Characterization of the germination of Bacillus megaterium spores lacking enzymes that degrade the spore cortex

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Introduction

Spores of Bacillus species are formed in the process of sporulation, and are both dormant and extremely resistant to environmental stresses (Nicholson et al. 2000; Setlow 2006). As a consequence, these spores can survive for extremely long periods in the absence of nutrients. However, if nutrients return to their environment, spores can rapidly return to life in the process of germination, which can take only a few min for spores of some species (Setlow 2003). Spore germination in Bacillus species is normally triggered by the binding of nutrient germinants to germinant receptors located in the spore’s inner membrane, and with Bacillus subtilis spores has been divided into two Stages, I and II. Events in Stage I include the release of much of the depots of small molecules from the spore core, including free amino acids and the huge amount (c. 20% of spore core dry wt.) of pyridine-2,6-dicarboxylic acid [dipicolinic acid (DPA)], predominantly as a 1 : 1 chelate with divalent cations, primarily Ca2+ (Ca-DPA) (Setlow et al. 2008). These small molecules are replaced in the core by water. Events in Stage I trigger the action of cortex lytic enzymes (CLEs) on the spore’s large peptidoglycan (PG) cortex that lies just slightly outside the spore core. The CLEs are specific for cortex PG, and CwlJ accelerates DPA release and is essential for Ca-DPA germination. The roles of these CLEs are similar in germination of Bacillus megaterium and Bacillus subtilis spores.

Abstract

Aims: To determine roles of cortex lytic enzymes (CLEs) in Bacillus megaterium spore germination.

Methods and Results: Genes for B. megaterium CLEs CwIJ and SleB were inactivated and effects of loss of one or both on germination were assessed. Loss of CwIJ or SleB did not prevent completion of germination with agents that activate the spore’s germinant receptors, but loss of CwIJ slowed the release of dipicolinic acid (DPA). Loss of both CLEs also did not prevent release of DPA and glutamate during germination with KBr. However, cwlJ sleB spores had decreased viability, and could not complete germination. Loss of CwlJ eliminated spore germination with Ca2+ chelated to DPA (Ca-DPA), but loss of CwIJ and SleB did not affect DPA release in dodecylamine germination.

Conclusions: CwIJ and SleB play redundant roles in cortex degradation during B. megaterium spore germination, and CwIJ accelerates DPA release and is essential for Ca-DPA germination. The roles of these CLEs are similar in germination of Bacillus megaterium and Bacillus subtilis spores.

Significance and Impact of the Study: These results indicate that redundant roles of CwIJ and SleB in cortex degradation during germination are similar in spores of Bacillus species; consequently, inhibition of these enzymes will prevent germination of Bacillus spores.
This increased core water content then allows protein movement and enzyme action in the spore core leading to spore outgrowth (Cowan et al. 2003; Setlow 2003). Among early events in spore outgrowth are the; (i) degradation of the small, acid-soluble spore proteins (SASP), some of which saturate spore DNA and protect it from damage; and (ii) initiation of ATP production and then macromolecular biosynthesis (Setlow et al. 2001; Setlow 2003, 2006, 2007).

Two redundant CLEs, CwlJ and SleB, are essential for cortex hydrolysis in Stage II of B. subtilis spore germination (Setlow 2003). CwlJ appears to normally be activated during germination by the Ca-DPA released in Stage I (Paidhungat et al. 2001), and can also be activated by high concentrations of exogenous Ca-DPA, a good germinant for spores of Bacillus species (Setlow 2003). The precise mechanism of activation of the other redundant CLE, SleB, is not known; while SleB is not activated by Ca-DPA, it may become active because of a change in the level of strain on cortex PG (Setlow 2003). Notably, B. subtilis spores lacking either CwlJ or SleB germinate relatively normally and have essentially normal viability. However, while spores lacking both CwlJ and SleB also go through Stage I of germination relatively normally, they cannot degrade their cortex PG (Setlow et al. 2001). Consequently, the viability of cwfl sleB B. subtilis spores is ≤0-01% that of wild-type spores. cwfl sleB B. subtilis spores also cannot initiate outgrowth following germination, neither generating ATP nor degrading their large SASP depot (Setlow et al. 2001). However, viable cwfl sleB B. subtilis spores can be recovered, if their cortex PG is degraded by lysozyme in a hypertonic medium (Setlow 2003). While spore germination and the roles of CLEs in this process have been reasonably well characterized in B. subtilis, this is not the case for any other spore former. Consequently, it is not clear if conclusions from B. subtilis can be extended to spores of other Bacillus species. Therefore, in this work we have examined the role of CLEs in Bacillus megaterium spore germination, and found that the conclusions drawn for B. subtilis spore germination can indeed be extended to spores of other Bacillus species.

