Activity and Regulation of Various Forms of CwlJ, SleB, and YpeB Proteins in Degrading Cortex Peptidoglycan of Spores of *Bacillus* Species *In Vitro* and during Spore Germination

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Germination of *Bacillus* spores requires degradation of a modified layer of peptidoglycan (PG) termed the spore cortex by two redundant cortex-lytic enzymes (CLEs), CwlJ and SleB, plus SleB's partner protein, YpeB. In this study, *in vitro* and *in vivo* analyses have been used to clarify the roles of individual SleB and YpeB domains in PG degradation. Purified mature *Bacillus cereus* SleB without its signal sequence (SleB^M) and the SleB C-terminal catalytic domain (SleB^C) efficiently triggered germination of decoated *Bacillus megaterium* and *Bacillus subtilis* spores lacking endogenous CLEs; previously, SleB's N-terminal domain (SleB^N) was shown to bind PG but have no enzymatic activity. YpeB lacking its putative membrane anchoring sequence (YpeB^M) or its N- and C-terminal domains (YpeB^N and YpeB^C) alone did not exhibit degradative activity, but YpeB^N inhibited SleB^M and SleB^C activity *in vitro*. The severe germination defect of *B. subtilis* *cwlJ sleB* or *cwlJ sleB ypeB* spores was complemented by ectopic expression of full-length *sleB* [*sleB*(FL)] and *ypeB* [*ypeB*(FL)], but normal levels of SleB^{FL} in spores required normal spore levels of YpeB^{FL} and vice versa. *sleB*(FL) or *ypeB*(FL) alone, *sleB*(FL) plus *ypeB*(C) or *ypeB*(N), and *sleB*(C) or *sleB*(N) plus *ypeB*(FL) did not complement the cortex degradation defect in *cwlJ sleB ypeB* spores. In addition, ectopic expression of *sleB*(FL) or *cwlJ*(FL) with a Glu-to-Gln mutation in a predicted active-site residue failed to restore the germination of *cwlJ sleB* spores, supporting the role of this invariant glutamate as the key catalytic residue in SleB and CwlJ.

Spores of *Bacillus* species are formed in sporulation and are metabolically dormant and extremely resistant to a variety of conditions and agents including heat, radiation, desiccation, and toxic chemicals (1, 2). As a consequence of their dormancy and resistance, spores can persist in the environment for long periods, most likely for years. However, spores sense their environment, and when conditions are likely to support growth, spores can return to life through germination followed by outgrowth (3, 4). Spore germination has long been a subject of research because of its intrinsic interest and because spores lose their resistance properties upon germination and are thus easy to kill. Since spores of a number of *Bacillus* species are agents of food spoilage and food poisoning as well as human diseases, blocking spore germination or stimulating all spores in populations to germinate rapidly could have applied importance.

A crucial event in spore germination is the degradation of a thick peptidoglycan (PG) layer termed the cortex, which has several spore-specific modifications, including the presence of muramic acid- δ -lactam (MAL) and a number of muramic acid residues that have only a single L-alanine residue (4, 5). Cortex hydrolysis allows the spore core to expand and hydrate to levels found in vegetative cells. If the degradation of cortex PG is blocked, spore viability is tremendously reduced, since spore metabolism and macromolecular synthesis cannot begin (6–8). In spores of *Bacillus* species, there are two redundant cortex-lytic enzymes (CLEs), CwlJ and SleB (9–12), both of which are specific for PG containing MAL, and *cwlJ sleB* double mutant spores exhibit extremely low viability (6–9, 13, 14).

The CLE SleB is a lytic transglycosylase, and its action generates large fragments from the spore cortex, with these fragments degraded further by other enzymes (15–18). However, how SleB is

held inactive in the dormant spore and activated during germination is unknown. SleB amino acid sequences as well as *in vitro* studies indicate that after processing the N-terminal signal peptide, the resulting mature SleB (SleB^M) has two domains, an N-terminal PG-binding domain (SleB^N) that has no catalytic activity but binds well to PG containing MAL, and a C-terminal catalytic domain (SleB^C) (18–21). The structures of *Bacillus anthracis* and *Bacillus cereus* SleB^C have been determined by X-ray crystallography at ~ 1.9 -Å resolution (19, 21). These structures reveal that SleB^C has significant structural similarity to many bacterial and phage lytic transglycosylases, including a conserved catalytic glutamate residue, but with a different topological arrangement of its various structural elements. Sequence alignment and secondary-structure prediction suggest that the CLE CwlJ also has a conserved catalytic glutamate residue in a putative active site similar to that in SleB^C (19, 21). Analyses of the activity of *B. cereus* SleB domains *in vitro*, including SleB^C and SleB^N as well as a derivative (SleB^{E/Q}) with the putative catalytic glutamate changed to glutamine, indicate that neither SleB^N nor SleB^{E/Q} have activity on *B. subtilis* spore cortex PG *in vitro* (21). In contrast, *B. cereus* SleB^M and SleB^C show high hydrolytic activity on cortex PG *in vitro*, with the activity of SleB^C being higher (21). However, analyses of *Ba*-

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cillus megaterium SleB domains *in vivo* indicate that the viability and germination of *B. megaterium* *cwlJ* *sleB* spores can be restored to normal levels by expression of SleB^N, SleB^C, or even a Glu-to-Ala derivative (SleB^{E/A}) (22). These results are thus in contrast to results from assays of purified SleB forms *in vitro*.

In addition to these apparently contradictory results from *in vitro* and *in vivo* studies of the roles of the SleB domains, two additional factors complicate the assessment of SleB domain function *in vivo*. The first is that the YpeB protein encoded by the gene downstream of *sleB* in a bicistronic operon is essential for SleB assembly into spores (15, 16). YpeB's N-terminal region consists of a short hydrophobic peptide likely responsible for anchoring the protein in the membrane (15). YpeB's C-terminal region (YpeB^C) contains three PepSY repeats reported to act as negative regulators of some eubacterial metalloproteases (23), and a truncated form of YpeB is formed rapidly in spore germination (16). Perhaps YpeB prevents premature or misplaced activation of SleB and proteolysis of YpeB during germination results in SleB activation. The second complicating factor is that spores of *Bacillus* species contain a third CLE, SleL (initially called YaaH), which hydrolyzes fragmented PG substrates containing MAL (24–26). While SleL alone cannot initiate cortex PG hydrolysis *in vivo*, perhaps SleL can facilitate cortex hydrolysis initiated by SleB and/or CwlJ sufficiently to increase the rate of spore germination. Indeed, in the absence of CwlJ alone, the rate of release of spores' dipicolinic acid (DPA) during germination of individual spores is greatly slowed (8, 27), although it is not known if this slowing is due to lack of SleL action on large cortex PG fragments normally generated by CwlJ.

In an attempt to resolve these complications and to further probe the relationship between SleB and YpeB, we have analyzed the activity of various *B. cereus* SleB domains on cortex PG *in vitro* either alone or in combinations. The ability of various SleB and YpeB forms and their combinations to restore viability to *B. subtilis* spores lacking CwlJ and SleB or all three CLEs, and in some cases YpeB, was also tested, and levels of different forms of SleB and YpeB in spores of these strains were determined. The results of these studies clarify the activity and role of SleB and YpeB forms in cortex PG hydrolysis during germination of *Bacillus* spores.

MATERIALS AND METHODS

Strains used and spore preparation and purification. *Bacillus* strains used in this work are listed in Table 1. *B. subtilis* strains are isogenic with PS832, a laboratory 168 strain that is *trp*⁺. *B. megaterium* strains are isogenic with strain QM B1551. Spores of *B. subtilis* or *B. megaterium* strains were prepared at 37°C on 2× Schaeffer's-glucose medium agar plates or at 30°C in supplemented nutrient broth, respectively, and spores were harvested and purified as described previously (8, 28). All spores used in this work were >97% free of growing or sporulating cells or germinated spores.

