Spore Germination Mediated by *Bacillus megaterium* QM B1551 SleL and YpeB

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Previous work demonstrated that *Bacillus megaterium* QM B1551 spores that are null for the *sleB* and *cwlJ* genes, which encode cortex-lytic enzymes (CLEs), either of which is required for efficient cortex hydrolysis in *Bacillus* spores, could germinate efficiently when complemented with a plasmid-borne copy of *ypeB* plus the nonlytic portion of *sleB* encoding the N-terminal domain of SleB (*sleB*ΔN). The current study demonstrates that the defective germination phenotype of *B. megaterium* *sleB cwlJ* spores can partially be restored when they are complemented with plasmid-borne *ypeB* alone. However, efficient germination in this genetic background requires the presence of *sle*L, which in this species was suggested previously to encode a nonlytic endopeptidase. Recombinant *B. megaterium* SleL showed little, or no, activity against purified spore sacculi, cortical fragments, or decoated spore substrates. However, analysis of muropeptides generated by the combined activities of recombinant SleB and SleL against spore sacculi revealed that *B. megaterium* SleL is actually an N-acetylglicosaminidase, albeit with apparent reduced activity compared to that of the homologous *Bacillus cereus* protein. Additionally, decoated spores were induced to release a significant proportion of dipicolinic acid (DPA) from the spore core when incubated with recombinant SleL plus YpeB, although optimal DPA release required the presence of endogenous CLEs. The physiological basis that underpins this newly identified dependency between SleL and YpeB is not clear, since pulldown assays indicated that the proteins do not interact physically *in vitro*.

Members of the bacterial genera *Bacillus* and *Clostridium* form environmentally resistant spores in response to nutrient starvation. Subsequent exposure of spores to conditions that are amenable to vegetative growth and metabolism triggers a series of germination reactions that result ultimately in the emergence of new vegetative cells (1, 2). A major germination event concerns depolymerization of the thick layer of cortical peptidoglycan that surrounds the spore protoplast. The cortex serves to maintain the relatively dehydrated status of the spore core (27 to 55% water by weight versus 70 to 80% water in vegetative cells [3]), and its removal by lytic enzymes is essential for hydration of the core to levels that permit protein mobility and the resumption of metabolic activity (4). The cortex is composed of structurally distinct peptidoglycans comprising linear chains of alternating N-acetylglucosamine and N-acetylmuramic acid (NAM) residues, the latter characterized by enzymatically processed side chains that yield either single L-alanine residues (in ~25% of NAM residues) or the spore-specific muramic acid lactam (MAL; prevalent in ~50% of NAM residues) (5, 6). Both modifications result in reduced availability of peptide side chains for cross-linking of glycan chains in cortical peptidoglycan, conferring a peptidoglycan sacculus that is relatively loosely cross-linked compared to vegetative cell sacculi (7).

The MAL moiety serves also as a structural recognition element for the cortex-lytic enzymes (CLEs) that are activated during spore germination and whose role is to depolymerize the cortical peptidoglycan while leaving intact the MAL-deficient germinal cell wall material, which forms the cell wall of the emerging cell (8). Spores of all *Bacillus* species and certain members of the *Clostridia* employ two semiredundant CLEs, SleB and CwlJ, to degrade the spore cortex during germination (9–13). Where examined, the viability of *sleB cwlJ* null mutant spores, as determined by their colony-forming activity on rich medium, is diminished by several orders of magnitude compared to that of wild-type (wt) spores of several species of *Bacillus* (10, 14, 15). The crystal structure of the catalytic C-terminal domain of SleB has been determined recently (16, 17), revealing a protein fold that is reminiscent of those of several bacterium- and phage-lytic transglycosylases, which is consistent with the results of earlier molecular-genetic and biochemical studies aimed at characterizing this CLE hydrolytic-bond specificity (18–20). Sequence alignments, putative secondary-structure assignments, and site-directed mutagenesis experiments indicate that CwlJ is a lytic transglycosylase also (17, 21), although this has yet to be confirmed by biochemical analysis.

Knowledge of the structure and function of SleB and, to a lesser extent, of CwlJ is relatively detailed, although several crucial questions remain to be answered. The same cannot be said for another protein, YpeB, whose structural gene is borne by *Bacillus subtilis* and *Bacillus megaterium* immediately downstream of *sleB* in a bicistronic operon and which shares no detectable homology with any protein of known function. However, previously conducted immunochemical studies with *B. subtilis* revealed that YpeB is essential for the presence of SleB in the spore (22), although the physiological basis of this observation was not established. A more recent study revealed that the reciprocal arrangement, i.e., that SleB is required for YpeB’s presence in the spore, also holds true.
Additionall, YpeB, in particular, the putative N-terminal domain of SleB, was demonstrated to inhibit the lytic activity of SleB against decoated spore substrates, supporting the hypothesis that YpeB may have a role in maintaining SleB in an inactive state during spore dormancy. However, despite the apparent codependency for localization and perceived modulation of activity, evidence for direct physical interaction between the proteins was not detected in vitro.

Despite the aforementioned information indicating an absolute requirement for the presence of either SleB or CwlJ for the initiation of spore cortex hydrolysis across species of the Bacillus genus, a previous study conducted by the present authors challenged this assertion to a degree. In that work, the severe germination defect of B. megaterium QM B1551 sleB cwlJ spores was complemented by plasmid-borne ypeB and the portion of sleB encoding the N-terminal domain of the enzyme (sleB<sub>N</sub>), with the resultant spores displaying essentially wild-type germination phenotypes in germination buffer or on solid nutrient medium. These results were unexpected since the N-terminal domain of SleB is noncatalytic and has a role primarily in PG binding. Furthermore, analysis of peptidoglycan fragments released into germination exudates revealed the presence of anhydromuropeptides, indicating that a lytic transglycosylase other than SleB must be present in these spores. Similar complementation analyses have been conducted recently with B. subtilis sleB cwl<sub>J</sub> spores. In that case, however, the germination defect was not complemented by sleB<sub>N</sub> plus ypeB. Hence, the protein(s) and molecular mechanisms that permit rapid germination of B. megaterium sleB cwlJ pHT-sleB<sub>N</sub> ypeB spores may be unique to this species, which occupies a distinct phylogenetic clade within the Bacilli.

