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ORIGINAL ARTICLE

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

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Abstract

Aims: To determine roles of cortex lytic enzymes (CLEs) in *Bacillus megaterium* spore germination.
Methods and Results: Genes for *B. megaterium* CLEs CwlJ and SleB were inactivated and effects of loss of one or both on germination were assessed. Loss of CwlJ or SleB did not prevent completion of germination with agents that activate the spore's germinant receptors, but loss of CwlJ 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 Ca^{2+} chelated to DPA (Ca-DPA), but loss of CwlJ and SleB did not affect DPA release in dodecylamine germination.
Conclusions: CwlJ and SleB play redundant roles in cortex degradation during *B. megaterium* spore germination, and CwlJ accelerates DPA release and is essential for Ca-DPA germination. The roles of these CLEs are similar in germination of *B. megaterium* and *Bacillus subtilis* spores.
Significance and Impact of the Study: These results indicate that redundant roles of CwlJ 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.

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 Ca^{2+} (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 appear to recognize a cortex-specific PG modification, muramic acid-δ-lactam (Popham *et al.* 1996). Hydrolysis of the cortex then allows the core to swell significantly through water uptake, ultimately bringing the core water content from the 45% of wet wt. in Stage I germinated *B. subtilis* spores to *c.* 80% of wet wt., a value comparable to that in growing cells (Setlow *et al.* 2001).

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 *cwlJ sleB B. subtilis* spores is ≤0·01% that of wild-type spores. *cwlJ sleB B. subtilis* spores also cannot initiate outgrowth following germination, neither generating ATP nor degrading their large SASP depot (Setlow *et al.* 2001). However, viabile *cwlJ sleB B. subtilis* spores can be recovered, if their cortex PG is degraded by lysozyme in a hypertonic medium (Setlow *et al.* 2001).

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 *Hind*III and *Sac*1 sites at the upstream and downstream ends respectively. The product was digested and ligated between the *Hind*III and *Sac*1 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-Δ*sleB*::*kan* was isolated. The Δ*sleB*::*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-Δ*sleB*::*kan* was isolated. This plasmid was used to transform *B. megaterium* QM B1551 to Km^r and Tc^r 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 1 *Bacillus megaterium* strains and plasmids used

Bacteria/plasmid	Relevant genotype or phenotype	Source (reference)
Bacillus megaterium		
QM B1551	Wild-type	P.S. Vary
PS1462	Δ*sleB*::*kan* Km^r	This study
PS4164	*cwlJ*::pUCTV2 Sp^r Tc^r	This study
PS4165	*cwlJ*::pUCTV2 Δ*sleB*::*kan* Km^r Sp^r Tc^r	This study
Plasmid		
pUCTV2	Shuttle plasmid; ts *Bacillus ori* Tc^r	(Wittchen and Meinhardt 1995)
pGEM3Z	*Escherichia coli* cloning vector Amp^r	Promega Corporation*
pDG792	Km^r cassette	(Guérout-Fleury *et al.* 1995)
pDG1726	Sp^r cassette	(Guérout-Fleury *et al.* 1995)

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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}).

Km. A Km^r Tc^s transformant, PS4162, that had undergone a double homologous recombination and carrying an insertion–deletion mutation in *sleB*, was isolated after further incubation at 42°C on LB agar plates without antibiotic. The correct construction of this strain was confirmed by PCR.