Generation of *B. subtilis* strains with various CLE genes. Appropriate *B. subtilis* *sleB* and *ypeB* fragments, including *sleB*(N) (residues 1 to 123), *sleB*(C) (residues 182 to 305), the SleB signal peptide coding region (residues 1 to 33) fused to *sleB*(C) [SigPep gene-*sleB*(C)], *ypeB*(N) (residues 1 to 213), *ypeB*(C) (residues 214 to 450), and the region coding for the likely N-terminal YpeB membrane insertion sequence (residues 1 to 25) fused in frame to *ypeB*(C) [MemSeg gene-*ypeB*(C)] were amplified by PCR from strain PS832 chromosomal DNA, and overlap PCR pieced together two PCR fragments when necessary. The E203Q and E21Q mutations were introduced into *B. subtilis* *sleB* and *cwlJ* genes, respectively, using an overlap PCR method (29). The final PCR products included the promoter and appropriate ribosome binding sites from the *sleB*-*ypeB*

operon or the *cwlJ* gene and were cloned into plasmid pDG364, a plasmid used to integrate genes at the *B. subtilis* *amyE* locus by a double crossover (30), and the insertions in plasmids were confirmed by DNA sequencing. These plasmids were used to transform *B. subtilis* strains to an *amyE* chloramphenicol resistance (Cm^r) phenotype, and the presence of the appropriate mutations in the *sleB*, *ypeB*, or *cwlJ* genes in the resultant Cm^r strains was confirmed by PCR and DNA sequencing.

To construct a *B. subtilis* *sleL* (GenBank accession number CAB11792) null mutant, upstream (nucleotides [nt] −420 to +80 relative to the +1 *sleL* translation start site) and downstream (nt +1085 to +1585) DNA regions flanking the *sleL* gene were amplified by PCR from PS832 chromosomal DNA. The *sleL* upstream PCR product was cloned into a modified pBluescript II KS(−) vector; downstream from this insert is a Cm^r cassette. The *sleL* downstream PCR product was cloned into the resulting pBluescript plasmid in the region following the Cm^r cassette. The correct orientations of the inserts in the plasmid were confirmed by DNA sequencing. The mutagenized plasmid was used to transform *B. subtilis* strain FB113 (*cwlJ* *sleB*) to a Cm^r phenotype. Transformants in which most of the *sleL* coding region had been replaced by the Cm^r cassette were identified by PCR followed by DNA sequencing.

The *B. subtilis* *ypeB* null mutant was constructed by replacing the majority of the gene with a macrolide-lincosamide-streptogramin B resistance (MLS^r) cassette using a strategy similar to that described above. The upstream (nt −128 to +88 relative to the +1 *ypeB* translation start site) and downstream (nt +1260 to +1704) regions of the gene were amplified by PCR from PS832 chromosomal DNA, and the *erm* cassette was amplified from plasmid pFE140 (31). A three-way overlap PCR was used to generate a PCR fragment in which the *erm* cassette is between the upstream and downstream regions of the *ypeB* gene. This PCR fragment was cloned into the pGEM-T easy vector (Promega Corp., Madison, WI), giving plasmid pXY1247, which was used to transform *B. subtilis* strain FB113 to MLS^r. The deletion of the majority of the *ypeB* coding region in the transformants was confirmed by PCR.

Construction of the *B. megaterium* *cwlJ* *sleB* *sleL* strain. A plasmid designed to introduce an insertion-deletion at the *B. megaterium* *sleL* locus (GenBank accession number YP_003560590) was constructed by ligating a PCR fragment spanning the *sleL* open reading frame (ORF) with EcoRI-digested pGEM3Z (Promega Corp.). The resultant plasmid was then used as a template for an inverse PCR, using XhoI-tagged primers to introduce a deletion between positions 591 and 629 of *sleL*. The digested, purified inverse PCR product was ligated with a Tc^r cassette that was PCR amplified from plasmid pUCTV2 (32). The Δ *sleL*::Tet cassette was then PCR amplified using primers with EcoRI sites at the 5' ends and ligated with a similarly digested variant of plasmid pUCTV2, in which the original Tc^r cassette had been replaced with an erythromycin resistance cassette. Plasmid pUCTV- Δ *sleL*::Tet was introduced into *B. megaterium* strain GC103 *cwlJ* *sleB* by protoplast transformation, and a strain, GC122, in which *sleL* was replaced with Δ *sleL*::Tet was isolated after repeated culture on LB agar plates incubated at 42°C in the absence of antibiotics, and its genotype was confirmed by PCR and DNA sequencing.

Expression and purification of various forms of *B. cereus* SleB and YpeB. The *B. cereus* *sleB* and *ypeB* genes were amplified by PCR from *B. cereus* strain ATCC 14579 chromosomal DNA and cloned into a modified pET15b vector. SleB^M (residues 32 to 259), SleB^N (residues 32 to 123), and SleB^C (residues 136 to 259) were expressed in *Escherichia coli* and purified by Ni²⁺-nitrilotriacetic acid (NTA) affinity chromatography followed by tobacco etch virus (TEV) protease removal of the His₆ tag and cation exchange and gel filtration (SD75; GE Healthcare, Piscataway, NJ) chromatography (21). YpeB without the putative N-terminal membrane anchor sequence (YpeB^M; residues 23 to 447), the N-terminal domain of YpeB (YpeB^N; residues 23 to 204), and YpeB^C (residues 214 to 447) were expressed in *E. coli* and purified by Ni²⁺-NTA affinity chromatography followed by TEV protease cleavage of the His₆ tag and then by anion (YpeB^M and YpeB^N) or cation (YpeB^C) exchange and gel filtration (SD200; GE Healthcare) chromatography. All SleB and YpeB proteins

TABLE 1 List of bacterial strains used in this work and viability of their spores^a