Materials and methods

Strains used and construction of mutants

The B. megaterium strains and the plasmids used in this work are listed in Table 1. To construct the sleB insertion-deletion strain, a 1822-bp fragment of DNA (from position 164–1966 of the sleB sequence; GenBank accession number AB120121) encompassing the entire gene and some flanking sequence, was amplified by PCR using primers that incorporated HindIII and Sac1 sites at the upstream and downstream ends respectively. The product was digested and ligated between the HindIII and Sac1 sites of pGEM3Z and used to transform Escherichia coli from which plasmid pGEM-sleB, was isolated. An inverse PCR was then performed, using Nco1 and Xho1 tagged primers, to introduce a deletion between positions 1193 and 1358 of the sequence. Digested and purified PCR product was ligated with a kanamycin resistance cassette excised from plasmid pDG792 with the appropriate enzymes, and the ligation mixture was used to transform E. coli, from which plasmid pGEM-ΔsleB:kan was isolated. The ΔsleB:kan cassette was ampliﬁed by PCR using primers to incorporate EcoR1 sites at the upstream and downstream ends, the product digested, ligated with EcoR1 cut pUCTV2, transformed into E. coli and plasmid pUCTV-ΔsleB::kan was isolated. This plasmid was used to transform B. megaterium QM B1551 to Km resistant and Tc resistant via polyethylene glycol-mediated protoplast transformation (Christie et al. 2008). Colonies that had integrated the plasmid at the sleB locus were selected by culturing transformants overnight at the nonpermissive temperature (42°C) for plasmid replication on Luria–Bertani (LB) medium agar plates (Paidhungat et al. 2000) containing

<table>
<thead>
<tr>
<th>Bacteria/plasmid</th>
<th>Relevant genotype or phenotype</th>
<th>Source (reference)</th>
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<tbody>
<tr>
<td>Bacillus megaterium</td>
<td></td>
<td></td>
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<tr>
<td>QM B1551</td>
<td>Wild-type</td>
<td>P.S. Vary</td>
</tr>
<tr>
<td>PS1462</td>
<td>ΔsleB::kan Km r</td>
<td>This study</td>
</tr>
<tr>
<td>PS4164</td>
<td>cwfl::pUCTV2 Sp r Tc r</td>
<td>This study</td>
</tr>
<tr>
<td>PS4165</td>
<td>cwfl::pUCTV2 ΔsleB::kan Km r Sp r Tc r</td>
<td>This study</td>
</tr>
<tr>
<td>Plasmid</td>
<td>Shuttle plasmid; ts Bacillus ori Tc r</td>
<td>Wittchen and Meinhardt 1995</td>
</tr>
<tr>
<td>pUCTV2</td>
<td>Escherichia coli cloning vector Amp r</td>
<td>Promega Corporation*</td>
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<tr>
<td>pGEM3Z</td>
<td>Km r cassette</td>
<td>Guérout-Fleury et al. 1995</td>
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<tr>
<td>pDG792</td>
<td>Sp r cassette</td>
<td>Guérout-Fleury et al. 1995</td>
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<tr>
<td>pDG1726</td>
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*Email address: ukcustserve@promega.com.
Km r, resistance to kanamycin (5 mg l⁻¹); Sp r, resistance to spectinomycin (100 mg l⁻¹); Tc r, resistance to tetracycline (10 mg l⁻¹).
recombination resulting in loss of the Tcr cassette and the attempts, colonies that had undergone a second round of Sp to select colonies that had integrated the plasmid into Sac

Sac

incorporating Sac

D

inactivation was prepared by ligating a 900-bp PCR

Hind

sequence, GenBank accession number EU037904) with known sequence. Digested and purified PCR product was reduce a deletion between positions 1004 and 1018 of the cwlJ

PCR was performed on the resulting plasmid (pGEM-
pGEM3Z digested with the same enzymes. An inverse incubation overnight at 42

/C176

B1551 to Sp r Tetr as described above. Transformants were

to transform

with the same enzymes, and the ligation mixture was used to transform

E. coli

into

E. coli

and plasmid pUCTV-

spc

50 mmol l

Æ

glucose-25 mmol l

Æ

KPO4 buffer (pH 7-4) at 30°C; (ii) 50 mmol l

Æ

KBr-25 mmol l

Æ

KPO4 buffer (pH 7-4) at 30°C; (iii) 1 mmol l

Æ

dodecylamine – 16 mmol l

Æ

KPO4 buffer (pH 7-4) at 44°C; and (iv) 50 mmol l

Æ

Ca-DPA (pH 7.5) – 2.5 mmol l

Æ

Tris-HCl buffer (pH 7.5) at 30°C. Germination with glucose, KBr or Ca-DPA and spores at an OD