The structural gene for *B. megaterium* CwlJ was identified using degenerate and Vectorette (Sigma Genosys) PCR methodologies (primers available upon request). A plasmid designed to disrupt the *cwlJ* locus by insertional inactivation was prepared by ligating a 900-bp PCR amplicon (encompassing position 687–1587 of the *cwlJ* sequence, GenBank accession number EU037904) with *Hind*III and *Sac*1 sites at the 5′ and 3′ ends, with pGEM3Z digested with the same enzymes. An inverse PCR was performed on the resulting plasmid (pGEM-*cwlJ*), using Pst1 and BamH1 tagged primers, to introduce a deletion between positions 1004 and 1018 of the known sequence. Digested and purified PCR product was ligated with a Sp^r cassette excised from plasmid pDG1726 with the same enzymes, and the ligation mixture was used to transform *E. coli* giving plasmid pGEM-Δ*cwlJ*::*spc*. The Δ*cwlJ*::*spc* cassette was then PCR amplified using primers incorporating *Sac*1 recognition sites at the 5′ and 3′ ends, digested and ligated with *Sac*1 cut pUCTV2, transformed into *E. coli* and plasmid pUCTV-Δ*cwlJ*::*spc* was isolated. This plasmid was used to transform *B. megaterium* QM B1551 to Sp^r Tet^r as described above. Transformants were incubated overnight at 42°C on LB agar plates containing Sp to select colonies that had integrated the plasmid into the chromosome at the cloned locus via homologous recombination, as confirmed by PCR. Despite repeated attempts, colonies that had undergone a second round of recombination resulting in loss of the Tc^r cassette and the intact *cwlJ* gene could not be isolated. However, a single-crossover mutant (strain PS4164 (*cwlJ*) in which the coding region of *cwlJ* is separated from the putative upstream promoter region by the integrated plasmid, was genetically stable when cultured at 30°C in the absence of antibiotic. This was established by conducting a series of PCR reactions employing primers to specifically detect the absence of the intact *cwlJ* locus and replicating plasmid (to detect excision events), using DNA extracted from spore samples germinated in complex medium [supplemented nutrient broth (SNB) (Nicholson and Setlow 1990)] for 1 h as a template. The same procedure was used to disrupt *cwlJ* in strain PS4162 (*sleB*), to give the double mutant strain PS4165 (*cwlJ sleB*).

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 ($OD_{600\ nm}$) of *c.* 1·0 (*c.* 5×10^7 spores ml^{-1}) 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 $OD_{600\ nm}$ 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^{-1} D-glucose-25 mmol l^{-1} KPO_4 buffer (pH 7·4) at 30°C; (ii) 50 mmol l^{-1} KBr-25 mmol l^{-1} KPO_4 buffer (pH 7·4) at 30°C; (iii) 1 mmol l^{-1} dodecylamine – 16 mmol l^{-1} KPO_4 buffer (pH 7·4) at 44°C; and (iv) 50 mmol l^{-1} Ca-DPA (pH 7·5) – 2·5 mmol l^{-1} Tris-HCl buffer (pH 7·5) at 30°C. Germination with glucose, KBr or Ca-DPA and spores at an $OD_{600\ nm}$ of 1 was routinely followed by monitoring the $OD_{600\ nm}$ 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 $OD_{600\ nm}$ of 1·5 and at various times 1 ml aliquots of germinating cultures were centrifuged for 2 min in a microcentrifuge, and the $OD_{270\ nm}$ of the supernatant fluid was determined as a measure of spore DPA release (Setlow *et al.* 2003). The total amount of DPA in the starting spores was determined from the $OD_{270\ nm}$ 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

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 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 $OD_{600\ nm}$ 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.* 10^9 spores) or 2 ml germinated with glucose for 30 or 60 min at an $OD_{600\ nm}$ 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 $OD_{600\ nm}$ 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 H_2O, 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 Mn^{2+} 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 D_2O 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 $OD_{600\ nm}$ 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 OD_{600} 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 CwlJ 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 ($\Delta T_{release}$) is relatively uniform for individual spores in a population. However, the factors that determine values for T_{lag} and $\Delta T_{release}$ 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 $\Delta T_{release}$ 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