Strain	Relevant genotypic and phenotypic characteristics ^b	Viability	Source (reference)
<i>B. subtilis</i> ^c			
PS832	Wild type	— ^d	Laboratory stock
PS533	pUB110 Km ^r	100 ^e	31
FB113	<i>cwlJ sleB</i> Sp ^r Tc ^r	<0.001	21
BH60	<i>cwlJ sleB sleL</i> Cm ^r Sp ^r Tc ^r	0.001	This work
BH61	FB113 <i>amyE::cwlJ</i> Cm ^r	83	This work
BH62	FB113 <i>amyE::cwlJ</i> (E21Q) Cm ^r	<0.001	This work
BH63	FB113 <i>amyE::sleB</i> (E203Q)- <i>ypeB</i> (FL) Cm ^r	<0.001	This work
PS4264	Δ <i>cwlJ</i> Δ <i>sleB</i> Δ <i>ypeB</i> MLS ^r Sp ^r Tc ^r	0.001	PS4265→FB113 ^f
PS4265	Δ <i>ypeB</i> MLS ^r	78	This work
PS4266	FB113 <i>amyE::sleB</i> (FL)- <i>ypeB</i> (FL) Cm ^r	107	This work
PS4267 ^g	FB113 <i>amyE::sleB</i> (N)- <i>ypeB</i> (FL) Cm ^r	0.01	This work
PS4268 ^h	FB113 <i>amyE::sleB</i> (C)- <i>ypeB</i> (FL) Cm ^r	0.002	This work
PS4269	FB113 <i>amyE::ypeB</i> (FL) Cm ^r	<0.002	This work
PS4270 ^g	FB113 <i>amyE::sleB</i> (FL)- <i>ypeB</i> (N) Cm ^r	30	This work
PS4271 ^h	FB113 <i>amyE::sleB</i> (FL)- <i>ypeB</i> (C) Cm ^r	31	This work
PS4272	FB113 <i>amyE::sleB</i> (FL) Cm ^r	49	This work
PS4273 ⁱ	FB113 <i>amyE::SigPep</i> gene- <i>sleB</i> (C)- <i>ypeB</i> (FL) Cm ^r	<0.001	This work
PS4275	PS4264 <i>amyE::sleB</i> (FL)- <i>ypeB</i> (FL) Cm ^r	104	This work
PS4276 ^g	PS4264 <i>amyE::sleB</i> (N)- <i>ypeB</i> (FL) Cm ^r	0.01	This work
PS4277 ^h	PS4264 <i>amyE::sleB</i> (C)- <i>ypeB</i> (FL) Cm ^r	<0.001	This work
PS4278	PS4264 <i>amyE::ypeB</i> (FL) Cm ^r	0.001	This work
PS4279 ^g	PS4264 <i>amyE::sleB</i> (FL)- <i>ypeB</i> (N) Cm ^r	<0.001	This work
PS4280 ^h	PS4264 <i>amyE::sleB</i> (FL)- <i>ypeB</i> (C) Cm ^r	<0.001	This work
PS4281	PS4264 <i>amyE::sleB</i> (FL) Cm ^r	<0.001	This work
PS4282	PS4264 <i>amyE::SigPep</i> gene- <i>sleB</i> (C)- <i>ypeB</i> (FL) Cm ^r	<0.001	This work
PS4283	Δ <i>cwlJ</i> Δ <i>ypeB</i> MLS ^r Tc ^r	<0.01	This work
PS4287 ⁱ	PS4264 <i>amyE::sleB</i> (FL)-MemSeg gene- <i>ypeB</i> (C) Cm ^r	0.05	This work
PS4288 ⁱ	FB113 <i>amyE::sleB</i> (FL)-MemSeg gene- <i>ypeB</i> (C) Cm ^r	8	This work
<i>B. megaterium</i>			
QM B1551	Wild type	—	Hillel Levinson
GC103	Δ <i>cwlJ</i> Δ <i>sleB</i> Km ^r Sp ^r	—	30
GC122	Δ <i>cwlJ</i> Δ <i>sleB</i> Δ <i>sleL</i> Km ^r Sp ^r Tc ^r	—	This work

^a Spores of various strains were prepared, purified, and heat shocked, and their relative viability was determined as described in Materials and Methods. All values shown are averages of results with two independent spore preparations and are $\pm 25\%$ of the value shown.

^b Abbreviations for antibiotic resistance: Cm^r, chloramphenicol (5 μ g/ml for *B. subtilis*); Km^r, kanamycin (5 μ g/ml for *B. megaterium*); Sp^r, spectinomycin (100 μ g/ml); Tc^r, tetracycline (20 μ g/ml for *B. subtilis* and 10 μ g/ml for *B. megaterium*); MLS^r, lincomycin (25 μ g/ml) and erythromycin (1 μ g/ml).

^c Genes inserted at *amyE* locus in *B. subtilis* strains are *B. subtilis* genes.

^d —, not determined.

^e The viability of the wild-type spores (PS533) was set at 100%.

^f Chromosomal DNA from the strain to the left of the arrow was used to transform the strain to the right of the arrow to MLS^r.

^g In these strains, *sleB*(N) includes the *SleB*'s signal peptide coding sequence and *ypeB*(N) includes the membrane-anchoring helix coding sequence.

^h In these strains, there is no signal sequence preceding *sleB*(C) or membrane-anchoring sequence preceding *ypeB*(C).

ⁱ In these strains, the *sleB* signal peptide coding region (SigPep gene) is fused in frame to *sleB*(C) or the *ypeB* membrane-anchoring sequence coding region (MemSeg gene) is fused in frame to *ypeB*(C).

contained an additional four N-terminal residues (Gly-Gly-Gly-Arg) prior to the native protein sequence. For Ni²⁺-NTA pulldown assays, *ypeB*(N) was cloned into a modified pET15b vector with a Sumo protein fused at the N terminus after the His₆ tag and purified as above but without removing the His₆-Sumo tag; the Sumo tag was added to ensure that the sizes of His₆-Sumo-YpeB^N and SleB^M were different. For reciprocal affinity pulldown assays, *ypeB*(M) was cloned into a modified pET15b vector with a StrepII tag fused at the N terminus to replace the His₆ tag. Coexpression of Strep-tagged *ypeB*(M) and His₆-tagged *sleB*(M) was achieved by transforming *E. coli* with two plasmids containing distinct origins of replication (p15A for the *ypeB*(M) plasmid and pBR322 for the *sleB*(M) plasmid).

Expression and purification of *B. megaterium* SleB^M and YpeB^M. The *B. megaterium* *sleB* and *ypeB* genes were amplified by PCR from *B. megaterium* strain QM B1551 chromosomal DNA. A modified *sleB*(M) gene lacking the region encoding the signal peptide (residues 33 to 310)

was generated by replacing the 8 codons that encode residues R144 to K151, located in the putative unstructured linker region that joins the N- and C-terminal domains, (21) with sequence encoding the 8-amino-acid StrepII tag (WSHPQFEK). The resulting PCR amplicon was ligated into plasmid pNZ8148 for transformation of *Lactococcus lactis* NZ9000 cells (33) to Cm^r at 30°C on solid M17 medium (Oxoid, Hampshire, United Kingdom) plus 0.5% glucose. The presence of the modified *sleB* gene in plasmid from Cm^r colonies was confirmed by DNA sequencing. The *ypeB*(M) gene lacking the membrane anchor region was cloned with a C-terminal His₁₀ tag using a ligation-independent cloning (LIC) and vector backbone exchange (VBEx) protocol (34). Essentially, the *ypeB*(M) gene was amplified by PCR using primers with 5' extensions that facilitated the LIC procedure. SmaI-digested pREcLIC vector and the *ypeB*(M) PCR product were then treated with T4 DNA polymerase in the presence of dCTP and dGTP, respectively. The two reaction products were mixed together in a 1:3 molar ratio, which was used to transform *E. coli* Top10

cells (Life Technologies, Paisley, United Kingdom) with selection on LB medium plates for carbenicillin resistance. The resultant plasmids were digested by SfiI and ligated to the VBEx vector pERL for transformation of *L. lactis* into a Cm^r strain. Plasmids from Cm^r colonies were isolated and sequenced to confirm the correct construction.

SleB^M and YpeB^M proteins were expressed in *L. lactis* in 500-ml Schott bottles with 400 ml M17 medium plus 1% glucose. Cultures were inoculated with 4 ml of an overnight culture and grown at 30°C with 90 rpm agitation to an optical density at 600 nm (OD₆₀₀) of ~0.6. Protein expression was induced by addition of nisin (MoBiTec, Göttingen, Germany) to 1 ng ml⁻¹, and growth was continued at 30°C for 4 h. Cells were harvested, washed, and suspended in 8 ml breakage buffer (100 mM Tris-HCl [pH 8.0], 150 mM NaCl, 1 mM phenylmethylsulfonyl fluoride [PMSF], and 1 mM EDTA for Strep-SleB^M; 20 mM NaH₂PO₄ [pH 7.4], 500 mM NaCl, 1 mM PMSF, and 20 mM imidazole for YpeB^M-His₁₀) followed by three passages through a One-shot Cell Disrupter (Constant Systems Ltd., Northants, United Kingdom) at 40,000 lb/in². Cell lysates were clarified by centrifugation, and the supernatant fluid was filtered and loaded on Strep-Tactin Sepharose columns (IBA Life Sciences, Goettingen, Germany) or HisTrap HP columns (GE Healthcare). The eluted proteins were desalted via centrifugal ultrafiltration (Pierce Protein Concentrator, 9K MWCO; Thermo Scientific, Waltham, MA) prior to protein interaction analyses.