Æ

of 1 was routinely followed by monitoring the OD

Æ

of the culture (Cabrera-Martinez et al. 2003), which normally falls c. 65% upon completion of spore germination. Germination with dodecylamine, as well as some experiments with KBr and glucose as germinants was also monitored using spores at an OD

Æ

of 1 and at various times 1 ml aliquots of germinating cultures were centrifuged for 2 min in a microcentrifuge, and the OD

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of the supernatant fluid from a 1 ml aliquot of the germinating culture boiled for 30 min, cooled for 5 min on ice and centrifuged for 2.5 min in a microcentrifuge. Previous work has shown that >85% of the material absorbing at 270 nm released from B. subtilis spores during germination or from dormant spores by boiling is DPA (Cabrera-Martinez et al. 2003; Setlow et al. 2003), and this was confirmed for B. megaterium spores (data not shown). In all experiments, the extent of spore germination was also checked by phase contrast microscopy. All germination experiments were repeated at least twice, with essentially identical results.

Spore viability

Viability of various spore preparations was determined by spotting duplicate 10 µl aliquots of appropriate dilutions

Spore preparation, purification and storage

Spores of B. megaterium strains were prepared at 30°C in liquid SNB medium without antibiotics, cleaned by repeated centrifugation and washing with water, and stored in water at 4°C protected from light (Nicholson and Setlow 1990). All spore preparations used in this work were free (98%) of growing cells, germinated spores and cell debris as determined by phase contrast microscopy.

Spore germination

Unless otherwise noted B. megaterium spores were germinated at an Optical Density at 600 nm (OD

Æ

of c. 1·0 (c. 5 × 10^7 spores ml

Æ
) or 1·5. Germination by glucose or KBr, two germinants that act through the spore’s germinant receptors (Cortezzo et al. 2004; Christie and Lowe 2007), was preceded by a heat shock (60°C; 15 min) of spores at an OD

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of 10–50 in water, followed by cooling in ice for c. 10 min. However, germination with Ca-DPA or dodecylamine did not require a heat shock. Germination solutions used were: (i) 10 mmol l

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d-glucose–25 mmol l

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KPO4 buffer (pH 7-4) at 30°C; (ii) 50 mmol l

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Viability of various spore preparations was determined by spotting duplicate 10 µl aliquots of appropriate dilutions
of spores in water on LB medium plates with an appropriate antibiotic, or no antibiotic in the case of wild-type spores. The plates were incubated at 30°C for 24–48 h and colonies were counted. Lysozyme recovery of PS4165 (cwlJ sleB) spores was achieved by adding lysozyme to molten (48°C) LB agar to give a final concentration of 1 mg l⁻¹ prior to pouring plates. Plates were incubated and colonies counted as described above.

Measurement of DPA release from single germinating spores

The kinetics of DPA release from single germinating spores were determined at 30°C for spores germinating in glucose by laser tweezers Raman spectroscopy as described (Chen et al. 2006), and measuring the intensity of the 1017 cm⁻¹ Raman peak of DPA. Ten individual spores of each strain were examined and the DPA level in these spores was determined with respect to the peak heights of standard DPA solutions. DPA release during germination of individual spores was expressed as the parameters $T_{lag}$ and $\Delta T_{release}$ (Chen et al. 2006; Peng et al. 2009) $T_{lag}$ is the time between mixing of spores with germinant and the initiation of rapid DPA release, and $\Delta T_{release}$ is the time between $T_{lag}$ and the time at which ≥95% of spore DPA has been released.

Other analytical procedures

For ATP analyses, spores were germinated at an OD₆₀₀ nm of 5 with glucose and at various times 1 ml aliquots were added to 4 ml boiling propanol, the samples boiled for 5 min, cooled on ice for ≥30 min and the samples were flash evaporated (Setlow and Kornberg 1970). The dry residue was kept at 4°C until just prior to assays, when 0.5 ml cold water was added, samples held on ice for c. 15 min with frequent mixing, centrifuged, and ATP assayed in the supernatant fluid by the firefly luciferase assay using a luminometer to measure light production as described (Setlow et al. 2001; Coleman et al. 2007).