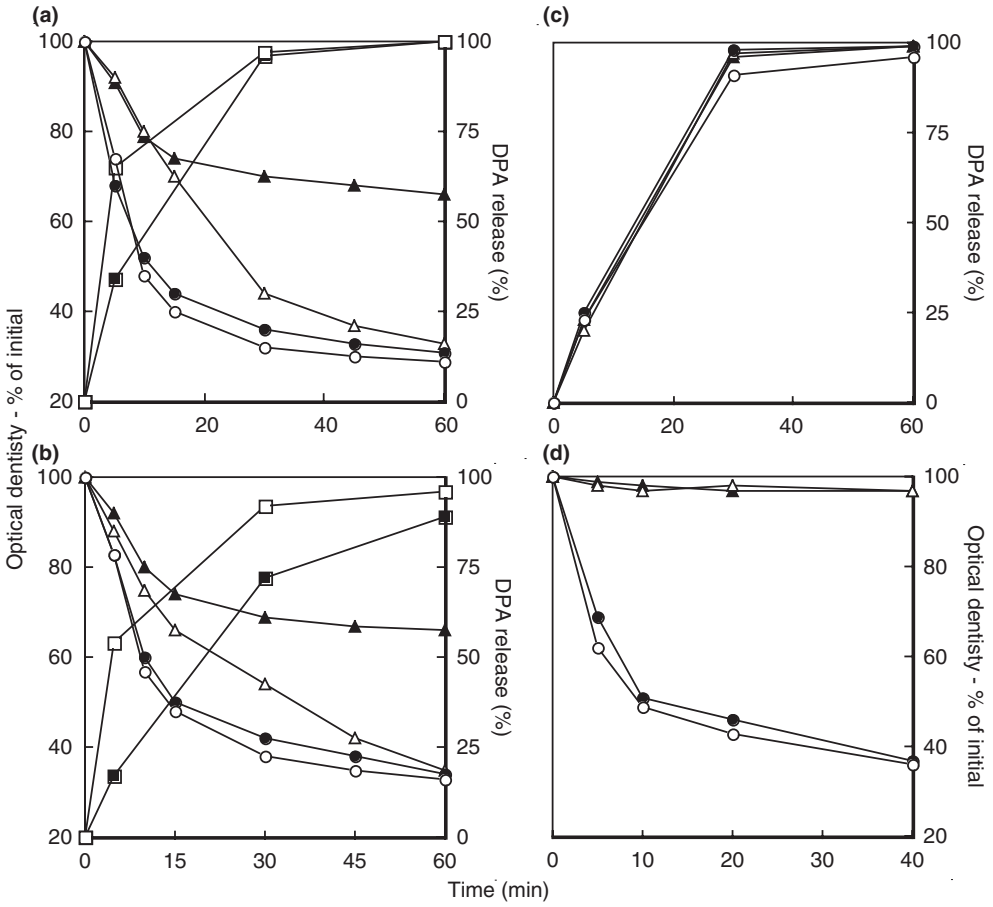

Figure 1 Germination of *Bacillus megaterium* spores of strains with and without CLEs. Spores were germinated with (a) glucose, (b) KBr, (c) dodecylamine or (d) Ca-DPA, and spore germination was measured by monitoring the $OD_{600\ m}$ of the culture (○, ●, △, ▲) or DPA release (□, ■) as described in the section Methods. The symbols used for the spores of the various strains are: ○, □, wild-type; △, PS4164 (*cwlJ*), ●, PS4162 (*sleB*); and ▲, ■, PS4165 (*cwlJ sleB*).

Table 2 Viability of spores of various *Bacillus megaterium* strains*

	Viability†	
	CFU ml^{-1} at $OD_{600\ nm}$ of 1	
Spores of strain	No lysozyme	With lysozyme
wild-type	7×10^7 (100)	8×10^7 (100)
PS4164 (*cwlJ*)	6×10^7 (86)	8×10^7 (100)
PS4162 (*sleB*)	4×10^7 (57)	7×10^7 (88)
PS4165 (*cwlJ sleB*)	5×10^4 (0·07)	2×10^7 (25)

*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 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 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%

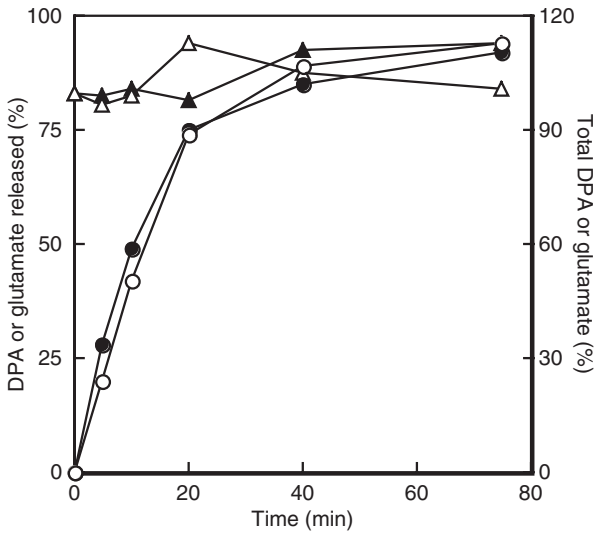

Figure 2 Release of DPA and glutamate during KBr germination of *cwlJ sleB Bacillus megaterium* spores. Spores of the *cwlJ sleB* (PS4165) *B. megaterium* strain were germinated with KBr and at various times the percentages of the total amounts of DPA and glutamate in the starting dormant spores released upon germination were determined as described in the section Methods, as were the levels of total DPA and glutamate. The symbols used are: ○, DPA released; △, total DPA; ●, glutamate released; and ▲, total glutamate.

