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A Gene (*sleB*) Encoding a Spore Cortex-Lytic Enzyme from *Bacillus subtilis* and Response of the Enzyme to L-Alanine-Mediated Germination

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The *Bacillus subtilis sleB* gene, which codes for the enzyme homologous to the germination-specific amidase from *Bacillus cereus*, was cloned and its nucleotide sequence was determined. Sequence analysis showed that it had an open reading frame of 918 bp, coding for a polypeptide of 305 amino acids with a putative signal sequence of 29 residues. Enzyme activity was not found in germination exudate of *B. subtilis* spores, which differs from the case of *B. cereus* enzyme. A *B. subtilis* mutant with an insertionally inactivated *sleB* gene revealed normal behavior in growth and sporulation. However, the *sleB* mutant was unable to complete germination mediated by L-alanine.

Transformation of a dormant resistant bacterial endospore into a metabolically active vegetative cell involves a complex series of sequential interrelated biochemical events (2, 6). This germination response is triggered in spores by germinants, and the underlying germination principal is believed to be universal for spores of all species (2).

In a late stage of the germination response, hydrolysis of spore peptidoglycan by spore cortex-lytic enzyme is a crucial event (2). Germination-specific amidases have been isolated from *Bacillus megaterium* KM and *C. perfringens* S40 spores (1, 5). Furthermore, we have recently indicated (7) that the germination-specific cortex-lytic enzyme released from *B. cereus* spores during germination triggered by L-alanine (3) is also an amidase. These amidases require in situ stressed spore cortex for hydrolytic activity. In addition, the enzymes share similar sensitivities to temperature, pH, ionic strength, and a variety of chemicals. However, analyses of the *C. perfringens* amidase gene, *sleC* (4), and the *B. cereus* amidase gene, *sleB* (7), suggested a diversity of structures of germination-specific amidases.

On the assumption that an enzyme homologous to the *B. cereus* amidase might play a key role in the germination process of *B. subtilis* spores, we screened for a *B. subtilis* homolog by using a heterologous probe derived from a cloned *B. cereus sleB* gene. Here, we report the cloning and sequencing of the enzyme from *B. subtilis*. The enzyme responded to germination triggered by L-alanine, the most universal germinant for spores from a diversity of origins. Cloning, sequencing, and molecular characterization of a germination-specific amidase of *B. cereus* spores has been published elsewhere (7).

Vegetative cells of *B. subtilis* 168 AJ12866 were used as the source of chromosomal DNA. The chromosomal DNA, prepared by the method of Wilson (14), was digested individually with restriction enzymes *Hin*dIII, *Pst*I, and *Eco*RI. Following digestion, DNA was separated by 0.8% agarose gel electrophoresis, transferred to nylon filters, and used for Southern blot analysis under less-stringent conditions (4× SSC, 0.1% sodium dodecyl sulfate [SDS], 55°C). To identify the *sleB* gene of *B. subtilis*, the 532-bp PCR product from the *sleB* gene of *B. cereus*, which corresponds to amino acid residues Glu-45 to Gly-222 of the *B. cereus* SleB, was used as a probe. The probe hybridized with a ~4.5-kb *Hin*dIII fragment of *B. subtilis* chromosomal DNA. *Hin*dIII fragments with sizes of 4 to 5 kb were isolated and were used to generate a partial library in pUC118. A plasmid clone containing 4.5-kb *Hin*dIII fragment, designated pBS45H, was obtained by colony hybridization. The restriction map for pBS45H is shown in Fig. 1. Southern blot analysis suggested that 2.2-kb *Nde*I fragment contains the *B. subtilis sleB* gene. Sequencing of a *Hin*dIII-*Nde*I region was carried out by the dideoxynucleotide chain termination method (9) with an AutoRead DNA sequencing kit (Pharmacia Biotech, Uppsala, Sweden) using the sequencing strategy shown in Fig. 1. All sequences reported were determined completely in both directions, and all restriction sites used in cloning fragments for sequencing were sequenced across.