Assays of cortex-lytic activity on decoated spores. The enzymatic activity of SleB and YpeB forms as well as chicken egg white lysozyme (Sigma, St. Louis, MO) was measured by monitoring the ability of the proteins to degrade cortex PG and thus trigger spore germination and release of the spore core's large DPA depot (~20% of core, dry weight) (21, 35). Spores of four *Bacillus* strains were used for this assay: (i) *B. subtilis* BH60 (*cwlJ sleB sleL*), (ii) *B. subtilis* FB113 (*cwlJ sleB*), (iii) *B. megaterium* GC122 (*cwlJ sleB sleL*), and (iv) *B. megaterium* GC103 (*cwlJ sleB*). Decoated spores were prepared by modifications to published procedures (28, 36). Briefly, purified *B. subtilis* spores (~15 mg, dry weight) were suspended in 5 ml of decoating buffer (0.1 M NaCl, 0.1 M NaOH, 1% sodium dodecyl sulfate [SDS], 0.1 M dithiothreitol [DTT]) at 70°C for 2 h and washed 10 times with distilled water by centrifugation. *B. megaterium* spores (~50 mg, dry weight) were decoated by incubation in 5 ml of extraction buffer (8 M urea, 1.5% SDS, 0.1 M β-mercaptoethanol, 50 mM Tris-HCl [pH 8.0]) at room temperature for 90 min and washed with 4 M urea and then the Tris buffer at least 4 times. The resulting spore pellets were stored in distilled water at an OD₆₀₀ of 2.0.

The hydrolytic activity of various proteins or SleB/YpeB mixtures (10 nM each or as indicated) on decoated spores was determined at 25°C in 200 μl of 25 mM K-HEPES buffer (pH 7.4), 50 μM TbCl₃, and 1 mM DTT with spores at an OD₆₀₀ of 0.5. Reactions were initiated by addition of spores, and DPA release was monitored by its fluorescence with Tb³⁺ in a multiwell fluorescence plate reader as described previously (21, 37). Each reaction mixture was tested in quadruplicate, and the reading detected at time zero was used as the background. The total DPA content of spores in the reaction mixture was determined from the maximum relative fluorescence units (RFU) of the same amount of spore suspensions boiled 30 min in water. The percentages of spores that had germinated/lysed by the end of reaction incubations were also checked by phase-contrast microscopy, and these analyses agreed with those of RFU values. All curves generated by plotting time versus RFU values were fitted using nonlinear regression to the exponential equation of the OriginPro 7.5 software program (OriginLab Corporation, Northampton, MA) to determine initial rates of DPA release (percentage of DPA release min⁻¹) for each reaction. Differences in hydrolytic activities of various SleB forms alone or in the presence of YpeB were analyzed for their significance by a two-tailed Student *t* test.

Assay of CwlJ, SleB, and YpeB function *in vivo*. To assay the functionality of CwlJ, SleB, and YpeB forms *in vivo*, genes expressing the proteins were integrated at the *amyE* locus of *B. subtilis* strains lacking *cwlJ* and *sleB* or *cwlJ*, *sleB*, and *ypeB*. The spores of the resultant strains at an OD₆₀₀ of 1.0 (~1.5 × 10⁸ spores/ml) were purified, heat shocked (30 min;

75°C), and cooled on ice, and 10-μl aliquots of serial 10-fold dilutions in water were spotted onto LB medium plates (38) containing one or two appropriate antibiotics. The plates were incubated for ~36 h at 30 to 37°C, and colonies were counted, since completion of spore germination, including cortex hydrolysis, is essential for colony formation from dormant spores. Spores of strains PS533 as well as FB113 and PS4264 were used as positive and negative controls, respectively.

Analysis of levels of various forms of SleB and YpeB in spores. Levels of different forms of SleB and YpeB in *B. subtilis* spores were determined by Western blot analysis using rabbit antisera against the mature *B. subtilis* proteins prepared as described previously (16). Spore inner membrane protein was isolated from decoated spores, and Western blot analyses on equal amounts of spore inner membrane protein were as described previously (16, 39). Control experiments with various amounts of purified *B. subtilis* SleB^M (residues 28 to 305), SleB^N (residues 28 to 123), SleB^C (residues 184 to 305), and YpeB^M (residues 23 to 450) showed that the anti-SleB^M serum was ≥30-fold less effective in detecting SleB^N and SleB^C (data not shown); various forms of *B. subtilis* SleB and YpeB were produced and purified using strategies similar to those used for *B. cereus* proteins. While purified *B. subtilis* YpeB^C and YpeB^N were not available for this analysis and the analogous *B. cereus* proteins were not detected by the antiserum against *B. subtilis* YpeB (data not shown), previous work has shown that a *B. subtilis* YpeB^{FL} degradation product of ~29 kDa is readily detected (16). After probing for SleB and YpeB, blots were also probed as described previously with antiserum against the C subunit of the GerB nutrient germinant receptor, which is also an inner spore membrane protein, to serve as an additional loading control (16, 39, 40).

Affinity pulldown assays. For Ni²⁺-NTA affinity pulldown assays with purified *B. cereus* proteins, 14 μM all proteins were incubated at 23°C for 10 min in 30 μl of binding buffer (50 mM Tris-HCl [pH 8.0], 200 mM NaCl, and 10 mM imidazole) prior to addition of 28 μl Ni²⁺-NTA resin (GE Healthcare). After 10 min, the resin was spun down, and then ~25 μl supernatant fluid, marked as the unbound (U) fraction in the figures, was removed. The resin was then washed three times with 0.6 ml of binding buffer, and the Ni²⁺ bound proteins were eluted with 30 μl buffer (50 mM Tris-HCl [pH 8.0], 200 mM NaCl, and 600 mM imidazole). One-third of each of the supernatant and eluted fractions (marked as the bound [B] fractions in the figures) was analyzed by SDS-PAGE and Coomassie blue staining.

For reciprocal affinity pulldown assays, His₆-tagged *B. cereus* SleB^M and Strep-tagged YpeB^M proteins were coexpressed in *E. coli*, and cell lysates were loaded on Ni²⁺-NTA or Strep-tactin columns at 4°C. The columns were washed extensively with buffer (20 mM Tris-HCl [pH 8.0], 150 mM NaCl), and proteins were eluted with 250 mM imidazole (for Ni²⁺-NTA) and 2.5 mM desthiobiotin (for Strep-tactin). Proteins were analyzed as described above.

Pulldown assays with purified *B. megaterium* proteins were carried out using streptavidin and cobalt-based resins. Typically, 500 μg of the bait protein was incubated with the appropriate affinity resin at 4°C for 1 h with gentle shaking and washed several times with binding buffer (20 mM NaH₂PO₄ [pH 7.4], 500 mM NaCl, 20 mM imidazole for His₁₀-tagged YpeB^M; 100 mM Tris-HCl [pH 8.0], 150 mM NaCl, 1 mM EDTA for Strep-tagged SleB^M) to remove unbound proteins. A similar amount of prey protein was added to the resin and incubated as described above. The binding buffers were adjusted to 500 mM imidazole (for cobalt) and 2.5 mM desthiobiotin (for streptavidin) to elute bound proteins. Washed (U) and eluted (B) fractions were concentrated approximately 10-fold and analyzed as described above.