The objective of the current study was therefore to attempt to gain insight into the molecular biology that supports efficient cortex hydrolysis in B. megaterium sleB cwlJ spores. Accordingly, we reveal a requirement in vivo for sleL and ypeB for efficient germination in this genetic background. Further, the protein counterparts of these genes are shown, when combined, to stimulate the release of significant amounts of dipicolinic acid (DPA) from a variety of decoated spore substrates. This was unexpected, since neither protein was thought to be lytic at the commencement of this study. We demonstrate here, however, that B. megaterium SleL is an N-acetylglucosaminidase. Finally, SleL does not appear to interact in vitro with YpeB, despite their apparent codependency for optimal activity.

**MATERIALS AND METHODS**

**Bacterial strains, culture conditions, and spore preparation.** Bacillus megaterium strains employed in this study (see Table S1 in the supplemental material) are isogenic with the wild-type (wt) QM B1551 strain. B. megaterium strains were routinely cultured at 30°C in LB medium, containing antibiotics where appropriate (see Table S1 in the supplemental material for concentrations). Bacillus cereus ATCC 10876 was used as a source of genomic DNA for B. cereus SleL-associated experiments. Plasmid isolation and propagation were achieved using Escherichia coli Top10 (Life Technologies Ltd., Paisley, United Kingdom), which was cultured routinely at 37°C in LB medium supplemented with carbenicillin (50 µg/ml). B. megaterium spores were prepared in supplemented nutrient broth (SNB) at 30°C. Spores were harvested after 48 h and purified as described previously (19). All spore preparations used in this work were predominantly (>99%) dormant phase-bright spores and were stored on ice at an optical density of 600 nm (OD<sub>600</sub>) of ~50.

**Construction of B. megaterium CLE mutant strains.** The construction of the B. megaterium sleB (15), sleB cwlJ and sleB sleL (19), and sleB cwlJ sleL (21) null mutant strains employed in this study has been described previously. Construction of pHT315-derived plasmids with the genes sleB<sub>E208A</sub> ypeB and ypeB sleB<sub>E208A</sub> (sleB with a mutation resulting in an E208A change in the enzyme) has also been described (19). Plasmid pHT-ypeB was constructed by first using PCR to amplify a DNA fragment that contained the entire B. megaterium ypeB open reading frame (ORF) plus ~200 bp of downstream sequence. A second PCR was conducted to amplify a DNA fragment that contained the putative sleB ypeB operon promoter and the ribosome binding site (RBS) that immediately precedes sleB. The DNA fragments were then fused by overlap PCR to create an ampiclon in which the ypeB ORF is placed under the control of sleB ypeB operon regulatory sequences. Finally, engineered BamHI sites at the 5’ ends of the ypeB ampiclons were digested and the fragments purified before being ligated with pHT315 linearized with the same enzyme. Plasmid pHT-ypeB was isolated from E. coli Top10 and then used to transform, via polyethylene glycol (PEG)-mediated protoplast transformation, B. megaterium sleB cwlJ and sleB cwlJ sleL strains to erythromycin resistance, creating strains GC123 and GC126, respectively. Plasmid pHT-sleL was constructed by PCR amplifying a 1.8-kb DNA fragment that encompassed the sleL ORF plus up- and downstream regulatory sequences, using B. megaterium QM B1551 genomic DNA as the template and primers that incorporate BamHI sites at their 5’ ends. The BamHI-digested ampiclon was then purified and ligated with similarly digested pHT315 and used to transform E. coli. Plasmid pHT-sleL was isolated and used subsequently to transform the B. megaterium sleB cwlJ sleL strain to erythromycin resistance, to create strain GC125. Plasmid pHT-ypeB sleL, where both the ypeB and sleL structural genes and their respective regulatory sequences are present, was constructed by digesting pHT-ypeB with EcoRI and then ligating the purified product with a similarly digested sleL ampiclon that contained EcoRI recognition sites at its 5’ end. The ligation mix was used to transform E. coli to carbencillin resistance, and plasmid pHT-ypeB sleL was isolated. Finally, the B. megaterium sleB cwlJ sleL strain was transformed to macrolide-licosamide-streptogramin B resistance (MLS<sup>R</sup>) with this plasmid to create strain GC127. All primers used in this work are available upon request.

**Spore germination and viability.** The progress of spore germination of various B. megaterium strains used in this work was monitored by determining the absorbance (at 600 nm) of heat-shocked (60°C, 10 min) spores suspended (OD<sub>600</sub> ~ 0.4) in 5 mM Tris-HCl, pH 7.8, plus 10 mM glucose, 10 mM proline, 10 mM leucine, and 50 mM potassium bromide (KBr), or, where indicated, 5% (wt/vol) beef extract (Oxoid, Basingstoke, United Kingdom). Germination assays were conducted in triplicate in 96-well plates incubated at 30°C in a PerkinElmer Envision-Xciti multi-label plate reader fitted with a 600-nm photometric filter. All experiments were conducted with at least two independently prepared batches of spores. Spore viability was assessed by plating serial dilutions of heat-shocked spores on solid LB medium, which were incubated at 30°C overnight before determination of the percent viability in comparison to that of parental QM B1551 spores, where 1 OD<sub>600</sub> unit is equal to ~10<sup>10</sup> CFU ml<sup>-1</sup>.