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.

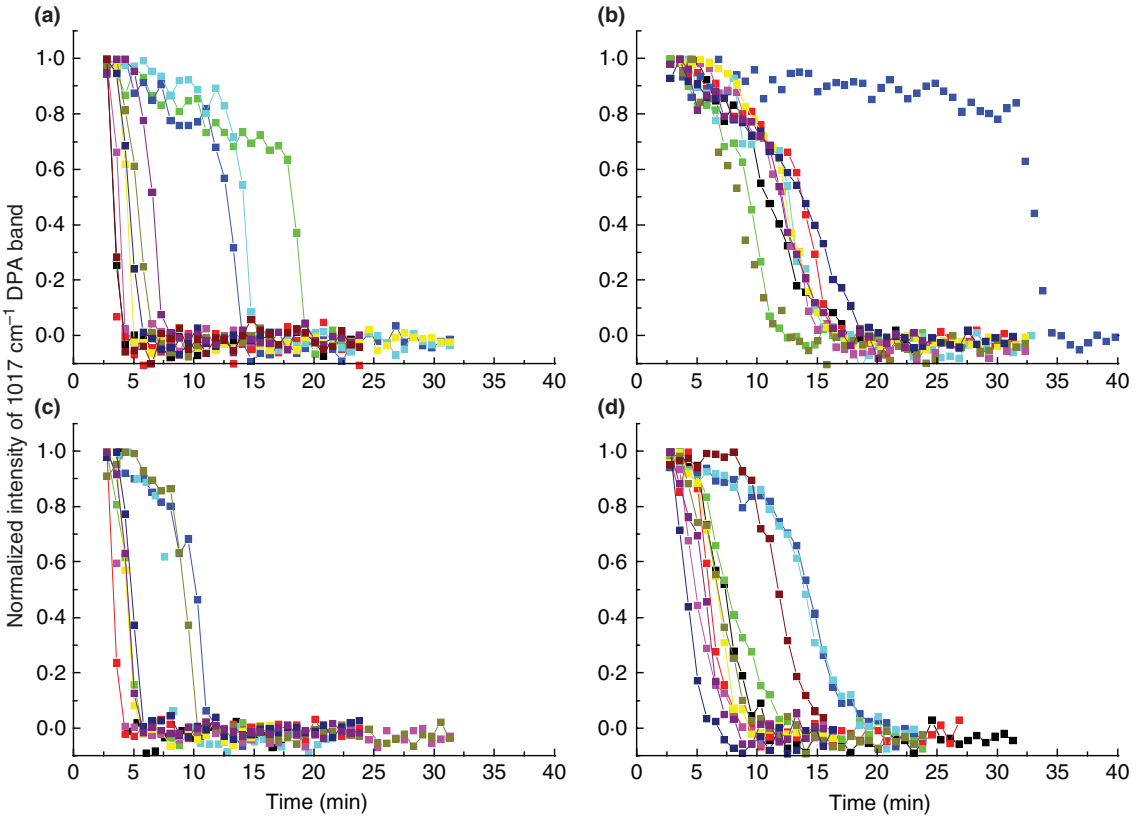

Figure 3 Dipicolinic acid release during glucose germination of individual *Bacillus megaterium* spores of strains with and without CLEs. Spores of (a) the wild type strain, (b) strain PS4164 (*cwlJ*), (c) strain PS4162 (*sleB*) and (d) strain PS4165 (*cwlJ sleB*) were germinated with glucose and DPA release from 10 individual spores of each strain was followed by laser tweezers Raman spectroscopy as described in the section Methods. Time zero is the time of mixing of spores with germinant, and the different symbols/colours in a–d denote data for different individual spores.

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

Spores of strain	T_{lag} (min)†	$\Delta T_{release}$ (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_{lag}, $\Delta T_{release}$ 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.

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

Spores examined	Core wet density (g cc^{-1})
Wild-type dormant	1·335
Wild-type germinated	1·170
cwlJ sleB dormant	1·335
cwlJ sleB germinated	1·254

*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).