The nucleotide sequence for cloned DNA is shown in Fig. 2. The nucleotide sequence consisting of 2,555 bp had three open reading frames which are transcribed in the same direction. The *B. subtilis sleB* gene, nucleotides 610 to 1527, was present between the N-terminally truncated *orf1* and the C-terminally truncated *orf3*, which encode unknown proteins. A putative ribosome-binding site, GGAGG (nucleotides 596 to 600), was found upstream of a possible start codon (ATG) at nucleotides 610 to 612. A possible candidate for promoter sequence was present upstream of *sleB*, namely the −35 region, AC (nucleotides 529 and 530), and the −10 region, AATAACCTC (nucleotides 549 to 557), with an 18-bp space between them. The termination codon TAG occurred at 918 bp from the initiation codon, so that *B. subtilis sleB* was indicated to encode a 305-residue protein with a molecular weight of 34,000. Between *sleB* and *orf3*, there was no obvious stem-loop structure, suggesting that *sleB* forms part of an operon. The nucleotide sequences 1517 to 1609 in Fig. 2, which contain coding regions for the last 3 residues of SleB and the amino-terminal 16 residues of Orf3, completely agreed with nucleotide sequences 1 to 93 in the 126 bp determined by Smith et al. (12) as a signal sequence-coding region for unidentified proteins of *B. subtilis*. In this region, a possible ribosome-binding site, GAGGTG (nucleotides 1550 to 1555), was found.

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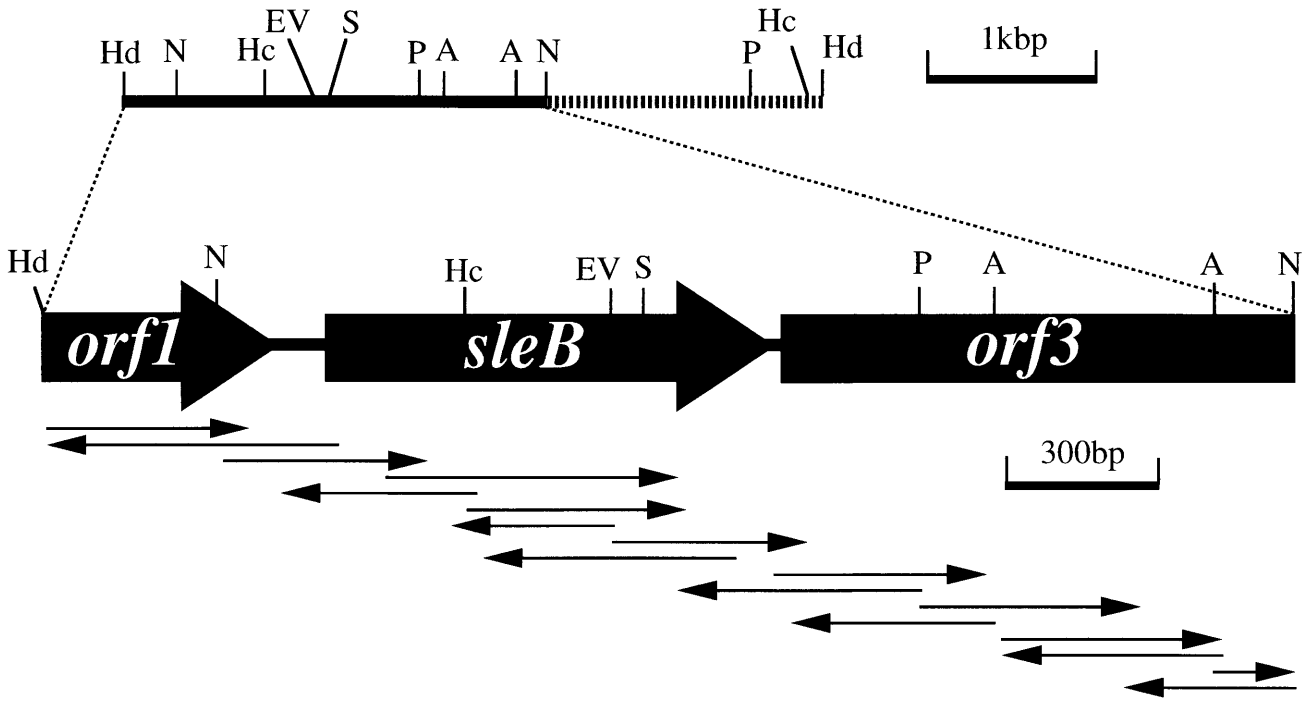