**RT-PCR analysis.** Reverse transcription-PCR (RT-PCR) was used to assess the expression of ypeB in various B. megaterium strains, essentially as described previously for other germination-associated genes (24). Briefly, cells (10<sup>3</sup>) were collected on an hourly basis following commencement of sporulation, as adjudged by plateauing of regular OD<sub>600</sub> measurements. Samples were treated with RNPprotect bacterial reagent (Qiagen, Manchester, United Kingdom), and RNA was then extracted and purified using an RNeasy mini kit (Qiagen). PCRs were conducted directly from RNA-containing samples using a One-Step RT-PCR kit (Qiagen), employing primers designed to amplify an ~500-bp fragment of ypeB. PCR products were electrophoresed on agarose gels and subjected to quantitative densitometry analysis using GelAnalyzer software.
Expression and purification of recombinant CLEs and YpeB. Expression and purification of B. megaterium SleL and YpeB proteins bearing C-terminal Streptag II peptides (SleL-strep and YpeB-strep, respectively) in Lactococcus lactis NZ9000 have been described previously (21). A plasmid designed to express mature B. megaterium SleL fused at its N terminus with maltose binding protein (MBP) was prepared via Gateway cloning from an appropriate PCR fragment amplified from B. megaterium genomic DNA and plasmid pDEST-HisMBP (25). The recombinant protein was expressed in E. coli BL21 cells, purified by Ni⁺⁺-nitrilotriacetic acid (NTA) chromatography, and subjected to overnight incubation at 4°C with tobacco etch virus (TEV; S219V) protease in 25 mM NaPO₄ (pH 7.0), 150 mM NaCl, 10 mM dithiothreitol (DTT), 2 mM EDTA, and 10% glycerol. A further round of Ni-NTA chromatography was conducted to purify SleL minus the MBP-His tag before buffer exchange to 30 mM NaPO₄ (pH 7.0), 1 mM EDTA, 1 mM DTT, and 0.1% (vol/vol) Triton X-100, and the protein was concentrated by centrifugal ultrafiltration. The purified protein was used immediately or mixed with 50% glycerol and stored at −80°C.

A combined ligation-independent cloning (LIC) and vector backbone exchange (VBEX) protocol (26) was used to prepare plasmids for the expression of B. megaterium SleL, YdhD, and YpeB and B. cereus ATCC 10876 SleL, all as C-terminal His₁₀ fusion proteins. The entire ORF of the corresponding structural gene for each protein was amplified by PCR, using B. megaterium QM B1551 and B. cereus ATCC 10876 genomic DNA as the templates and primers with 5’ sequence extensions that facilitate the LIC procedure. The SwaI-digested pREcLIC vector and the various purified PCR products were then treated with T4 DNA polymerase in the presence of dCTP (vector) and dGTP (PCR products), respectively. LIC-ready plasmid and PCR products were mixed together in 1:3 molar ratios and incubated for 5 min at room temperature, and then 1 μl of the respective reaction mixture was used to transform E. coli to carbenicillin resistance. Colonies were screened for the correct constructs by PCR, and the resultant plasmids were purified, mixed individually with equimolar aliquots of VBEX-compatible pERL digest, digested with SfiI, and then ligated with T4 DNA ligase. Ligation mixtures were used to transform electrocompetent L. lactis cells to chloramphenicol resistance, and the resultant Cle and YpeB expression strains were stored at −80°C as glycerol stocks until required.

Expression of recombinant proteins in L. lactis was conducted in 500-ml Schott bottles containing 400 ml of M17 medium with 1% glucose plus chloramphenicol (10 μg/ml). Precultured cell cultures were inoculated with 8 ml of an overnight culture of the appropriate expression strain and then incubated at 30°C and agitated at 90 rpm until an OD₆₀₀ of ~0.6 was attained. Recombinant protein expression was induced by addition of nisin (MoBiTec, Göttingen, Germany) to 1 ng ml⁻¹. Samples were collected every 30 min before addition of SleL (1 μg/ml) or cortical-fragment substrate. Decoated spores could be restored to chloramphenicol resistance, and the resultant Cle and YpeB expression strains were stored at −80°C as glycerol stocks until required.

Affinity pulldown assays. Pulldown assays with purified SleL-His₁₀ and YpeB-strep were carried out using streptavidin and cobalt-based resins, essentially as described previously (21). Bound and unbound fractions eluted from chromatography columns were concentrated approximately 10-fold by centrifugal ultrafiltration and then analyzed by SDS-PAGE and Coomassie blue staining.

In vitro assays of cortex-lytic activity. The enzymatic activities of SleL, YdhD, and YpeB were assessed against decoated spores, spore sacculi, and cortical-fragment substrates. Decoated B. subtilis spores were prepared by suspending spores (OD₆₀₀ ~ 30) in 1 ml of decoating buffer (0.1 M NaCl, 0.1 M NaOH, 0.1 M DTT, and 1% [wt/vol] SDS), followed by incubation at 70°C for 2 h and then extensive washing with distilled water. Decoated spore pellets were suspended in distilled water (OD₆₀₀ ~ 2) and stored frozen until use. Cortical fractions were prepared by subjecting decoated spores to five passages through the One-Shot cell disruptor (40 × 10⁶ lbf/in²). Disrupted spore fractions were recovered by centrifugation and resuspended in extraction buffer (1 M NaCl, 50 mM Tris-HCl [pH 8.0] plus 2% [wt/vol] SDS and 1% [vol/vol] 2-mercaptoethanol) and heated at 95°C for 1 h. Spore pellets were cooled, washed extensively with buffer (20 mM Tris-HCl, pH 8.0) to remove SDS, and then resuspended in the same buffer supplemented with 10 mM CaCl₂ and 0.1 mg/ml TPCK (tosylsoybul phenylalanine chloromethyl ketone)-trypsin (Sigma-Aldrich, Dorset, United Kingdom), followed by incubation at 37°C for 16 h. Trypsin was inactivated by addition of SDS (1% [wt/vol]) and the sample heated to 95°C for 15 min, followed by extensive washing with 5 mM Tris-HCl, pH 7.5. The cortical-fragment suspension was adjusted to an OD₆₀₀ of ~0.4 and stored at −20°C until use. Sacculi were prepared identically, except the cell disruption stage was omitted and nucleic acids were removed by digestion with 10 μg DNase and 50 μg RNase (Sigma-Aldrich) at 37°C for 2 h in 100 mM Tris-HCl (pH 7.8) plus 20 mM MgSO₄ prior to trypsin digestion.