Levels of SASP in dormant and germinated wild-type and cwlJ sleB spores were determined essentially as described (Nicholson and Setlow 1990; Setlow et al. 2001). Spores of each strain, either dormant (3 mg dry wt.; c. 10⁹ spores) or 2 ml germinated with glucose for 30 or 60 min at an OD₆₀₀ nm of c. 10, were harvested by centrifugation, and the pellet fractions frozen and lyophilized. The dry spores were disrupted by abrasion with glass beads in a dental amalgamator (Wig-L-Bug) for ten 1 min periods of shaking, the dry powder extracted with 1 ml of cold 3% acetic acid for 30 min on ice, and re-extracted with an additional ml of 3% cold acetic acid. After centrifugation, the pooled supernatant fluid was dialysed in Spectrapor No. 3 tubing against 1 change of 2 l of 1% acetic acid at 4°C for c. 24 h. The dialysed material was lyophilized, the dry residue dissolved in 30 µl of fresh 8 mol l⁻¹ urea plus 15 µl of acid gel diluent, and c. 5 µl aliquots were run on acrylamide gel electrophoresis at low pH and the gel stained with Coomassie Brilliant Blue.

For analysis of small molecules released during the germination of wild type and cwlJ sleB spores, germination was in KBr with spores at an OD₆₀₀ nm of 30. After various germination times, 1 ml aliquots were centrifuged and the supernatant fluid (S1) was removed and frozen, as were the pellet fractions. Subsequently, the pellet fractions were suspended in 1 ml H₂O, boiled for 30 min, cooled, centrifuged, the supernatant (P1) removed and the pellet re-extracted with 1 ml water (P2), the P1 and P2 supernatant fluids pooled and all supernatant fractions centrifuged to remove contaminating spores. Both the S1 and P1/2 supernatant fluids were then passed through a Chelex column to remove Mn²⁺ as described (Loshon et al. 2006), the column washed with water, and the run through and wash fractions were combined and lyophilized. The dry residue was dissolved in 700 µl D₂O containing 10 mg l⁻¹ 2,2-dimethyl-2-silapentane-5-sulfonic acid as an internal standard, NMR spectra were obtained, and levels of small molecules were determined from these spectra as described (Loshon et al. 2006; Setlow et al. 2008).

For analysis of the release of hexosamine containing fragments of cortex PG into the germination medium, heat activated spores of various strains were germinated at an OD₆₀₀ nm of 50 in 10 mmol l⁻¹ glucose and 10 mmol l⁻¹ Tris-HCl buffer (pH 7.8). After incubation for 40 min at 30°C, samples (3 ml) were centrifuged, and analyses of hexosamine in the supernatant fluid were carried out as described (Popham et al. 1996; Tennen et al. 2000).

The core wet densities of dormant and germinated B. megaterium spores were determined essentially as described (Lindsay et al. 1985; Popham et al. 1996), but spores were not decoated. c. 2 mg dormant spores of the wild-type and cwlJ sleB strains were analysed, as were c. 2 mg of these spores that were germinated for 60 min with glucose at an OD₆₀₀ of 7.5, harvested by centrifugation, washed twice with 1 ml water, and suspended in 100 µl dilute Nycodenz (Sigma Chemical Company, St Louis, MO, USA) before applying to Nycodenz density gradients.

Results

Germination of CLE mutant spores

Previous work with B. subtilis spores has shown that loss of either CwJ or SleB or both CLEs does not eliminate
spore germination with nutrient germinants or the artificial germinant dodecylamine, while loss of CwlJ alone eliminates spore germination with Ca-DPA (Paidhungat et al. 2001; Setlow et al. 2001; Setlow 2003). In addition, while loss of either CwlJ or SleB does not prevent completion of B. subtilis spore germination, loss of both CLEs abolishes Stage II of germination and cwlJ sleB B. subtilis spores are extremely inefficient in colony formation. However, despite the knowledge gained on the role of various CLEs in B. subtilis spore germination, it is not clear if the conclusions from work with B. subtilis spores can be extended to spores of other Bacillus species.

Bacillus megaterium also has both a cwlJ and a sleB gene on its chromosome, each of which give proteins that are 57 and 56% identical in amino acid sequence to the B. subtilis proteins. Interestingly, the chromosomal cwlJ gene contains a frame-shift mutation resulting in loss of 17 N-terminal amino acids compared to B. subtilis CwlJ. However, a blast search of the B. megaterium genome revealed no other cwlJ homologues on the chromosome, so presumably the truncated protein is functional (as it indeed is; see below). A possible CwlJ has been identified on a B. megaterium plasmid pBM400 (Scholle et al. 2003). However, it is unlikely that this protein is capable of cortex hydrolysis if it is even present in the spore, as the predicted protein has only 86 residues, while the chromosomally encoded protein has 121.