RESULTS

Cortex-lytic activity of various SleB and YpeB forms on decoated spores. Previous work showed that *B. cereus* SleB^M and SleB^C proteins can trigger germination of decoated *B. subtilis* *cwlJ sleB* spores, most likely by catalyzing spore cortex lysis and subsequent DPA release, while SleB^N has no activity (21). To further

TABLE 2 Enzymatic activity of *B. cereus* SleB forms and hen lysozyme on different decoated spores

Strain and relevant genotypic characteristics	Relative initial rate of DPA release ^a		
	SleB ^M	SleB ^C	Lysozyme
<i>B. subtilis</i>			
BH60 <i>cwlJ sleB sleL</i>	100 ± 8	150 ± 6 ^b	5 ± 1 ^b
FB113 <i>cwlJ sleB</i>	105 ± 1 ^c	725 ± 27 ^b	6 ± 2 ^b
<i>B. megaterium</i>			
GC122 <i>cwlJ sleB sleL</i>	121 ± 2 ^c	382 ± 5 ^b	1.0 ± 0.5 ^b
GC103 <i>cwlJ sleB</i>	306 ± 19 ^b	874 ± 26 ^b	4.1 ± 0.4 ^b

^a Rates of DPA release were determined as described in Materials and Methods. The initial rates of the reactions were calculated as the average and standard deviation from two or three independent measurements. The initial rate was set at 100 for SleB^M action on decoated BH60 spores. SleB^M, SleB^C, and lysozyme were all at 10 nM concentrations.

^b The *P* value for this number relative to that for BH60 spores with SleB^M was ≤0.005.

^c The *P* value for this number relative to that for BH60 spores with SleB^M was >0.05.

dissect SleB's catalytic determinants and to confirm that SleB alone is sufficient to trigger spore germination, we assayed purified *B. cereus* SleB proteins on decoated *B. subtilis* and *B. megaterium* spores lacking various CLEs (Table 2). As expected, SleB^M or SleB^C caused rapid release of the majority of DPA from decoated spores and these proteins were much more effective than lysozyme (Fig. 1A and Table 2). Consistent with previous observations (21), SleB^C was more active than SleB^M in triggering DPA release from the decoated spores, and these differences were highly significant (*P* ≤ 0.005). However, SleB^C's activity was significantly lower with *cwlJ sleB sleL* spores than with *cwlJ sleB* spores, and SleB^M was less active with the *B. megaterium* triple mutant spores. In contrast to results with decoated spores, neither SleB^M nor SleB^C triggered DPA release from intact *B. subtilis cwlJ sleB* spores, even when 15-fold-higher concentrations than those that gave rapid DPA release from decoated spores were used (data not shown).

Despite being highly conserved in most *Bacillus* species, the exact role of YpeB, SleB's partner protein, is unclear. Besides the presumed N-terminal membrane anchor and the C-terminal PepSY repeats, most of the N-terminal region of YpeB (YpeB^N) is predicted to be highly helical but exhibits no sequence homology to other proteins. As the PepSY domain often has inhibitory activity in a propeptidase, we analyzed the potential activity of individual YpeB domains in cortex hydrolysis. However, high levels of purified YpeB^M, YpeB^N, or YpeB^C alone exhibited no measurable degradative activity on decoated spores (Fig. 1B to E). Note that high levels of lysozyme added to these decoated spores caused rapid DPA release (Fig. 1A and data not shown).

Effects of various YpeB forms on SleB activity on decoated spores. Although YpeB forms exhibited no activity on decoated spores, it was possible that YpeB or its domains might have effects on SleB's activity. Consequently, we tested the effects of YpeB forms on SleB^M and SleB^C activity on decoated spores (Tables 3 and 4). YpeB^M generally had insignificant effects on SleB^M activity, with significant (*P* ≤ 0.05) stimulation of SleB^M activity on *B. megaterium* spores only at the highest YpeB^M levels tested. However, high levels of YpeB^M gave very significant (*P* ≤ 0.005) inhibition of SleB^C activity on all spores tested. The effects of YpeB^C on SleB activity were generally similar to those of YpeB^M. However, YpeB^N strongly inhibited (*P* ≤ 0.05 to 0.005) SleB^M and SleB^C

activity on decoated *B. subtilis* spores. YpeB^N also gave strong inhibition (*P* ≤ 0.05 to 0.005) of SleB^M and SleB^C activity on *B. megaterium cwlJ sleB* spores, but less inhibition with *B. megaterium cwlJ sleB sleL* spores. All three YpeB forms also more strongly (*P* ≤ 0.005) inhibited SleB^C activity with *B. megaterium cwlJ sleB* spores. In contrast, with spores of the two *B. subtilis* genotypes, the effects of the YpeB forms on the activity of both SleB^M and SleB^C were very similar.

Lack of physical interaction between *B. cereus* or *B. megaterium* SleB and YpeB. Given the significant effects of YpeB on SleB activity and the coupled transcription of these two proteins' genes in a bicistronic operon, it was possible that YpeB and SleB forms interact directly. We thus assessed the interactions between various forms of YpeB and SleB using affinity pulldown assays. However, purified *B. cereus* His₆-YpeB^M, His₆-Sumo-YpeB^N, or His₆-YpeB^C did not pull down any untagged SleB forms (Fig. 2A to C). Similarly, reciprocal pulldown assays found no interaction between purified *B. megaterium* YpeB^M-His₁₀ and Strep-SleB^M or between YpeB^M-His₁₀ and Strep-SleB^N or Strep-SleB^C (Fig. 2D and data not shown). Finally, reciprocal pulldown assays from extracts of *E. coli* or *L. lactis* cells coexpressing *B. cereus* SleB^M and YpeB^M with different tags also found no evidence of SleB-YpeB interactions (Fig. 2E and data not shown). Thus, YpeB interacts weakly if at all with SleB *in vitro*.

Functionality of various forms of CwlJ, SleB, and YpeB *in vivo*. The results presented above and previously (21) indicated that purified SleB^C retains activity on cortex PG *in vitro* while SleB^N and YpeB forms do not. However, there was no YpeB requirement for SleB catalysis *in vitro*, although YpeB is somehow required for SleB's localization/assembly in spores and thus SleB activity in spore germination (16). SleB^{E/Q} is also inactive *in vitro* (21), but in *B. megaterium* spores, SleB^{E/A} is reported to complement the *cwlJ sleB* double mutation defect (22).

Given the discrepancies between results noted above *in vitro* and *in vivo*, we further assessed the ability of various SleB and YpeB forms to restore the viability of *B. subtilis cwlJ sleB* spores (39) (strain FB113; Table 1). All mutant strains with different genetic backgrounds showed a sporulation efficiency comparable to that of the wild-type strain (data not shown). As expected, ectopic expression of *sleB*(FL) plus *ypeB*(FL) complemented the *cwlJ sleB* double mutation defect even if *ypeB* was also deleted (strains PS4266 and PS4275). In contrast, ectopic expression of *sleB*(N) plus *ypeB*(FL) or *ypeB*(FL) alone did not restore *cwlJ sleB* or *cwlJ sleB ypeB* spores' viability (strains PS4267, PS4269, PS4276, and PS4278). *sleB*(C) plus *ypeB*(FL) was also ineffective in restoring *cwlJ sleB* or *cwlJ sleB ypeB* spores' viability (strains PS4268 and PS4277), but the *sleB*(C) gene in these strains did not have SleB's N-terminal signal sequence. However, even when the *sleB*(C) construct was prepared with the N-terminal SleB signal peptide, SigPep gene-*sleB*(C) plus *ypeB*(FL) did not restore *cwlJ sleB* or *cwlJ sleB ypeB* spores' viability (strains PS4273 and PS4282). These results suggest that both the N-terminal PG binding and the C-terminal catalytic domains of SleB are necessary for SleB function in spore germination.