FIG. 1. Restriction map of the cloned *Hin*dIII fragment harboring the *sleB* gene of *B. subtilis*. The upper part of the figure shows a restriction map of the 4.5-kb *Hin*dIII chromosomal fragment, and the positions of open reading frames for *orf1*, *sleB*, and *orf3* are represented in the middle part. Horizontal arrows in the expanded section show the direction and extent of sequencing. Hd, *Hin*dIII; N, *Nde*I; Hc, *Hin*cII; EV, *Eco*RV; S, *Sma*I; P, *Pvu*II; A, *Acc*I.

The sequence starting from Phe-30 of *B. subtilis* SleB was quite similar to the N-terminal amino acid sequence of the mature enzyme of *B. cereus* SleB, FSNQVIQRGASGEKVI ELQ (3). The preceding 29-amino-acid sequence had most of the characteristics of a signal peptide. At the N terminus are two basic residues, followed by an alignment containing a high proportion of nonpolar residues. The −3 and −1 rules (13) also predict a cleavage site between Ala-29 and Phe-30 of *B. subtilis* SleB. These suggest that the mature enzyme is a 276-residue protein with a molecular weight of 30,848. Except for the insertions of residues 108 to 111 and residues 139 to 182 of *B. subtilis* SleB, a predicted amino acid sequence of the enzyme had high homology with the *B. cereus* enzyme (7). Both proteins contained a motif consisting of the fusion of two repeated amino acid sequences (Fig. 2, shaded sequences). The motif was also found in noncatalytic regions of various peptidoglycan hydrolases with a range of substrate specificities (4, 7), suggesting that the region is probably involved in the recognition of some repeated peptidoglycan unit of cell wall and spore cortex. A cysteine residue at the C-terminal region, which is indicated to be functionally important in *B. cereus* SleB (7), is also conserved in the *B. subtilis* enzyme as residue Cys-304.

In contrast to the *B. cereus* enzyme, however, enzyme activity was not found in germination exudate of *B. subtilis* spores and in extract from spores disrupted in 0.25 M potassium phosphate, pH 7.0. To establish the involvement of *B. subtilis* SleB in germination process, we cloned a chloramphenicol resistance cassette from the plasmid pCX18 (10) between the *Hin*cII and *Sma*I sites within *B. subtilis sleB* in pBS45H and integrated this insertion-deletion mutant into the *B. subtilis* chromosome by double-crossover recombination. Occurrence of the expected recombination event was verified by analysis of the PCR product amplified from the *sleB* gene, corresponding to the sequence encoding amino acid residues Asp-42 to Asp-264 of *B. subtilis* SleB, in chromosomal DNA of the resultant mutant, SL-1. The mutant was normal in heat resistance and spore-forming ability (Table 1), and the mutation did not affect the development from spores, i.e., germination and vegetative propagation, in Schaeffer medium (data not shown). Furthermore, as shown in Fig. 3A, when germination was measured by monitoring the decrease in the A_{600} of a spore suspension upon the addition of 10 mM L-alanine, spores of strain SL-1 responded to the germinant. However, the A_{600} values of the spore suspension slowly decreased by 60% compared with the wild-type strain, and microscopic observation revealed that the mutant did not darken completely compared with the wild type, as shown in Fig. 4. With the decrease in A_{600} of spore suspensions of the mutant, dipicolinic acid was released from spores in an amount not greatly different from that released by the wild-type strain, although there was a difference in the rate of dipicolinic acid liberation between the two strains (Fig. 3B). This means that there is no effect of the mutation on release of