Enzyme assays were conducted in 96-well plates by suspending decoated spores, sacculi (the OD₆₀₀ for both was ~0.5), or cortical fractions (OD₆₀₀ ~ 0.2) in buffer (5 mM Tris-HCl [pH 7.8] plus 1 mM DTT) to which the respective protein(s) (1 μg/ml each) was added. Ninety-six-well plates were incubated (37°C) in a PerkinElmer Envision-Xcite multilabel plate reader and agitated orbitally for 10 s every 2 min, and absorbance (600 nm) measurements were made over a 3-h period. DPA released by decoated spores was measured colorimetrically (27), with the percentage released calculated by comparing enzyme-treated supernatant values to the total DPA released by boiling (for 5 min) an equivalent amount of dormant spores. A DPA calibration curve, prepared from a similar colorimetric assessment of DPA solutions of known concentrations (0 to 100 μg ml⁻¹), was used to determine DPA concentrations released from spores.

Statistical analysis. Differences in the hydrolytic activities of various proteins, germinative rates, and levels of spore viability were analyzed for their significance via two-sample Student t tests and one-way analysis of variance (ANOVA) where appropriate.

Muropeptide analysis. Protocols for extraction, purification, and subsequent high-performance liquid chromatography-mass spectrometry (HPLC-MS) analysis of peptidoglycan fragments generated by in vivo cleavage were essentially identical to those described previously (19). For in vitro experiments intended for subsequent muropeptide analyses, B. subtilis sacculi (OD₆₀₀ = 60) were resuspended in 30 mM NaPO₄ (pH 7.0), 1 mM DTT, 1 mM EDTA, and 0.1% (vol/vol) Triton X-100 to which SleB (1 μM) was added. This reaction mixture was incubated at 30°C for 30 min before addition of SleL (1 μM). The reaction was allowed to continue for a further 90 min at 30°C. Soluble peptidoglycan fragments were recovered from reaction supernatants by centrifugation, subjected to sodium borohydride reduction, and analyzed by HPLC-MS as described previously (19).

RESULTS
Analysis of B. megaterium ypeB and sleL in vivo. Previous work revealed that an essentially wild-type germination phenotype could be restored to B. megaterium sleB cwlJ spores when they were complemented with plasmid-borne copies of cwlJ and ypeB, arranged as a bicistronic operon under the control of the native sleB ypeB promoter (19). This was unexpected, since neither sleB nor ypeB was thought to be associated directly with lytic activity during spore germination, and indeed, further evidence to support noncatalytic roles for both polypeptides has been obtained since (17, 21). Similarly, sleB cwlJ spores complemented with ypeB and

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TABLE 1 Germination and viability of *B. megaterium* CLE mutant spores complemented with the plasmid-borne *sleB* variants, *ypeB*, and *sleL*.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Rate of spore germination (%)&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Viability (%)&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>QM B1551</td>
<td>Wild type</td>
<td>65</td>
<td>100</td>
</tr>
<tr>
<td>GC103</td>
<td>Δ<em>sleB</em> Δ<em>cwlJ</em></td>
<td>17</td>
<td>0.005</td>
</tr>
<tr>
<td>GC106</td>
<td>Δ<em>sleB</em> Δ<em>cwlJ</em> pHT-<em>sleB&lt;sup&gt;β&lt;/sup&gt;</em> <em>ypeB</em></td>
<td>63&lt;sup&gt;c&lt;/sup&gt;</td>
<td>70&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>GC114&lt;sup&gt;e&lt;/sup&gt;</td>
<td>Δ<em>sleB</em> Δ<em>cwlJ</em> pHT-<em>sleB&lt;sup&gt;β&lt;/sup&gt;</em>-<em>ypeB&lt;sup&gt;208A&lt;/sup&gt;</em></td>
<td>40&lt;sup&gt;d&lt;/sup&gt;</td>
<td>60&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>GC120</td>
<td>Δ<em>sleB</em> Δ<em>cwlJ</em> pHT-<em>ypeB</em></td>
<td>48&lt;sup&gt;d&lt;/sup&gt;</td>
<td>20&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>GC104</td>
<td>Δ<em>sleB</em> Δ<em>cwlJ</em></td>
<td>59</td>
<td>50</td>
</tr>
<tr>
<td>GC124</td>
<td>Δ<em>sleB</em> Δ<em>cwlJ</em> pHT-<em>ypeB</em></td>
<td>40&lt;sup&gt;e&lt;/sup&gt;</td>
<td>10&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>GC112&lt;sup*e&lt;/sup&gt;</td>
<td>Δ<em>sleB</em> Δ<em>cwlJ</em>Δ<em>cwlJ</em></td>
<td>16</td>
<td>0.001</td>
</tr>
<tr>
<td>GC125</td>
<td>Δ<em>sleB</em> Δ<em>cwlJ</em>Δ<em>cwlJ</em> pHT-<em>sleL</em></td>
<td>15</td>
<td>0.02</td>
</tr>
<tr>
<td>GC126</td>
<td>Δ<em>sleB</em> Δ<em>cwlJ</em>Δ<em>cwlJ</em> pHT-<em>ypeB</em></td>
<td>18</td>
<td>0.01</td>
</tr>
<tr>
<td>GC127</td>
<td>Δ<em>sleB</em> Δ<em>cwlJ</em>Δ<em>cwlJ</em> pHT-<em>ypeB</em>Δ<em>cwlJ</em></td>
<td>49&lt;sup&gt;f&lt;/sup&gt;</td>
<td>49&lt;sup&gt;f&lt;/sup&gt;</td>
</tr>
<tr>
<td>GC128</td>
<td>Δ<em>sleB</em> Δ<em>cwlJ</em>Δ<em>cwlJ</em> pHT-<em>ypeB</em>Δ<em>cwlJ</em>Δ<em>cwlJ</em></td>
<td>12</td>
<td>0.02</td>
</tr>
<tr>
<td>GC129</td>
<td>Δ<em>sleB</em> Δ<em>cwlJ</em>Δ<em>cwlJ</em> pHT-<em>sleB&lt;sup&gt;β&lt;/sup&gt;</em> pHT-<em>ypeB</em></td>
<td>20</td>
<td>0.01</td>
</tr>
</tbody>
</table>

<sup>a</sup> Rates of spore germination are given relative to the OD<sub>600</sub> loss (65%) of wild-type QM B1551 spores after incubation for 60 min at 30°C in 5 mM Tris-HCl, pH 7.8, plus GPLO (10 mM glucose, proline, and leucine, plus 50 mM KBr), which was set equal to 100%. Values presented are the averages of results from three independent experiments conducted using two different batches of spores. Standard deviation from the mean was <10%.