Construction of mutations in B. megaterium chromosomal cwlJ and sleB was straightforward and gave strains PS4164 (cwlJ), PS4162 (sleB) and PS4165 (cwlJ sleB), in addition to the wild-type strain. All four strains sporulated equally efficiently, as determined from the percentage of cells containing phase bright spores c. 18 h after cells were inoculated on plates (data not shown). Analysis of these spores indicated that the wild-type and sleB spores germinated approximately in parallel with glucose or KBr, when spore germination was assessed by following the OD_{600 nm} of cultures. Germination of cwlJ spores was slower, and the cwlJ sleB spores lost much less of their OD_{600 nm} and more slowly during glucose or KBr germination (Fig. 1a,b). However, comparison of DPA release by wild-type and cwlJ sleB spores germinating with glucose or KBr indicated that spores of both strains released almost all their DPA, although DPA release from the cwlJ sleB spores was slower (Fig. 1a,b).

Using the germinant dodecylamine that does not trigger germination via the germinant receptors (Setlow et al. 2003), spores of all four strains exhibited very similar rates of germination as assessed by monitoring DPA release directly (Fig. 1c). In contrast to these results, while Ca-DPA triggered the germination of both the wild-type and sleB spores at similar rates, both the cwlJ and cwlJ sleB spores exhibited no germination with this agent (Fig. 1d).

Analysis of the viability of spores of the four B. megaterium strains showed that cwlJ and sleB spore viability was slightly lower than that of the wild-type spores, but cwlJ sleB spore viability was <0·1% that of wild-type spores (Table 2), indicating that these spores cannot complete spore germination. However, inclusion of low levels of lysozyme in plates on which cwlJ sleB spores were applied increased the viability of these spores more than 300-fold, to only 4-fold lower than that of wild-type spores (Table 2).

Small molecule release during spore germination

Previous work has shown that with B. subtilis spores release of other small molecules, including glutamate and arginine, parallels DPA release during germination, and that lack of CwlJ and SleB does not eliminate the release of these small molecules (Setlow et al. 2008). Wild-type B. megaterium spores also release their large depot of glutamate (they have little arginine) in parallel with DPA during KBr germination (Setlow et al. 2008), and this was also the case for cwlJ sleB B. megaterium spores (Fig. 2).

The data cited above for small molecule release was for spore populations, not individual spores. Thus it was of interest to determine if loss of various CLEs would have an effect on the DPA release kinetics of individual spores. This was of special interest because: (i) the results noted above suggested that CwlJ alone accelerated the rate of DPA release during spore germination; and (ii) the kinetics of DPA release from individual germinating spores appear quite heterogeneous, in particular in the lag time (T_{lag}) following addition of a germinant and prior to the initiation of rapid DPA release (Chen et al. 2006; Peng et al. 2009). In contrast, the actual time needed for rapid DPA release (ΔT_{release}) is relatively uniform for individual spores in a population. However, the factors that determine values for T_{lag} and ΔT_{release} are not known. Strikingly, average T_{lag} values for spores of all four B. megaterium strains germinating in glucose were extremely similar, when results from ten individual spores of each strain were compared, and even more so when the value for the one very slow to germinate cwlJ spore was discarded (Fig. 3a-d; Table 3). However, the average ΔT_{release} times were 2·5–4·5-fold slower for the cwlJ and cwlJ sleB spores (Table 3).

Spore core wet density, ATP production and cortex and SASP degradation during germination and outgrowth of wild-type and cwlJ sleB spores

Previous work has shown that the defect in cwlJ sleB B. subtilis spore germination is in the inability of these spores to degrade cortex PG and thus complete Stage II
of germination (Setlow et al. 2001; Setlow 2003). That this might be the case for cwlJ sleB B. megaterium spores was then tested by measuring the wet density of the core during germination of wild-type B. subtilis spores the core’s wet density, reflective of the core water content, decreases in Stages I and II of germination (Setlow et al. 2001). There is a partial increase in core water content, and thus a decrease in the core wet density, in Stage I as Ca-DPA is replaced by water, followed by a further increase in core water content in Stage II as the core expands when the restraining cortical PG is degraded (Popham et al. 1996; Setlow et al. 2001).

However, this second rise in core water content, and the concomitant decrease in core wet density to that of a growing cell is not seen in cwlJ sleB B. subtilis spores, presumably because the cortex cannot be degraded (Setlow et al. 2001). We obtained similar results for the core wet density of dormant and germinated wild-type and cwlJ sleB spores of B. megaterium spores by buoyant density gradient centrifugation (Lindsay et al. 1985; Popham et al. 1996). During germination of wild-type B. subtilis spores the core’s wet density, reflective of the core water content, decreases in Stages I and II of germination (Setlow et al. 2001). There is a partial increase in core water content, and thus a decrease in the core wet density, in Stage I as Ca-DPA is replaced by water, followed by a further increase in core water content in Stage II as the core expands when the restraining cortical PG is degraded (Popham et al. 1996; Setlow et al. 2001).