FIG. 2. Nucleotide sequence of 2,555 bp of the cloned *Hin*dIII-*Nde*I fragment and deduced amino acid sequence. The deduced amino acid sequences of *orf1* (nucleotides 1 to 471, N terminally truncated), *sleB* (nucleotides 610 to 1527), and *orf3* (nucleotides 1562 to 2555, C terminally truncated) are given above the nucleotide sequence. The numbers of the nucleotides and amino acids are shown on the right. A candidate for a promoter sequence (−35 and −10 regions) and putative ribosome-binding sites (rbs) are underlined. An inverted repeat, a possible ρ-independent transcriptional terminator, is indicated by dashed arrows. Asterisks indicate termination codons. A horizontal arrow at amino acid Phe-30 of SleB denotes a putative N terminus of the mature form by homology with the protein from *B. cereus*. Shaded amino acid sequences represent postulated recognition sites for some repeated unit of spore peptidoglycan.

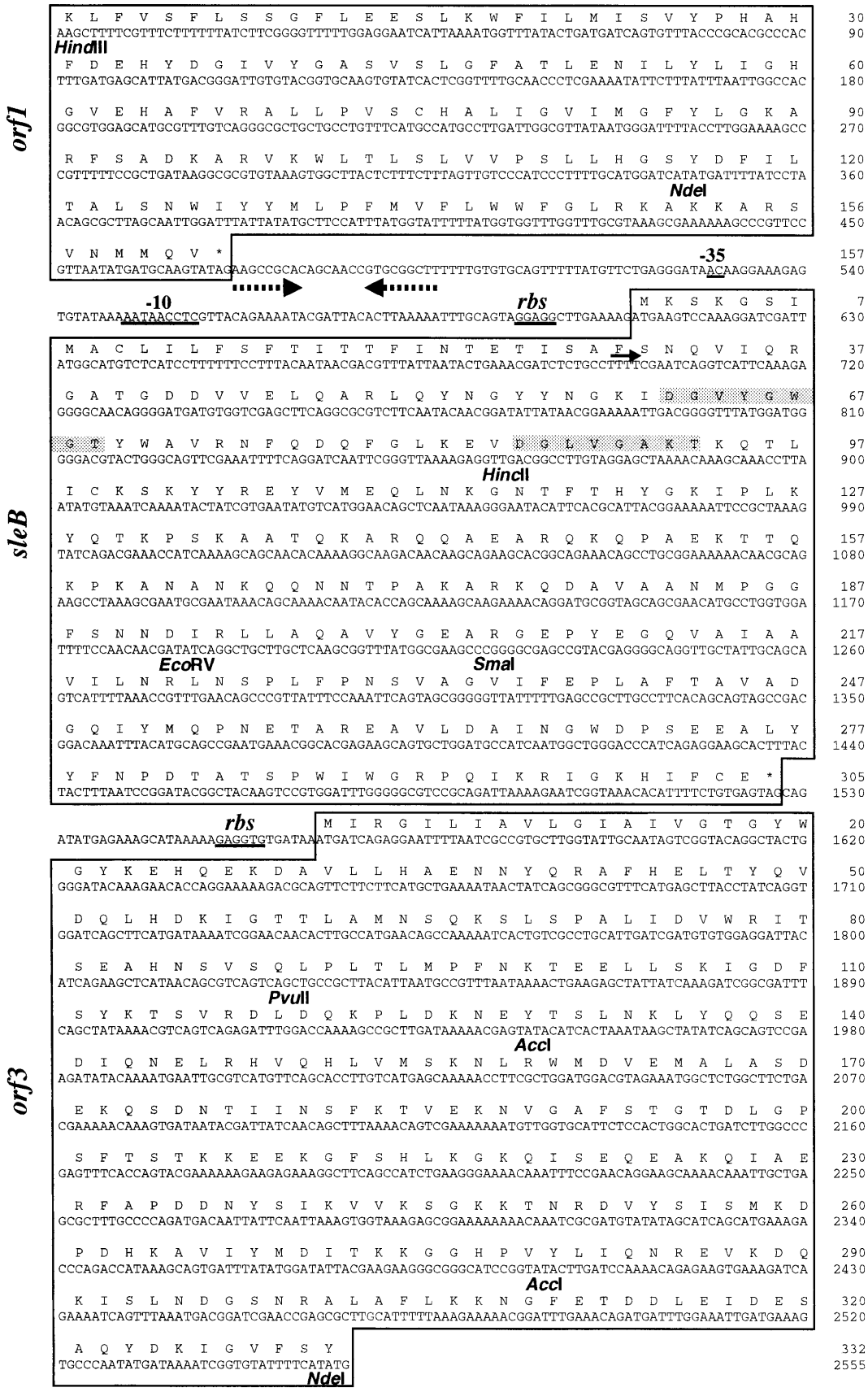

TABLE 1. Effect of insertional inactivation of the *sleB* gene on sporulation[a]

Strain	CFU (10^8 ml^{-1}) of:		% Sporulation
	Spores	Viable cells	
168	2.65	5.29	50.1
SL-1	1.17	1.77	66.1

[a] A single colony of vegetative cells of *B. subtilis* 168 or *B. subtilis* SL-1 from a freshly streaked plate was inoculated into Schaeffer medium and grown at 37°C for 16 h. Six hundred microliters of the culture was inoculated into 6 ml of Schaeffer medium and incubated with shaking at 37°C. Portions of the cultures were taken at 2 h (T_2) and 24 h (T_{24}) after the end of exponential growth. The diluted T_2 culture