<sup>b</sup> Spore viability values were determined as described in Materials and Methods and are presented relative to that obtained for the wild-type QM B1551 strain, which was set at 100%.

<sup>c</sup> Spores of this strain and all CLE triple mutant-derivative spores were germinated in 5% (wt/vol) beef extract for 60 min at 30°C. The OD<sub>600</sub> loss for parental QM B1551 spores under these conditions was 65%, which represents 100% spore germination.

<sup>d</sup> The P value for this number relative to that for GC103 spores in the same column was <0.001.

<sup>e</sup> The P value for this number relative to that for GC104 spores in the same column was <0.001.

<sup*f</sup> The P value for this number relative to that for GC122 spores in the same column was <0.001.

<sup>g</sup> This strain carries a plasmid encoding the C-terminal domain of SleB with the mutation E208A (pHT-*sleB<sup>β</sup>*<sup>208A</sup>* *ypeB*).

Hence, it seems that the placement of *ypeB* on a multicopy plasmid, which resulted in apparent gene expression levels that are comparable with those of the wt strain, may be an important factor in supporting cortex hydrolysis in the *sleB* CLE<sub>β</sub> background. In addition, analysis of peptidoglycan fragments released into the germination exudate by GC123 spores revealed the presence of the anhydromuropeptides G8 and G9 (Fig. 3), further evidence that *B. megaterium* has a lytic transglycosylase in addition to SleB and CwlJ and that this unidentified enzyme is probably responsible for the major cortex lytic activity in this strain.

Having established that *ypeB* and, by inference, YpeB seem to be required for cortex hydrolysis to occur during germination in the *sleB* CLE<sub>β</sub> background, we then examined the role of *sleL* in the germination of various CLE mutant strains. SleL has been characterized from mutational analyses as a probable nonlytic epimerase in *B. megaterium* and *B. subtilis* (19, 22) but was identified from in vitro analyses with *B. cereus* and *B. anthracis* orthologues as an

with a gene for the C-terminal portion of SleB in which catalytic glutamate 208 was changed to alanine also displayed a considerably enhanced germinative response compared to that of the parental *sleB* CLE<sub>β</sub> spores (19) (Table 1). In an attempt to dissect these germinative responses further, a *sleB* CLE<sub>β</sub> strain complemented with only plasmid-borne *ypeB* under the control of its promoter was constructed. Spores of this strain (GC123) lost 48% of their initial OD<sub>600</sub> value when incubated in germination buffer, significantly (P < 0.001) greater than the OD<sub>600</sub> losses observed typically (~17%) in *sleB* CLE<sub>β</sub> spores (Table 1 and Fig. 1). The ability of these spores to form colonies on solid growth medium was also significantly (P < 0.001) greater than that of *sleB* CLE<sub>β</sub> spores (20% versus 0.005%, with respect to wt values), indicating that the presence of *ypeB* alone can restore significant cortex hydrolysis to *sleB* CLE<sub>β</sub> spores.

Unfortunately, antisera against *B. megaterium* YpeB are not available, and so we could not determine the abundance of this protein in *sleB* CLE<sub>β</sub> or *sleB* CLE<sub>β</sub> spores complemented with plasmid-borne *ypeB*. However, RT-PCR analyses indicated that *ypeB* is expressed in strains in which the *sleB* ORF is disrupted with an antibiotic-resistance cassette (Fig. 2), albeit at levels that appear to be diminished compared to those of wt and GC123 strains. Denaturing gel analysis revealed an approximately 10-fold decrease in the abundance of *ypeB* RT-PCR products during the analysis period for strain GC103 (Δ*sleB* Δ*cwlJ*<sub>β</sub>) compared to the levels in both the wt and GC123 (Δ*sleB* Δ*cwlJ*<sub>β</sub> pHT-*ypeB*) (data not shown).

Hence, it seems that the placement of *ypeB* on a multicopy plasmid, which resulted in apparent gene expression levels that are comparable with those of the wt strain, may be an important factor in supporting cortex hydrolysis in the *sleB* CLE<sub>β</sub> background. In addition, analysis of peptidoglycan fragments released into the germination exudate by GC123 spores revealed the presence of the anhydromuropeptides G8 and G9 (Fig. 3), further evidence that *B. megaterium* has a lytic transglycosylase in addition to SleB and CwlJ and that this unidentified enzyme is probably responsible for the major cortex lytic activity in this strain.

Having established that *ypeB* and, by inference, YpeB seem to be required for cortex hydrolysis to occur during germination in the *sleB* CLE<sub>β</sub> background, we then examined the role of *sleL* in the germination of various CLE mutant strains. SleL has been characterized from mutational analyses as a probable nonlytic epimerase in *B. megaterium* and *B. subtilis* (19, 22) but was identified from in vitro analyses with *B. cereus* and *B. anthracis* orthologues as an
Analyzed as B. megaterium SleL or YpeB protein was directly responsible for the lytic activity observed in sleB cwlJ spores complemented with appropriate genes. This was approached in the first instance by assaying for DPA release from decoated B. subtilis spore substrates incubated with various recombinant proteins. Proteins employed included B. megaterium SleL, YpeB, and the chromosomally encoded SleL homologue YdhD (BMQ_1726). Previous work has shown that YdhD is a spore-associated protein in B. subtilis (31), although it does not appear to have an essential role in spore germination (22). Analysis of a B. megaterium strain (GC130) engineered to express green fluorescent protein (GFP) under the control of the putative YdhD promoter revealed green fluorescence in the mother–cell compartment during sporulation (see Fig. S1 in the supplemental material). Similarly, faint green fluorescence was observed in B. megaterium YdhD-GFP spores (data not shown). Hence, B. megaterium YdhD is probably inherent to spores and may be an additional CLE involved in spore germination. The activity of recombinant B. cereus SleL in these assays, both individually and when coincubated with B. megaterium YpeB, was examined also.