However, this second rise in core water content, and the concomitant decrease in core wet density to that of a growing cell is not seen in cwlJ sleB B. subtilis spores, presumably because the cortex cannot be degraded (Setlow et al. 2001). We obtained similar results for the core wet density of dormant and germinated wild-type and cwlJ sleB B. megaterium spores, as the decrease in the core wet density of these cwlJ sleB spores was only c. 50%

Table 2 Viability of spores of various Bacillus megaterium strains*

<table>
<thead>
<tr>
<th>Spores of strain</th>
<th>No lysozyme</th>
<th>With lysozyme</th>
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<tbody>
<tr>
<td>wild-type</td>
<td>7 x 10^7</td>
<td>8 x 10^7</td>
</tr>
<tr>
<td>PS4164 (cwlJ)</td>
<td>6 x 10^7</td>
<td>8 x 10^7</td>
</tr>
<tr>
<td>PS4162 (sleB)</td>
<td>4 x 10^7</td>
<td>7 x 10^7</td>
</tr>
<tr>
<td>PS4165 (cwlJ sleB)</td>
<td>5 x 10^4</td>
<td>2 x 10^3</td>
</tr>
</tbody>
</table>

*Spores at an OD_{600 nm} of 1 were heat shocked, diluted in water and spore viabilities were determined on plates without and with lysozyme (1 mg l^-1) as described in the section Methods.

†Values in parentheses are the percent viability relative to that of wild-type spores plated on the same medium.

CFU, colony forming units.

of dormant and germinated wild-type and cwlJ sleB spores by buoyant density gradient centrifugation (Lindsay et al. 1985; Popham et al. 1996). During germination of wild-type B. subtilis spores the core’s wet density, reflective of the core water content, decreases in Stages I and II of germination (Setlow et al. 2001). There is a partial increase in core water content, and thus a decrease in the core wet density, in Stage I as Ca-DPA is replaced by water, followed by a further increase in core water content in Stage II as the core expands when the restraining cortical PG is degraded (Popham et al. 1996; Setlow et al. 2001).

However, this second rise in core water content, and the concomitant decrease in core wet density to that of a growing cell is not seen in cwlJ sleB B. subtilis spores, presumably because the cortex cannot be degraded (Setlow et al. 2001). We obtained similar results for the core wet density of dormant and germinated wild-type and cwlJ sleB B. megaterium spores, as the decrease in the core wet density of these cwlJ sleB spores was only c. 50%
of that found with germinated wild-type spores (Table 4). Core wet density was measured here instead of total spore wet density, because changes in total spore wet density during germination are due almost completely to changes in core wet density.

That the cortex is not degraded during the germination of the cwlJ sleB spores was also shown directly by assays of hexosamine containing PG fragments released into the medium during spore germination. In contrast to wild-type, cwlJ and sleB spores that released similar amounts of cortex PG hexosamine during glucose germination, the cwlJ sleB spores released ≤10% of the hexosamine released by spores of the other three strains (data not shown). This is similar to what was found previously when hexosamine release during the germination of spores of these same B. subtilis strains was examined (Popham et al. 1996; Tennen et al. 2000).

The results given above strongly suggested that cwlJ sleB spores have a major defect, since while they initiate germination relatively normally: (i) they almost never give rise to colonies most likely because degradation of the cortex PG during Stage II of germination does not take place; and (ii) their core wet density does not return to that of growing cells. Presumably the latter defect does not raise the core water content sufficiently to allow protein movement and enzyme action in the spore core (Cowan et al. 2003). If this is indeed the case, then these cwlJ sleB spores will be incapable of entering outgrowth that follows completion of spore germination, and thus will not produce ATP and will not degrade their SASP, two events that normally take place early in spore outgrowth (Setlow et al. 2001; Setlow 2003). Indeed, while wild-type spores rapidly produced ATP and degraded their SASP when spore germination was initiated with glucose, the cwlJ sleB spores did not (Figs 4 and 5).