was immediately plated on LB plates and grown at 37°C for 16 h. Colonies were counted as viable cells. The colony-forming ability of spores was determined for the T_{24} culture heat-treated at 80°C for 10 min to kill vegetative cells. Culture of strain SL-1 was carried out in medium containing 34 μg of chloramphenicol per ml.

dipicolinic acid, suggesting that such release occurs prior to the onset of cortex hydrolysis.

The above results indicate that the *B. subtilis sleB* gene is certainly expressed in the spore and that product of the gene responds to, at least, L-alanine-triggered germination. Recently, Sekiguchi et al. (11) indicated that the CwlD (a putative cell wall hydrolase)-deficient mutant lacks the ability to germinate. SleB has no homology with CwlD. Taken together, these results strongly indicate the presence of multiple pathways in bacterial germination, which depend on germinant-specific spore cortex-lytic enzyme(s). In addition, the lack of liberation of the SleB enzyme from germinated spores suggests an inter-

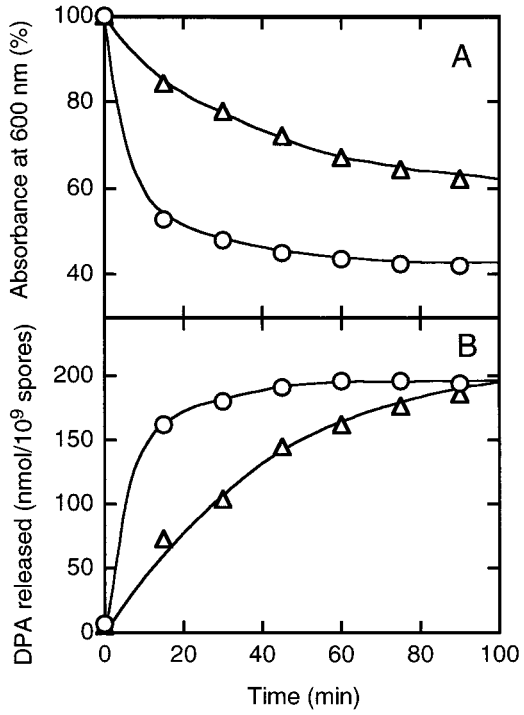

FIG. 3. Spore germination of *B. subtilis* 168 (○) and *B. subtilis* SL-1 (△). Germination of spores was monitored by A_{600} at the indicated times after addition of germination buffer (10 mM L-alanine, 0.2M KCl, 20 mM Tris-HCl, pH 7.0) at 37°C and expressed as relative absorbance (panel A). Dipicolinic acid released into the supernatant of spore suspension during germination (panel B) was also measured according to the method of Sacks (8). Initial spore concentration was 3×10^8 spores per ml.