Incubation of decoated wt B. subtilis spores with recombinant B. megaterium SleL led to the release of DPA at a significantly (P < 0.001) but only marginally greater level than that of DPA released from spores incubated without any protein (Table 2). DPA release induced by YpeB and YdhD was largely indistinguishable from no-protein control values. Spores incubated with B. cereus SleL, however, released 17% of their total DPA content, significantly higher (P < 0.001) than control spore DPA levels but still markedly less than that of spores incubated with an equivalent amount of lysozyme (98%). Unexpectedly, incubation of decoated spores with equimolar concentrations of YpeB plus B. megaterium SleL or B. cereus SleL, compared to incubation with SleL alone, led to significant (P < 0.001) increases in DPA release (79% and 90%, respectively). Coincubation with both proteins was required to induce substantial DPA release, since decoated B. subtilis spores incubated with YpeB, which was removed subsequently by washing the spores in buffer prior to incubation with SleL, released only a small quantity (<3%) of their DPA content. Incubation of the C-terminal portion of YpeB with B. megaterium SleL led to an increase in DPA release (21%) compared to that with SleL individually (9%). However, the combination of SleL plus the N-terminal portion of YpeB appeared to be nonstimulatory, as did YdhD plus YpeB, which gave only a modest increase in DPA release (11%) compared to that with YdhD alone (6%). In virtually all cases examined, the amount of DPA released from decoated CLE triple mutant (BH60) B. subtilis spores was reduced compared to the amount released by wild-type spores treated with the same individual and combined recombinant proteins (Table 2). Incubation with B. megaterium SleL plus YpeB, for example, led to only a 25% DPA release in decoated B. subtilis BH60 spores compared to a 79% release in decoated wild-type B. subtilis spores. These data are indicative of endogenous CLE activity contributing to DPA release in wild-type spores incubated with recombinant proteins. In contrast, lysozyme stimulated comparable amounts of DPA release from wild-type and BH60 spores.

In further experiments with decoated spore substrates, B. subtilis cwlJ spores, whose cortex lacks the MAL moiety that serves as a recognition element for CLEs, were incubated with both SleL homologues, YpeB, and combined SleL and YpeB. As anticipated,
TABLE 2 DPA release from decoated *B. subtilis* spores incubated with various recombinant SleL, YdhD, and YpeB proteins

<table>
<thead>
<tr>
<th>Protein(s)</th>
<th><em>B. subtilis</em> wild type</th>
<th><em>B. subtilis</em> sleB cwlJ</th>
<th>B. subtilis cwlD</th>
<th>B. subtilis gerA gerB gerK</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>4</td>
<td>3</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>SleL</td>
<td>9</td>
<td>6</td>
<td>7</td>
<td>5</td>
</tr>
<tr>
<td>SleL, YdhD</td>
<td>17</td>
<td>9</td>
<td>7</td>
<td>9</td>
</tr>
<tr>
<td>YpeB</td>
<td>3</td>
<td>7</td>
<td>9</td>
<td>9</td>
</tr>
<tr>
<td>SleL plus YpeB</td>
<td>79<strong>ef</strong></td>
<td>25<strong>ef</strong></td>
<td>21<strong>e</strong></td>
<td>11<strong>e</strong></td>
</tr>
<tr>
<td>SleL, YdhD plus YpeB</td>
<td>90<strong>ef</strong></td>
<td>33<strong>e</strong></td>
<td>24<strong>e</strong></td>
<td>12<strong>e</strong></td>
</tr>
<tr>
<td>YpeB</td>
<td>3</td>
<td>8</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>SleL plus YpeB</td>
<td>21<strong>e</strong></td>
<td>19<strong>e</strong></td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Lysozyme</td>
<td>98</td>
<td>96</td>
<td>96</td>
<td>99</td>
</tr>
<tr>
<td>Heat-treated SleL and YpeB</td>
<td>3</td>
<td>1</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>Sequential incubation with YpeB and then SleL</td>
<td>ND</td>
<td>1</td>
<td>3</td>
<td>ND</td>
</tr>
</tbody>
</table>

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**a** *B. megaterium* proteins were used except where indicated (a “Bc” subscript indicates a *B. cereus* protein).

**b** Decoated spores (OD600 ~ 0.5) were incubated with a 1 μM concentration of the respective purified proteins for 3 h at 37°C, and then the amount of DPA retained and released by the spores was assessed by colorimetric detection as described in Materials and Methods. The percentage of DPA released was normalized against colorimetric values obtained from spores boiled in water. Values presented are the averages of results from three independent experiments, where the standard deviation was <15% of the mean.

**c** One-way ANOVA indicated that the percentage of DPA released from the indicated SleL-associated reactions differed significantly (P < 0.001) from the percentage of DPA released from control (no-protein) reactions.

**d** Two-sample t tests indicated that the percentage of DPA released from wild-type *B. subtilis* spores differed significantly (P < 0.001) from the percentage of DPA released from *B. subtilis* sleB cwlJ sleL spores treated with the corresponding proteins.

**e** Two-sample t tests indicated that the percentage of DPA released from *B. subtilis* cwlD spores differed significantly (P < 0.005) from the percentage of DPA released from *B. subtilis* gerA gerB gerK spores treated with the corresponding proteins.

**f** ND, not determined.

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neither SleL homologue nor YpeB showed appreciable activity against decoated *cwlD* spores, with ≤7% of the DPA being released (Table 2). However, unexpectedly, DPA release was enhanced in *cwlD* spores incubated with YpeB plus either *B. megaterium* SleL or *B. cereus* SleL, resulting in 21% and 24% DPA release, respectively. These values are significant compared to that of negative-control spores (P < 0.001, as determined by one-way analysis of variance) but are still considerably less than that for *cwlD* spores incubated with lysozyme, which release essentially all of their DPA content. Further analysis of *B. subtilis* cwlD spores treated with SleL plus YpeB by phase-contrast microscopy revealed, as with BH60 spores (Fig. 4), a mixture of phase-dark/gray and phase-bright spores, indicating that DPA release is being stimulated in only a fraction of the decoated spore population. This may reflect incomplete chemical decoating of the *cwlD* spores, although the observation that lysozyme stimulates complete DPA release is counter to this idea. Similar DPA release values were obtained with decoated *B. subtilis* gerA gerB gerK spores, which lack all three functional nutrient germinant receptors. Intriguingly, however, the amount of DPA released from these receptorless spores when coincubated with SleL (from either species) plus YpeB was significantly (P < 0.005) less than the amount of DPA released from *cwlD* spores treated with the same protein combinations (Table 2).