Discussion

The results reported in this communication indicate that CwlJ and SleB function redundantly in degrading the B. megaterium spore’s cortex during germination, as has been found with B. subtilis spores (Setlow 2003). The absence of either CwlJ or SleB did not affect B. megaterium spore viability appreciably, while the lack of both enzymes lowered spore viability >99.9%. Since lysozyme treatment of the germinated cwlJ sleB spores restored the viability of these spores, this is strong evidence that the defect in the germination of cwlJ sleB spores is in cortex degradation, since exogenous lysozyme is capable of degrading the cortex of spores of Bacillus species, and can also restore the viability to B. subtilis spores in which the cortex cannot be degraded by endogenous enzymes (Popham et al. 1996; Setlow et al. 2001). The lack of cortex degradation by B. megaterium cwlJ sleB spores was also shown directly by the lack of release of hexosamine containing cortical PG fragments during these spore’s germination. While the cwlJ sleB B. megaterium spores exhibited low viability, they did initiate germination with two germinants, KBr and glucose, that trigger germination by activating one or more of the spore’s germinant receptors (Cortezzo et al. 2004; Christie and Lowe 2007; Christie et al. 2008). Indeed, germination of cwlJ or sleB B. megaterium spores with glucose or KBr was relatively normal as assessed by following the OD$_{600}$ nm of germinating cultures, although the OD$_{600}$ nm of germinating cwlJ sleB cultures fell only about one-half that of germinating cultures of the other three strains that fell 65–70%. However, DPA release during this germination of cwlJ sleB spores was relatively similar to that with wild-type spores, albeit slower. Presumably the loss of some OD$_{600}$ nm during germination of cwlJ sleB spores with glucose or KBr is because of the decreased refractive index of the germinated cwlJ sleB spore core reflected in its lower core wet density caused by DPA release and replacement of the DPA with water. However, with spores of strains retaining at least one CLE the OD$_{600}$ nm fell 65–70% during germination. This larger change reflects both DPA release as well as cortex hydrolysis and the subsequent swelling of the spore core.
In addition to cwlJ and sleB, the *B. megaterium* genome also contains a homolog of the *Bacillus cereus* sleL gene (also called yaaH; accession number EU008333). *B. cereus* SleL has been reported to be active in hydrolysis of cortex PG (Chen et al. 2000). However, mutation of *B. megaterium* sleL had no discernible spore germination phenotype alone or in combination with cwlJ or sleB mutations (data not shown). Thus SleL cannot play an essential role in spore cortex hydrolysis during germination, although this enzyme might further reduce the sizes of cortical PG fragments released by other CLEs as suggested previously (Chen et al. 2000).

It was notable that DPA release during germination of cwlJ sleB spores with glucose or KBr was slower when DPA release from spore populations was examined, as has

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**Table 3** Parameters of DPA release from single germinating *Bacillus megaterium* spores of various strains*

| Spores of strain | \( T_{\text{lag}} \) (min)‡ | \( \Delta T_{\text{release}} \) (min)‡ | [DPA] at \( t_0 \) § |
|------------------|----------------|----------------|----------------|---|
| Wild-type        | 6 ± 5.2        | 2.5 ± 0.8      | 280 ± 64       |
| PS4164 (cwlJ)    | 4.5 ± 1.9      | 11.6 ± 2.5     | 328 ± 76       |
| PS4162 (sleB)    | 4.3 ± 2.2      | 2.4 ± 0.9      | 304 ± 62       |
| PS4165 (cwlJ sleB) | 5.2 ± 3.2 | 6.0 ± 1.4      | 289 ± 52       |

*Parameters for DPA release from ten individual germinating *B. megaterium* spores from each of the four strains were taken from the data in Fig. 3, and \( T_{\text{lag}}, \Delta T_{\text{release}} \) and spore DPA levels were determined as described in the section Methods. Values are given ± standard deviation values. DPA concentrations in spores are given as mmol l\(^{-1}\) ± SD.

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**Table 4** Core wet densities of dormant and wild-type and cwlJ sleB *B. megaterium* spores*

<table>
<thead>
<tr>
<th>Spores examined</th>
<th>Core wet density (g cc(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type dormant</td>
<td>1:335</td>
</tr>
<tr>
<td>Wild-type germinated</td>
<td>1:170</td>
</tr>
<tr>
<td>cwlJ sleB dormant</td>
<td>1:335</td>
</tr>
<tr>
<td>cwlJ sleB germinated</td>
<td>1:254</td>
</tr>
</tbody>
</table>