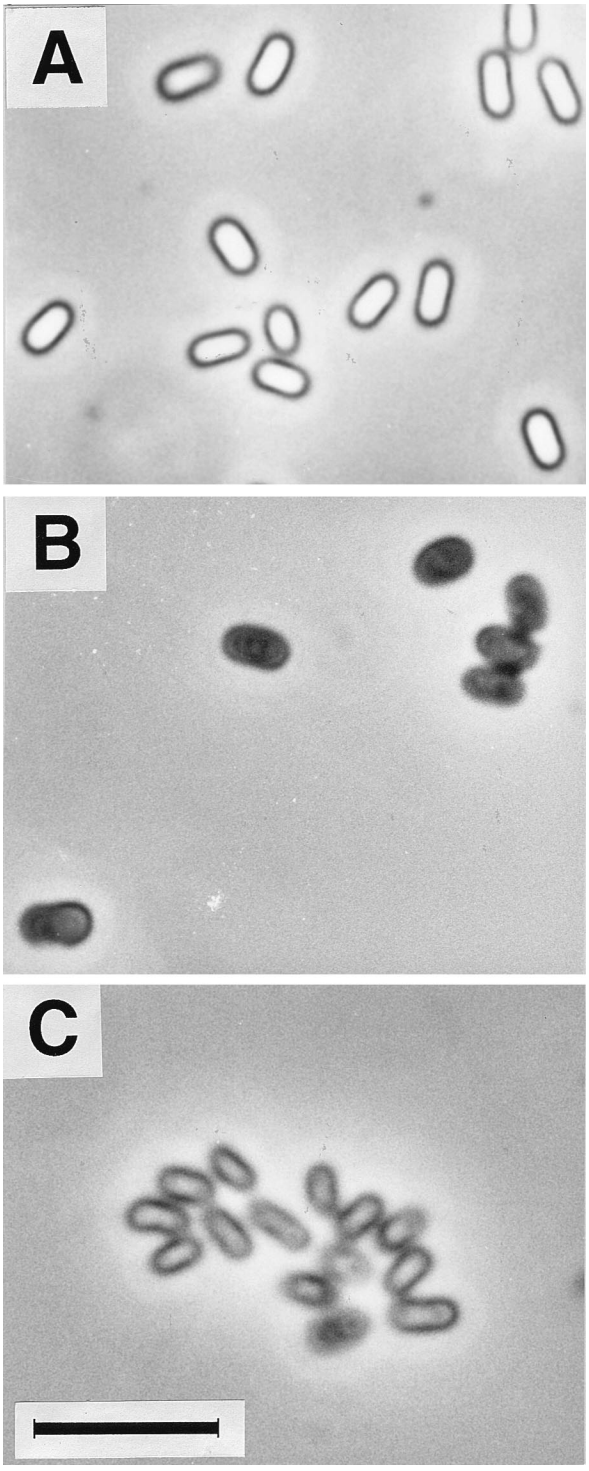

FIG. 4. Phase-contrast microscopy of *B. subtilis* 168 and *B. subtilis* SL-1 treated with L-alanine. Spores of both strains were germinated at 37°C for 2 h as shown in Fig. 3 by addition of germination buffer (10 mM L-alanine, 0.2 M KCl, 20 mM Tris-HCl, pH 7.0), further incubated at room temperature for 20 h, and then observed by phase-contrast microscopy. (A) Dormant spores of *B. subtilis* 168. (B) *B. subtilis* 168. (C) *B. subtilis* SL-1. Dormant spores of strain SL-1 were phase-bright (>98%), as were those of the wild-type strain. All pictures are shown at the same magnification. Bar, 5 μm.

action of *B. subtilis* SleB in an active form with some spore ingredient.

Nucleotide sequence accession number. The nucleotide sequence reported here has been given DDBJ/EMBL/GenBank accession number D79978.

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