In order to investigate the apparent lytic activity of combined SleL and YpeB, a range of other peptidoglycan-containing substrates were examined. First, cortical fragments purified from decoated, disrupted *B. subtilis* spores were incubated with the various recombinant proteins, both individually and in combination with YpeB. Resultant losses in optical density (OD600) of cortical-fragment suspensions indicated that *B. cereus* SleL, as expected, is associated with cortical-fragment lytic activity; the OD600 of the suspension was reduced by an amount equivalent to that achieved with lysozyme (Fig. 5). Similarly, *B. megaterium* YdhD also possesses cortical-fragment lytic activity, albeit at an apparently reduced rate compared to that of *B. cereus* SleL. In contrast, neither *B. megaterium* SleL nor YpeB, individually or combined, displayed detectable cortical-fragment lytic activity.

Finally, purified *B. subtilis* spore sacculi were then examined as a substrate for the various proteins. Optical density measurements in these experiments revealed that none of the recombinant spore proteins, including SleL and YpeB mixtures, resulted in appreciable losses in OD600 over a 90-min period (typically ≤5%), whereas incubation with lysozyme was observed to result in a rapid decrease (~45%) in the OD600 of the suspension (Fig. 6). As expected, incubation of sacculi with recombinant *B. megaterium* SleB also resulted in a decrease in the OD600 of the suspension (data not shown). In an attempt to identify whether *B. megaterium* SleL might show activity against SleB-generated products, both...
recombinant proteins were coincubated with sacculi and the products released to the reaction supernatant collected for HPLC-MS analysis. A similar experiment was conducted using B. cereus SleL plus B. megaterium SleB. Muropeptide analyses conducted with the latter supernatants, which were not subjected to mutanolysin digestion, revealed the generation of several N-acetylglucosaminidase-derived products (Fig. 7 and see Table S2 in the supplemental material), including anhydro-trisaccharides and tetra- and hexasaccharide muropeptides with glucosamine at the reducing end of the glycan chain. The reducing sugar of several major muropeptides purified during this work was not to be reduced to the equivalent alcohol during these experiments, despite being subjected to sodium borohydride treatment, resulting in a 2-Da difference in the expected masses of the products.

Muropeptide analysis of B. megaterium SleL/SleB reactions revealed the presence of anhydro-trisaccharides, resulting from the digestion of SleB-generated anhydro-muropeptides, albeit at considerably reduced abundances compared to those of B. cereus SleL-derived products, revealing that B. megaterium SleL is an N-acetylg glucosaminidase.

Protein interactions. The requirement for both SleL and YpeB to stimulate significant release of DPA from decoated-spore substrates indicated that the two proteins might physically interact to achieve their combined effect. In order to investigate this possibility, recombinant versions of B. megaterium SleL and YpeB, with His10 and Strep-tag II affinity tags, respectively, were expressed and purified as described in Materials and Methods and then subjected to pulldown assays using appropriate chromatography resins. Potential interactions between B. megaterium SleL and SleB were probed in a similar manner since sneB cwIJ spores complemented with sneB5 plus ypeB show a more efficient germinative response than those complemented with ypeB alone (Table 1). However, as with previous pulldown assays investigating potential interactions between SleB and YpeB (21), Coomassie blue-stained gels associated with reciprocal pulldown assays indicated that neither B. megaterium SleL nor SneL and SneB physically interact in vitro (Fig. 8).

DISCUSSION

A major objective of the current work was to gain insight into underlying genetic and physiological mechanisms that underpin...
efficient cortex hydrolysis and spore germination in *B. megaterium* *sleB* *cwlf* null mutant spores. Previous research demonstrated that *B. megaterium sleB cwlf* spores complemented with plasmid-borne *sleB* and *ypeB* could germinate essentially as efficiently as wild-type spores, probably due to the presence of an as-yet-unidentified lytic transglycosylase, whose presence was detected via muropeptide analysis of spore germination exudates (19). The current study extends these findings, revealing that germination in this genetic background requires only plasmid-borne *ypeB* and that the nonlytic peptidoglycan-binding domain encoded by *sleB* can be dispensed with, at least to a degree; i.e., OD600 loss and spore viability are diminished when only *ypeB* is present (spore viability is ~20% in *sleB* *cwlf* pHt-*ypeB* spores versus 70% in pHt-*sleB* *ypeB* spores and 0.005% in *sleB* *cwlf* spores). In addition, germinative analysis of various multiple CLE null mutant strains revealed that intact *sleL* is essential for efficient germination in the *sleB* *cwlf* genetic background, since *sleB* *cwlf* *sleL* spores complemented with pHt-*sleB* *ypeB* showed a severe germination defect (0.02% viability).

A major conclusion of the present work, the identification of *B. megaterium SleL* as an N-acetylglucosaminidase, provides a strong rationale to support the genetic requirement for intact *sleL* in the *sleB* *cwlf* background. Hence, it seems reasonable to assume that in the absence of *SleB* and *Cwlf* in *B. megaterium* spores, *SleL* is required to make the initial cuts to the cortex during stage 2 of germination. Thereafter, additional enzymes, including an as-yet-unidentified lytic transglycosylase and perhaps YdhD (demonstrated to be a cortical fragment lytic enzyme [CFLE] in this work), contribute to efficient cortex hydrolysis. This idea is somewhat what at odds with *SleL*’s previous functional characterization as purely a CFLE, at least for the *B. cereus* and *B. anthracis* homologues. This may well be its principal role, including in *B. megaterium*, but the apparent ability of *SleL* to cleave intact cortical peptidoglycan raises a question about the mechanism(s) that maintains the protein in an inactive state in the dormant spore. We do not yet have the answers to this, but the observed synergy in function that is apparent when *SleL* is coincubated with *ypeB* against decoated-spore substrates perhaps indicates that protein interactions are important here, possibly in addition to any changes in cortical stress that occur during germination. However, pulldown assays conducted in the present work with *SleL* and *ypeB* do not support this idea. It may be, however, that such interactions are too weak to detect *in vitro* or occur only *in vivo* under conditions conferred by the developing spore.