Analysis of the ability of YpeB domains to enable SleB to complement the *cwlJ sleB* mutations initially gave confusing results, as *sleB*(FL) either alone or plus *ypeB*(N) or *ypeB*(C) gave strong complementation of the viability defect in *cwlJ sleB* spores (strains PS4270, PS4271, and PS4272). This was in contrast to the essential requirement for YpeB for normal *B. subtilis* spore germination via

FIG 1 Enzymatic activity of various SleB and YpeB proteins and lysozyme in degrading the cortex of decoated spores. (A) DPA release from decoated spores of *B. subtilis* *cwlJ* *sleB* *sleL* strain BH60 upon adding 10 nM SleB^M and SleB^C proteins and various amounts of lysozyme. The percentage of DPA release was normalized against the maximum RFU obtained from the spores boiled in water as described in Materials and Methods. Fluorescence measurements on a reaction mixture in the absence of the enzymes (Control) were used as the negative control. (B, C) DPA release from decoated spores of BH60 (B) or *B. megaterium* GC122 (with deletion of *cwlJ*, *sleB*, and *sleL*) (C) upon adding various amounts of the *B. cereus* YpeB^M protein. (D, E) DPA release from decoated BH60 spores upon adding various amounts of *B. cereus* YpeB^N (D) or YpeB^C (E) proteins. All readings in panels B to E were virtually zero and overlapped the control values (comparing vertical axes in panels A and B to E).

SleB seen previously (15, 16) and in the current work (strain PS4283). A likely explanation for these results is that *ypeB* is not deleted in strain FB113 (*cwlJ* *sleB*) (41), although it has been separated from the upstream *sleB* promoter by introduction of an antibiotic resistance cassette replacing most of the *sleB* coding sequence. It is thus possible that low levels of YpeB are produced in the absence of SleB (see below). Indeed, when much of the *ypeB* coding region was deleted from the *cwlJ* *sleB* strain, *sleB*(FL) alone or *sleB*(FL) plus either *ypeB*(N) or *ypeB*(C) was ineffective in com-

plementing the viability defect of the *cwlJ* *sleB* *ypeB* spores, even if YpeB's membrane anchor sequence was fused to YpeB^C (strains PS4279 to PS4281 and PS4287). Loss of chromosomal *cwlJ* and *ypeB* genes also eliminated the viability of these spores (strain PS4283), as expected (7, 15). Thus, YpeB and both of its domains are indispensable for maintaining SleB function during spore germination.

As found *in vitro* with *B. cereus* SleB^{E157Q} (21) and *in vivo* with *B. anthracis* *sleB*(E151A) (19), the corresponding *B. subtilis*

TABLE 3 Effects of *B. cereus* YpeB forms on *B. cereus* SleB forms' action on decoated *B. subtilis* spores^a

YpeB form added and concn (nM)	Relative initial rate of DPA release from spores of strain:			
	BH60 <i>cwlJ sleB sleL</i>		FB113 <i>cwlJ sleB</i>	
	SleB ^M	SleB ^C	SleB ^M	SleB ^C
None	100 ± 8	100 ± 4	100 ± 1	100 ± 4
YpeB ^M				
10	114 ± 12 ^b	103 ± 8 ^b	103 ± 7 ^b	83 ± 5 ^c
20	115 ± 16 ^b	71 ± 3 ^c	107 ± 5 ^b	72 ± 2 ^d
50	99 ± 14 ^b	40 ± 5 ^d	111 ± 6 ^c	50 ± 5 ^d
YpeB ^N				
10	28 ± 2 ^d	29 ± 2 ^d	30 ± 2 ^d	49 ± 2 ^d
20	26 ± 1 ^d	24 ± 1 ^d	25 ± 1 ^d	33 ± 2 ^d
50	11 ± 2 ^d	9.1 ± 2.1 ^d	6.5 ± 0.4 ^d	6.7 ± 1.2 ^d
YpeB ^C				
10	109 ± 2 ^b	85 ± 4 ^c	102 ± 9 ^b	83 ± 2 ^c
20	116 ± 5 ^c	79 ± 5 ^c	105 ± 9 ^b	73 ± 7 ^d
50	118 ± 3 ^c	45 ± 5 ^d	98 ± 6 ^b	36 ± 2 ^d

^a Rates of DPA release were determined as described in Materials and Methods. The initial rates of the reactions were calculated as the average and standard deviation from two or three independent measurements. The initial rates in the absence of YpeB were set at 100 for both SleB^M and SleB^C and for the two strains of spores used. SleB concentrations were all 10 nM.

^b The *P* value for this number relative to that for spores in the same column with SleB^M or SleB^C and without any YpeB forms was >0.05.

^c The *P* value for this number relative to that for spores in the same column with SleB^M or SleB^C and without any YpeB forms was ≤0.05.

^d The *P* value for this number relative to that for spores in the same column with SleB^M or SleB^C and without any YpeB forms was ≤0.005.

sleB(E203Q) mutant plus *ypeB*(FL) did not complement *B. subtilis* *cwlJ sleB* spores (strain BH63), further indicating that the invariant Glu157 in *B. cereus* SleB, and presumably all SleBs, is a key catalytic residue, as suggested from structural and bioinformatic analyses. Comparable analysis has suggested that an analogous conserved glutamate residue (Glu21 in *B. cereus* and *B. anthracis* CwlJ1 [19, 21]) is the key catalytic residue in CwlJ. It was not possible to test the effect of alterations in this glutamate residue on CwlJ action *in vitro*, since soluble active CwlJ protein is not available. As expected, ectopic expression of *cwlJ* from its own promoter restored *cwlJ sleB* spores' viability (strain BH61). However, ectopic CwlJ^{E21Q} expression was ineffective (strain BH62), supporting the notion that this conserved glutamate is CwlJ's key catalytic residue (19, 21). Note that CwlJ also has a likely partner protein termed GerQ that is essential for CwlJ assembly in spores (12). However, in *B. subtilis*, *gerQ* is not cotranscribed with *cwlJ*.

Levels of SleB and YpeB in spores of strains expressing *sleB* and *ypeB* variants. An obvious concern in the interpretation of results in which ectopic expression of *sleB* and *ypeB* variants were used to complement the viability defect in CLE-deficient spores was whether the SleB and YpeB variants were present in dormant spores. Consequently, we used rabbit antisera against *B. subtilis* SleB^M and YpeB^{FL} proteins in Western blot analysis of spore inner membrane proteins, since SleB and YpeB are associated with spores' inner membrane (16). Initial analyses showed that the two antisera could readily detect purified mature *B. subtilis* SleB and YpeB, but detection of SleB^N and SleB^C was at least >30-fold less sensitive than SleB^M (Fig. 3 and data not shown). However, the

TABLE 4 Effects of *B. cereus* YpeB forms on *B. cereus* SleB forms' action on decoated *B. megaterium* spores^a

YpeB fragment added and concn (nM)	Relative initial rate of DPA release from spores of strain:			
	GC122 <i>cwlJ sleB sleL</i>		GC103 <i>cwlJ sleB</i>	
	SleB ^M	SleB ^C	SleB ^M	SleB ^C
None	100 ± 2	100 ± 1	100 ± 8	100 ± 11
YpeB ^M				
10	125 ± 4 ^b	103 ± 4 ^b	114 ± 15 ^b	59 ± 8 ^c
20	132 ± 3 ^c	99 ± 1 ^b	136 ± 13 ^c	47 ± 4 ^d
50	161 ± 3 ^c	64 ± 1 ^d	164 ± 14 ^c	21 ± 2 ^d
YpeB ^N				
10	71 ± 5 ^c	116 ± 1 ^c	81 ± 13 ^c	81 ± 1 ^c
20	61 ± 6 ^c	115 ± 1 ^c	58 ± 8 ^d	68 ± 6 ^c
50	45 ± 2 ^d	56 ± 2 ^d	10.4 ± 0.2 ^d	16 ± 1 ^d
YpeB ^C				
10	121 ± 1 ^c	108 ± 2 ^b	102 ± 6 ^b	58 ± 8 ^c
20	126 ± 2 ^c	101 ± 3 ^b	122 ± 13 ^c	43 ± 3 ^d
50	142 ± 6 ^c	78 ± 1 ^c	132 ± 11 ^c	19 ± 3 ^d

^a Rates of DPA release were determined as described in Materials and Methods. The initial rates of the reactions were calculated as the averages and standard deviations from two or three independent measurements. The initial rates in the absence of YpeB were set at 100 for both SleB^M and SleB^C for the spores of the two strains used. SleB concentrations were all 10 nM.

