Bacillus subtilis* Sporulation: Regulation of Gene Expression and Control of Morphogenesis, *Microbiol. Rev.*, 1993, vol. 57, no. 1, pp. 1–33.
2. Haldenwang, W.G., The Sigma Factors of *Bacillus subtilis*, *Microbiol. Rev.*, 1995, vol. 59, no. 1, pp. 1–30.
3. Gusev, M.V. and Mineeva, L.A., *Mikrobiologiya* (Microbiology), Moscow: Mosk. Gos. Univ., 1992, p. 28.
4. Schlegel, H.G., *Allgemeine Mikrobiologie*, 6th ed., Stuttgart: Georg Thieme, 1985. Translated under the title *Obshchaya mikrobiologiya*, Moscow: Mir, 1987.
5. Leshchinskaya, I.B., Balaban, N.P., Kapranova, M.N., and Golubenko, I.A., *Sovremennye metody izucheniya nukleinovykh kislot i nukleaz mikroorganizmov* (Modern Methods of Investigating Microbial Nucleic Acids and Nucleases), Kazan: Kazan State Univ., 1980.
6. Kaverzneva, E.D., A Standard Method for Measuring the Proteolytic Activity of Complex Proteases, *Prikl. Biokhim. Mikrobiol.*, 1971, vol. 7, no. 2, pp. 225–228.
7. Lyublinskaya, L.A., Voyushina, T.L., and Stepanov, V.M., Pyroglutamylpeptide *p*-nitroanilides as Chromogenic Substrates of Serine Proteinases, *Bioorg. Khim.*, 1987, vol. 13, no. 6, pp. 748–753.
8. Znamenskaya, L.V., Vershinina, O.A., Vershinina, V.I., Krasnov, S.I., Kostrov, S.V., Akimkina, T.V., Leshchinskaya, I.B., and Khartli, R.V., The Expression of the Secretory Guanyl-Specific Ribonuclease Genes of *Bacillus intermedius* and *Bacillus pumilis* in *Bacillus subtilis*, *Mol. Genet. Mikrobiol. Virusol.*, 1999, no. 1, pp. 12–17.
9. Hulett, F.M., Kim, E.E., Bookstein, C., Kapp, N.V., Edwards, C.W., and Wyckoff, H.W., *Bacillus subtilis* Alkaline Phosphatase III and IV: Cloning, Sequencing, and Comparisons of Deduced Amino Acid Sequences with the *Escherichia coli* Alkaline Phosphatase Three-Dimensional Structure, *J. Biol. Chem.*, 1991, vol. 266, pp. 1077–1084.
10. Liu, W. and Hulett, F.M., *Bacillus subtilis* PhoP Binds to the PhoB Tandem Promoter Exclusively within the Phosphate Starvation–Inducible Promoter, *J. Bacteriol.*, 1997, vol. 179, no. 20, pp. 6302–6310.
11. Hoch, J.A., *spo0* Genes, the Phosphorelay, and the Initiation of Sporulation, *Bacillus subtilis* and Other Gram-Positive Bacteria: Biochemistry, Physiology, and *Molecular Biology*, Sonenshein, A.L. *et al.*, Eds., Washington: Am. Soc. Microbiol., 1993, pp. 747–755.
12. Senesi, S., Lippi, P., Rela, L., and Falcone, G., Cellular Location and Physiological Role of Phosphomonoesterases in *Bacillus subtilis*, *Microbiologica*, 1981, vol. 4, pp. 371–382.
13. Sharipova, M.R., Shakirov, E.V., Balaban, N.P., Gabdrakhmanova, L.A., Shilova, M.A., Rudenskaya, G.N., and Leshchinskaya, I.B., The Cellular Location of Proteolytic Enzymes of *Bacillus intermedius*, *Mikrobiologiya*, 2000, vol. 69, no. 5.
14. Perego, M., Higgins, C.F., Pearce, S.R., Gallagher, M.P., and Hoch, J.A., The Oligopeptide Transport System of *Bacillus subtilis* Plays a Role in the Initiation of Sporulation, *Mol. Microbiol.*, 1991, vol. 5, pp. 173–185.