The physiological basis for the apparent dependency between *sleL* and *ypeB* for efficient spore germination in the *sleB* *cwlf* genetic background is complicated further by the facts that these genes are expressed in different cellular compartments during sporulation and that their products are localized to different regions of the mature spore. *SleL*, for example, is expressed in the mother cell and is localized to the inner spore coat, whereas *ypeB* is forespore expressed and localized predominantly to the spore inner membrane (18, 22, 32). Hence, the mechanism that underpins this dependency is a topic for further research.

In addition, the identification of *B. megaterium SleL* as an N-acetylglucosaminidase is in stark contrast to the strong correlation *in vivo* between the presence of intact *sleL* and nonlytic epimerase activity observed during germination, at least in *B. megaterium* and *B. subtilis* spores (19, 22). However, a plausible route to the generation of epimerase products, essentially as glucosaminidase reaction intermediates, has been suggested (33), although at no point were epimerase-derived muropeptides detected during *in vitro* studies with *B. megaterium SleL* during this work. Further work will be required to examine the source of epimerase products generated during the germination of *B. megaterium* and *B. subtilis* spores.

Finally, some of the most intriguing observations from the current work stem from incubation of *SleL* plus *ypeB* with decoated-spore substrates. As mentioned above, the presence of *ypeB* seems to confer an apparent increase in the efficiency of *SleL*’s lytic activity against these substrates, although coincubation with both enzymes is required. This effect was observed with *B. megaterium* and *B. cereus* *SleL* homologues, with a DPA release increase from 9% to 79% for *B. megaterium* *SleL* when it was coincubated with *ypeB* and a DPA release increase from 17% to 90% for *B. cereus* *SleL* when it was combined with *B. megaterium* *ypeB* against decoated wild-type *B. subtilis* spores. However, similar experiments conducted with CLE triple mutant spores indicate that maximal release of DPA requires stimulation of endogenous CLEs. Similarly, the coincubation of *SleL* and *ypeB* did not confer detectable lytic activity against purified spore sacculi, nor did, in the case of *B. megaterium*, the incubation of *SleL* against cortical fragments. However, it may be that *SleL* is an infrequent cutter of spore peptidoglycan and that even in the presence of *ypeB*, perhaps acting in a chaperone-type capacity to enhance *SleL* function, lytic activity is insufficient to hydrolyze spore sacculi to detectable levels. Clearly, however, it is adequate at stimulating DPA release and endogenous CLE activity in decoated spores.

Regardless of *SleL*’s lytic activity against the aforementioned substrates, we do not expect combined *SleL/YpeB*, or indeed any CLE, to induce cortex lysis-mediated DPA release from *B. subtilis* *cwld* spores, since they lack the MAL moiety that serves as a substrate recognition element for CLEs. However, these spores were observed to release 21% and 24% of their total DPA when decoated and incubated with *B. megaterium* *SleL* plus *ypeB* and *B. cereus* *SleL* plus *ypeB*, respectively, values that are broadly in line with the amount of DPA released from *B. subtilis* CLE triple mu-

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**FIG 8** *B. megaterium* *SleL* does not interact *in vitro* with *YPEB* (A) or *SleL* (B). Co2+ -NTA and streptavidin affinity pulldown assays were used to probe for interactions between *SleL*-*His10* and *YPEB-strep* and between *SleL*-*His10* and *SleB-strep*. Co2+ -NTA (HisPur) or streptavidin-resin (Strep-Tactin)-bound proteins were incubated with candidate interacting proteins, and eluted bound (B) and unbound (U) fractions were analyzed by SDS-PAGE and Coomassie blue staining as described in Materials and Methods.
tant spores. One explanation for this observation is that SleL, in the presence of YpeB, recognizes and hydrolyzes MAL-deficient cortical peptidoglycan. Alternatively, the combined presence of these proteins triggers an endogenous CLE that is active, perhaps only to a degree, against non-MAL-containing peptidoglycan.

Similarly, B. subtilis spores that lack all three functional nutrient germinant receptors showed a significant reduction in the amount of DPA released when decoated and incubated with B. megaterium or B. cereus Sle, plus YpeB (11% or 12%, respectively). It is not clear why DPA release appears to be diminished in these spores, since endogenous CLEs are present but appear not to have been activated to any great extent. Similarly, heat-denatured Sle. plus YpeB induced negligible amounts of DPA release, indicating that DPA release appears to be diminished in spores containing SleL plus YpeB (11% or 12%, respectively). It is not clear why DPA release appears to be diminished in spores containing SleL plus YpeB (11% or 12%, respectively). It is not clear why DPA release appears to be diminished in spores containing SleL plus YpeB (11% or 12%, respectively).

To conclude, efficient germination of B. megaterium sleB cwJ spores is dependent upon the presence of ypeB and sleL. Expression of ypeB from a multicopy plasmid, apparently to levels comparable with those of wt sporulating cells, appears not to have been activated to any great extent. Similarly, heat-denatured Sle+. plus YpeB induced negligible amounts of DPA release, indicating that stimulation of DPA release from spores containing germinant receptors is not due to the presence of contaminant amino acids or other molecules with germinant capacity in the protein preparations. Moreover, exposure to lysozyme induced complete DPA release upon cortex hydrolysis, indicating that even in the absence of the germinant receptors, DPA efflux channels are functional in these spores. However, since CLEs are expected to hydrolyze only cortical peptidoglycan, whereas lysozyme will additionally degrade the germ cell wall, perhaps the germinant receptors have a role to play in mediating DPA release when the germ cell wall remains intact. Clearly, further work is required to clarify this aspect of the work.

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