^b The *P* value for this number relative to that for spores in the same column with SleB^M or SleB^C and without any YpeB forms was >0.05.

^c The *P* value for this number relative to that for spores in the same column with SleB^M or SleB^C and without any YpeB forms was ≤0.05.

^d The *P* value for this number relative to that for spores in the same column with SleB^M or SleB^C and without any YpeB forms was ≤0.005.

sensitivity of detection of *B. subtilis* YpeB^C and YpeB^N is not known.

The anti-SleB serum detected SleB in wild-type spores as a 31-kDa band, the size expected for SleB^M, and this band was much fainter with spores lacking a *sleB* gene (Fig. 3A and B, lanes 1 and 2); note that this lower-intensity, faint, 31-kDa band was present in all spores lacking *sleB* (lanes 2 and 4 to 11) and is presumably a nonspecific band. SleB in spores ectopically expressing *sleB* and *ypeB* in a *cwlJ sleB* double mutant or *cwlJ sleB ypeB* triple mutant background was also readily detected (Fig. 3A and B, lanes 3), as was SleB in spores ectopically expressing SleB^{E203Q} and YpeB (Fig. 3A, lane 12). However, in *cwlJ sleB* or *cwlJ sleB ypeB* backgrounds, levels of SleB^M above the background were not detected in spores expressing *sleB*(FL) unless *ypeB*(FL) was coexpressed, but not when *ypeB*(N) or *ypeB*(C) were coexpressed (Fig. 3A and B, lanes 7 to 9 and 11). SleB^N or SleB^C also was not detected, even if YpeB^{FL} was coexpressed (Fig. 3A and B; lanes 4, 5, and 10), but this could be due to the lack of reactivity of the anti-SleB serum with SleB^N or SleB^C. Overall, these results are consistent with the notion that the entire YpeB protein is required for SleB stabilization or localization to the spore inner membrane.

The anti-YpeB serum also readily detected YpeB in spores, much as the expected ~51-kDa mature protein (Fig. 3A and B, lanes 1 and 3). Note that a much lower intensity band comigrating with mature YpeB^M was seen in spores of all strains (Fig. 3A and B); presumably this is a nonspecific band, since it was seen even in spores lacking *ypeB* (Fig. 3A and B, lanes 2). In addition to mature YpeB, spores expressing *ypeB* plus *sleB* had significant amounts of ~30- and 33-kDa bands that were at minimal levels, if detected at

FIG 2 YpeB does not interact with SleB. (A to C) *B. cereus* proteins. Ni²⁺-NTA affinity pulldown assays were used to characterize the interaction of YpeB His₆-YpeB^M (A), His₆-Sumo-YpeB^N (B), or His₆-YpeB^C (C) with SleB (SleB^M, SleB^N, or SleB^C). The indicated proteins were incubated, reactions were precipitated with Ni²⁺-NTA resin, and bound (B) and unbound (U) proteins were analyzed by SDS-PAGE and Coomassie blue staining as described in Materials and Methods. (D) *B. megaterium* proteins. Streptavidin resin-bound Strep-SleB^M was incubated with YpeB^M-His₁₀, YpeB^M-His₁₀ bound to cobalt resin was incubated with Strep-SleB^M, and bound (B) and unbound (U) proteins were analyzed by SDS-PAGE and Coomassie blue staining as described in Materials and Methods. Similar results were obtained with Strep-tagged *B. megaterium* SleB^N and SleB^C proteins when incubated with *B. megaterium* YpeB^M-His₁₀ (data not shown). (E) *B. cereus* proteins. Reciprocal affinity pulldown assay with coexpressed Strep-YpeB^M and His₆-SleB^M. Cell lysates were incubated with Strep-Tactin or Ni²⁺-NTA beads and eluted with desthiobiotin or imidazole, respectively, and eluates were analyzed by SDS-PAGE and Coomassie blue staining as described in Materials and Methods.

all, in strains that should express no or low YpeB levels (Fig. 3A and B, compare lanes 1 and 3 with lanes 2, 4 to 6, and 10). The source of the smaller YpeB species is not clear, but it could be due to proteolysis during spore inner membrane isolation. Indeed, previous work showed that YpeB is rapidly cleaved during spore

germination to an ~29-kDa fragment (16), so perhaps such cleavage also takes place during disruption of dormant spores by lysozyme.

In the *cwlJ* *sleB* strain, ectopic expression of *sleB* [or *sleB*(E203Q)] plus *ypeB* gave high levels of YpeB in spores (Fig. 3A,

FIG 3 Levels of SleB and YpeB in spores of various strains. Equivalent amounts of inner membrane protein from spores of various strains lacking normal chromosomal copies of *cwlJ* and *sleB* (A) or *cwlJ*, *sleB*, and *ypeB* (B), except for the wild-type strain (PS5333 spores; lane 1), were run on SDS-PAGE, and proteins were subjected to Western blot analysis with antisera against *B. subtilis* SleB (upper panels) or YpeB (middle panels); the blots probed for SleB were stripped and reprobed for YpeB. The blots were subsequently stripped and reprobed for GerBC as an additional loading control (lower panels); the intensities of all bands were analyzed densitometrically using ImageJ, and the differences among all intensities were within 15% of that of the wild-type band (data not shown). Note that in panel A, the lanes for strain BH63 were from a separate blot.

lanes 3 and 12). In addition, *cwlJ sleB* spores expressing *sleB*(FL) alone or plus *ypeB*(N) or *ypeB*(C) had levels of the 51-kDa species above the low levels in spores lacking *ypeB* (Fig. 3A, lanes 7 to 9), consistent with weak expression of *ypeB* even when the normal chromosomal *sleB* was deleted. In general, minimal levels, if any, of the YpeB^N and YpeB^C domains were detected in the *cwlJ sleB* spores expressing these proteins (but see below), and in the absence of wild-type *sleB*(FL) levels, wild-type YpeB levels were also not present (Fig. 3A, lanes 4 to 6). These findings were also made with spores of the *cwlJ sleB ypeB* strain (Fig. 3B, lanes 4 to 10), except that the intensity of the 51-kDa band was not elevated in spores ectopically expressing *sleB*(FL) alone or with YpeB domains when the chromosomal *ypeB* gene was deleted (Fig. 3B,

lanes 7 to 9). Interestingly, while *ypeB*(C) expressed at *amyE* did not give high levels of YpeB^C, with at most a small amount of a 31-kDa species in *cwlJ sleB* spores (Fig. 3A, lane 8), in both *cwlJ sleB* and *cwlJ sleB ypeB* spores, expression of MemSeg gene-*ypeB*(C) at *amyE* resulted in significant levels of a ~32-kDa band, the size predicted for MemSeg-YpeB^C (Fig. 3A and B; lanes 11). This observation is consistent with protein expression in *E. coli*, where purified YpeB^C was more stable than YpeB^N (Fig. 2B and C). However, even when *sleB*(FL) was coexpressed with MemSeg gene-*ypeB*(C) at *amyE*, SleB was not accumulated in these spores (Fig. 3A and B, lanes 11), consistent with the lack of germination of these spores (Table 1; strains 4287 and 4288).