*The core wet densities of dormant spores and spores germinated 60 min with glucose were determined as described in the section Methods. Note that DPA release even from cwlJ sleB spores was complete after 60 min of germination with glucose (Fig. 1a).
from \textit{cwlJ} \textit{B. subtilis} spore populations germinating with nutrients, although not from germinating \textit{sleB} \textit{B. subtilis} spores (Ishikawa \textit{et al.} 1998), and the rate of decrease in OD\textsubscript{600} nm during germination of \textit{cwlJ} \textit{B. megaterium} spores with either KBr or glucose was also slower than with wild-type or \textit{sleB} spores. The release of DPA from individual \textit{cwlJ sleB} \textit{B. megaterium} spores germinating with glucose was also significantly slower than from germinating wild-type or \textit{sleB} spores. The difference in DPA release from these types of spores appeared to be solely in AT\textsubscript{release}, and \textit{cwlJ} spores also exhibited this slower AT\textsubscript{release} during glucose germination while \textit{sleB} spores did not. In contrast, values of T\textsubscript{lag} prior to rapid DPA release during glucose germination were essentially identical for \textit{B. megaterium} spores with or without CLEs. That the presence of CwlJ significantly accelerates AT\textsubscript{release} for DPA has also been seen during nutrient germination of individual \textit{B. subtilis} spores, while the absence of SleB has no effect (Peng \textit{et al.} 2009).

The reason(s) why the absence of CwlJ and not SleB increased values of AT\textsubscript{release} for DPA during spore germination are not clear. These two proteins are located in different regions of the spore cortex, CwlJ on the outer surface and SleB predominantly on the inner surface (Bagyan and Setlow 2002; Chirakkal \textit{et al.} 2002). Thus CwlJ may digest the cortex from the outside in, with SleB acting from the inside out. Another difference in these two CLEs is that CwlJ is activated perhaps directly by Ca-DPA, either added exogenously or released from the spore during germination (Paidhungat \textit{et al.} 2001). This initiation of DPA release in germination may result in immediate stimulation of cortex hydrolysis and initiation of cortex lysis may then further accelerate the rate of DPA release. DPA release in germination appears to require the SpoVA proteins, which are most likely located in the spore’s inner membrane, perhaps comprising a DPA channel in this membrane (Tovar-Rojo \textit{et al.} 2002; Vepachedu and Setlow 2004, 2005, 2007). However, how cortex lysis could affect the flux of DPA through such a channel is not known. In contrast to direct activation of CwlJ by DPA, SleB is not activated by DPA, but perhaps by some change in the strain or stress on cortical PG because of release of all or most of the spore’s DPA and its replacement with water (Setlow 2003). Consequently, perhaps SleB normally initiates cortex hydrolysis later than does CwlJ, and this may be another reason that action of CwlJ and not SleB accelerates the rate of DPA release during spore germination.

The lack of ATP production and SASP degradation during germination of \textit{cwlJ sleB} \textit{B. megaterium} spores as well as a core wet density of germinated \textit{cwlJ sleB} spores in between that of dormant \textit{cwlJ sleB} spores and fully
germinated wild-type spores is similar to what has been found for germinated cwlJ sleB B. subtilis spores (Setlow et al. 2001). Presumably the lack of cortex hydrolysis during germination of cwlJ sleB B. megaterium spores precludes sufficient uptake of water into the spore core to allow action of core enzymes that are essential for ATP generation and SASP hydrolysis.

As found with B. subtilis spores (Paidhungat et al. 2001), CwlJ was essential for the germination of B. megaterium spores with Ca-DPA. This suggests that Ca-DPA also triggers B. megaterium spore germination by activating CwlJ. This further suggests that release of endogenous Ca-DPA also activates CwlJ, and that this is one mechanism whereby events in Stage I of B. megaterium spore germination trigger Stage II events, as is also the case in B. subtilis spore germination (Paidhungat et al. 2001).

However, spore germination with dodecylamine, which appears to proceed by direct activation of Ca-DPA release by this non-nutrient germinant, does not require any CLE with B. megaterium spores, as is also the case with B. subtilis spores (Setlow et al. 2003). That DPA release in dodecylamine germination of B. megaterium spores was essentially identical with both wild-type and cwlJ sleB spores further indicates that at least some basal function of the channels for DPA release from spores is independent of cortex hydrolysis.

The general agreement in the involvement of the CLEs CwlJ and SleB in the germination of B. megaterium and B. subtilis spores as seen in the current work is a further indication that the process of spore germination is very similar in two species. Clear CwlJ and SleB homologs are also present in all spore-forming members of the Bacillales whose completed genome sequences are compiled in the NCBI database, with sequence identities to the B. subtilis proteins of ≥54% and similarities of ≥67%. This compilation includes members of Anoxybacillus, Bacillus, Geobacillus, Lysinibacillus and Oceanobacillus species, and further suggests that the roles of CLEs in spore germination are similar in all members of these species. If inhibitors could be developed to inhibit CwlJ and SleB, both of which probably recognize the muramic acid-δ-lactam in spore cortex PG (Popham et al. 1996), such inhibitors would greatly decrease the completion of germination of spores of Bacillus and related species. This in turn could greatly reduce the ability of spores of these species to cause food poisoning or disease.

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References


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