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

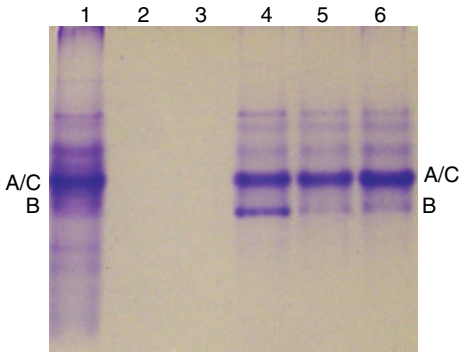

Figure 4 SASP levels in dormant and germinated *Bacillus megaterium* spores with or without CwlJ and SleB. Dormant spores of the wild type strain (lane 1) and strain PS4165 (*cwlJ sleB*) (lane 4), wild-type spores germinated 30 (lane 2) or 60 min (lane 3) with glucose, and *cwlJ sleB* spores (strain PS4165) germinated 30 (lane 5) or 60 min (lane 6) with glucose were disrupted, extracted, extracts processed, lyophilized, re-dissolved, aliquots run on polyacrylamide gel electrophoresis at low pH and the gel stained with Coomassie Blue as described in the section Methods. The labels a/c and b to the left of lane 1 and right of lane 6 denote the migration position of the two major *B. megaterium* α/β-type SASP A and C (which co-migrate) and the only γ-type SASP, B respectively. The direction of migration of the gel was from the top to the bottom of the figure.

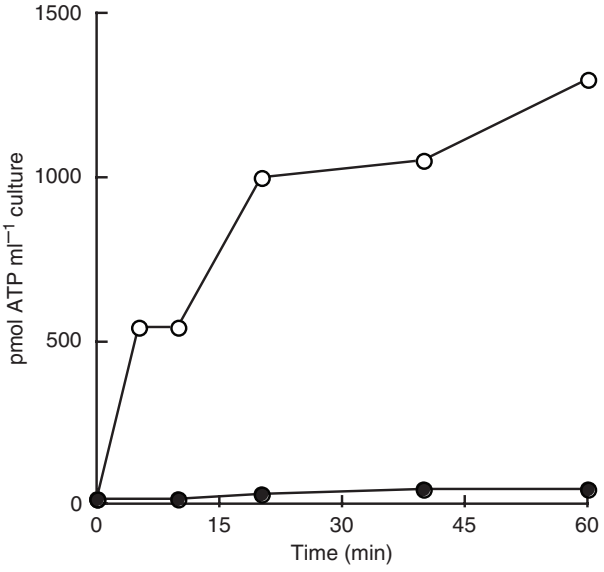

Figure 5 ATP production following germination of wild-type and *cwlJ sleB Bacillus megaterium* spores. Spores of the wild-type (○) and *cwlJ sleB* (PS4165) (●) strains were germinated in glucose and samples taken for ATP extraction, processed and assayed for ATP as described in the section Methods.

also been found for *cwlJ sleB B. subtilis* spores germinating with nutrients, although DPA is fully released from germinating *cwlJ sleB* spores of both species (Ishikawa *et al.* 1998; Setlow *et al.* 2001). DPA release is also slower from *cwlJ B. subtilis* spore populations germinating with nutrients, although not from germinating *sleB B. subtilis* spores (Ishikawa *et al.* 1998), and the rate of decrease in $OD_{600\ nm}$ during germination of *cwlJ B. megaterium* spores with either KBr or glucose was also slower than with wild-type or *sleB* spores. The release of DPA from individual *cwlJ sleB B. megaterium* spores germinating with glucose was also significantly slower than from germinating wild-type or *sleB* spores. The difference in DPA release from these types of spores appeared to be solely in $\Delta T_{release}$, and *cwlJ* spores also exhibited this slower $\Delta T_{release}$ during glucose germination while *sleB* spores did not. In contrast, values of T_{lag} prior to rapid DPA release during glucose germination were essentially identical for *B. megaterium* spores with or without CLEs. That the presence of CwlJ significantly accelerates $\Delta T_{release}$ for DPA has also been seen during nutrient germination of individual *B. subtilis* spores, while the absence of SleB has no effect (Peng *et al.* 2009).

The reason(s) why the absence of CwlJ and not SleB increased values of $\Delta T_{release}$ 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 *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 *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 *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 *cwlJ sleB B. megaterium* spores as well as a core wet density of germinated *cwlJ sleB* spores in between that of dormant *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 *Bacillaces* whose completed genome sequences are complied 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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