DISCUSSION

The results presented in this work have led to a number of new conclusions about the hydrolysis of cortex PG during germination of spores of *Bacillus* species. The first is that the redundant *B. subtilis* CLEs CwlJ and SleB both have key catalytic glutamate residues (19, 21). This has been shown for *B. cereus* SleB *in vitro* and for *B. anthracis* SleB *in vivo* (19, 21) and is consistent with the high-resolution structure of *B. cereus* and *B. anthracis* SleB^C. CwlJ activity has not yet been characterized *in vitro*, but this enzyme's predicted structure's similarity to that of SleB led to the suggestion that CwlJ and SleB have similar active sites, including a catalytic glutamate (19, 21). This does appear to be the case, suggesting that CwlJ may have a catalytic activity similar to that of SleB, but this suggestion requires further work to confirm it. The second new conclusion is that YpeB has no degradative activity on PG, certainly not enough to trigger germination of decoated spores *in vitro* or of CLE-deficient spores *in vivo*. This is perhaps not surprising given the lack of similarity of the YpeB primary sequence to known PG hydrolytic enzymes.

The third new conclusion is that SleL is not essential for cortex PG hydrolysis by exogenous SleB, as with decoated *B. megaterium* or *B. subtilis* spores lacking SleL as well as CwlJ and SleB, cortex hydrolysis and subsequent DPA release from these spores can be induced by either SleB^M or SleB^C. However, the presence of *sleL* in *B. megaterium cwlJ sleB* or *B. subtilis cwlJ sleB* spores resulted in more-rapid cortex hydrolysis and DPA release due to the added SleB^C and to SleB^M's more rapid DPA release from decoated *B. megaterium cwlJ sleB* spores. These results are consistent with SleL playing an auxiliary role in cortex PG hydrolysis, although there are several caveats to this conclusion, as follows: (i) it appears possible that SleL is lost or inactivated when *B. subtilis* spores are incubated in 0.25 N NaOH, similar to the NaOH concentration used in spore decoating, as production of likely SleL products during spore germination is greatly reduced (42); (ii) the great majority, but perhaps not all, of the SleL in *B. anthracis* spores is located in spores' outer layers, perhaps the coat/cortex boundary, where it would presumably be removed by decoating procedures, and a decoating treatment removes most SleL from *B. cereus* spores (8, 24–26, 41). Thus, it is possible that SleL is either inactive or not present in the decoated *B. subtilis* spores used in the current work for analysis of the activity of SleB forms. SleL alone appears incapable of initiating significant cortex hydrolysis in intact spores *in vivo* or when added to decoated spores *in vitro* (8, 14, 26, 41), but perhaps the presence of SleL in spores somehow modifies the overall coat structure, such that SleL deletion compromises the coat's integrity, leading to more-effective decoating and thus improved access of exogenous CLEs to the cortex PG.

A fourth conclusion is that wild-type YpeB^{FL} levels are required for accumulation of wild-type SleB^{FL} levels in spores and vice versa, as ectopic expression of either *ypeB*(FL) or *sleB*(FL) resulted in minimal levels of SleB^{FL} or YpeB^{FL} in CLE-deficient spores. However, ectopic *sleB*(FL) expression alone restored much germination to *cwlJ sleB* spores, but not to *cwlJ sleB ypeB* spores. These observations suggest that low levels of YpeB^{FL} are accumulated in *cwlJ sleB* spores even in the absence of *sleB*(FL) and a small amount of ectopically expressed *sleB*(FL) can thus be assembled in these spores; the low YpeB^{FL} levels are likely due to minimal transcription of the intact *ypeB* gene in these strains. The germination of *cwlJ sleB* spores ectopically expressing *sleB*(FL) further suggests that even a very low SleB^{FL} level is sufficient for spore germination, since the level of SleB^{FL} in *cwlJ sleB* spores ectopically expressing *sleB*(FL) was close to the background level seen in spores lacking *sleB*.

A fifth conclusion is that *sleB* and *ypeB* need not be expressed in an operon for SleB to be localized in spores and trigger spore germination. In strain FB113, lacking both *cwlJ* and *sleB*, there clearly was some *ypeB* transcription, by read-through from either the *sleB-ypeB* operon's promoter or some other promoter, and this YpeB synthesis allowed the germination of *cwlJ sleB* spores when *sleB* was synthesized ectopically at the *amyE* locus.

A sixth conclusion is that neither SleB^N, SleB^C with or without a fused signal peptide, or SleB^{E203Q} is able to complement the severe germination defect of *B. subtilis cwlJ sleB* spores. A similar result has been obtained in *B. anthracis* with SleB^{E151A} (19). These results are in contrast to the complementation of the *cwlJ sleB* double mutation defect of *B. megaterium* spores by all three of these SleB forms (22). The reason for this discordance between the results with *B. subtilis* and *B. anthracis* spores and the results with *B. megaterium* spores is not clear. However, certainly a logical possibility is that *B. megaterium* spores contain a CLE in addition to CwlJ, SleB, and SleL and that this additional CLE, which appears likely to also be a lytic transglycosylase (22), requires YpeB for its activity or its localization. Further work will be required to identify this additional putative CLE in *B. megaterium* spores.

A final conclusion from this work concerns the effects of various YpeB domains on SleB activity *in vitro* and *in vivo*. YpeB^M had only small effects on SleB^M activity on decoated spores but inhibited SleB^C activity up to 5-fold. YpeB^C also had little effect on SleB^M activity but inhibited SleB^C significantly, in particular in *B. subtilis* spores that retained *sleL*. However, YpeB^N was the most effective in inhibiting either SleB^M or SleB^C. With *B. megaterium cwlJ sleB sleL* spores, inhibition of SleB^C by YpeB^N was at most only ~50%, but with *B. megaterium cwlJ sleB* spores and all *B. subtilis* spores, inhibition by YpeB^N was much greater.

There is one major feature of SleB activity that is not really understood: why is SleB not active in dormant spores, where it is present as SleB^M, and how does SleB^M become active upon triggering of spore germination by activation of germinant receptors? One possibility is that YpeB is involved in regulating SleB activity, and this is consistent with the strong inhibition of SleB^M and SleB^C by YpeB^N. In addition, perhaps the absence of SleB from *ypeB* spores is to ensure these spores do not germinate spontaneously during sporulation by SleB action due to the absence of the inhibitory YpeB. However, purified YpeB^M and SleB^M exhibited no detectable interaction *in vitro*. How can these observations and possibilities be reconciled? One possibility is that YpeB and SleB associate only in the milieu of the spore inner membrane, where

both proteins are largely localized (16), or only when SleB is bound to PG. Perhaps YpeB may even bind to PG, although this has never been tested and YpeB has no recognizable PG-binding domains. If YpeB can indeed associate with SleB somehow, it is possible that destabilization of their association and subsequent activation of SleB during spore germination could result from the proteolytic processing of YpeB^N seen in both protein expression experiments and Western blot analysis (16). In addition, ectopic expression of *sleB*(FL) plus either *ypeB*(N) nor *ypeB*(C) did not restore viability to *B. subtilis cwlJ sleB ypeB* spores, although whether YpeB^N and YpeB^C are stably incorporated in spores at any significant level is not clear, although at least MemSeg-YpeB^C is. However, even though present at significant levels in CLE-deficient spores, MemSeg-YpeB^C did not facilitate the accumulation of SleB expressed ectopically as tested by Western blot analysis and measurements of these spores' germination. It is also not known if SleB^N and SleB^C are present at normal or even any levels in mature spores, even if SleB^C is fused to the SleB signal peptide. Clearly, determination of the function of various SleB and YpeB domains and the nature of their relationship requires further work.

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