The protease CspB is essential for initiation of cortex hydrolysis and dipicolinic acid (DPA) release during germination of spores of Clostridium perfringens type A food poisoning isolates

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The genome of the Clostridium perfringens food poisoning isolate SM101 encodes a subtilisin-like protease, CspB, upstream of the sleC gene encoding the enzyme essential for degradation of the peptidoglycan cortex during spore germination. SleC is an inactive pro-SleC in dormant spores that is converted to active SleC during spore germination and Csp proteases convert pro-SleC to the active enzyme in vitro. In this work, the germination and viability of spores of a cspB deletion mutant of strain SM101, as well as cspB expression, were studied. The cspB gene was expressed only during sporulation, and only in the mother cell compartment. cspB spores were unable to germinate significantly with either a rich nutrient medium, KCl, or a 1:1 chelate of Ca\(^2+\) and dipicolinic acid (DPA); the viability of these spores was ~10^4-fold lower than that of wild-type spores, although cspB and wild-type spores had similar viability on plates containing lysozyme, and cspB spores could not process inactive pro-SleC into active SleC during spore germination. Germination of cspB spores was blocked prior to DPA release and cortex hydrolysis, and germination and viability defects in these spores were complemented by an ectopic cspB. These results indicate that Csp proteases are essential to generate active SleC and allow cortex hydrolysis early in C. perfringens spore germination. However, Csp proteases likely play another role in spore germination, since cspB spores did not release DPA upon exposure to germinants, while sleC spores have been shown previously to release DPA, albeit slowly, upon exposure to germinants.

INTRODUCTION

Clostridium perfringens is an anaerobic, spore-forming pathogenic bacterium, and many strains cause gastrointestinal (GI) diseases in humans and animals (McClane, 2007). C. perfringens spores are metabolically dormant, resistant to many environmental insults and, once conditions are favourable, these spores can germinate, outgrow, return to vegetative growth and then release toxins and cause disease (McClane, 2007; McDonnell, 1986; Paredes-Sabja et al., 2008b).

Abbreviations: Ca-DPA, Ca\(^2+\)-dipicolinic acid chelate; CLE, cortex-lytic enzyme; DPA, dipicolinic acid; FP, food poisoning; GUS, \(\beta\)-glucuronidase; PG, peptidoglycan.

Two supplementary tables, listing bacterial strains and plasmids and primers used in this study, and a supplementary figure, showing sequence alignment of Csp proteins of C. perfringens strains, are available with the online version of this paper.
of cortex hydrolysis through the activation of the cortex-lytic enzyme (CLE) CwlJ, which is present in dormant spores as the mature, potentially active enzyme (Paidhungat et al., 2001). In C. perfringens, the CLE SleC alone is sufficient to degrade the spore peptidoglycan (PG) cortex, and Ca-DPA does not appear to directly activate cortex hydrolysis (Paredes-Sabja et al., 2008a, b, 2009a). SleC is present in dormant C. perfringens spores, although not in the mature potentially active form, but rather as an inactive zymogen, pro-SleC, which is cleaved into active SleC early in spore germination (Urakami et al., 1999). These findings suggest that signalling pathways differ in at least some aspects of germination in spores of Bacillus and Clostridium species. Ultimately, removal of the PG cortex eliminates a physical constraint, allowing the core to expand and hydrate to levels found in vegetative cells (Popham et al., 1996), thus restoring enzyme activity and metabolism and allowing spore outgrowth (Cowen et al., 2003; Setlow, 2003).

Work with C. perfringens S40 has identified three germination-specific serine proteases (Csp proteases) that belong to the subtilisin subfamily (Shimamoto et al., 2001; Siezen & Leunissen, 1997). The majority of subtilisins are synthesized as pre-pro-enzymes, subsequently secreted into an external medium in a pro-form following the removal of the pre-peptide (or signal peptide), and activated autocatalytically by cleavage of the pre-peptide (Shinde & Inouye, 1996). Mature subtilisins possess three conserved residues (Asp, His, Ser) that form the catalytic triad of these enzymes (Siezen & Leunissen, 1997), and the three Csp proteins in C. perfringens S40 possess this catalytic triad as well as the oxyanion binding hole (Shimamoto et al., 2001). The Csp proteins are synthesized in the mother cell compartment of the sporulating cell and subsequently translocated into the PG cortex of the developing forespore, and during this period there is processing at both the N and C termini, generating pro-SleC (Masayama et al., 2006; Shimamoto et al., 2001). In vitro studies have shown that a fraction from germinated C. perfringens S40 spore extracts containing the three Csp proteins can process pro-SleC to SleC, thus activating the enzyme for degradation of the cortex of decoated spores (Kumazawa et al., 2007; Okamura et al., 2000; Shimamoto et al., 2001). Surprisingly, genome sequencing (Myers et al., 2006; Shimizu et al., 2002) indicates that C. perfringens type A food poisoning (FP) isolates encode only one Csp, CspB, although non-FP isolates such as S40 carry a cspABC triocistronic operon encoding all three Csp proteins (Fig. 1a). However, the precise function of Csp proteins in C. perfringens spore germination is unclear. Consequently, in the current work we have constructed a cspB mutant of a C. perfringens FP isolate to elucidate the role of CspB in spore germination.

METHODS

Bacterial strains and plasmids. C. perfringens and plasmids used in this study are described in Supplementary Table S1.

Fig. 1. (a) Genomic arrangement and (b) expression of cspB in C. perfringens SM101. (a) The arrangement of the csp–sleC locus in the C. perfringens FP strain SM101 and the non-FP strains F4969 and S40 and the location of primers used to amplify the upstream region of cspB from strain SM101 are indicated. (b) GUS specific activity from the cspB–gusA fusion in C. perfringens SM101 grown in TGY vegetative (△) and DS sporulation (●) media was determined as described in Methods. Data are the mean of three independent experiments, and time zero denotes the time of inoculation of cells into either TGY or DS medium.

Construction of cspB–gusA fusion plasmid and β-glucuronidase (GUS) assay. A 441 bp DNA fragment upstream of cspB in C. perfringens SM101, which included the 170 bp intergenic region between cspB and CPR2568 that most likely contains the cspB gene promoter, was PCR-amplified using primers CPP393/CPP397 (all primers used in this work are listed in Supplementary Table S2). The forward and reverse primers had SalI and PstI sites, respectively. This PCR fragment was digested with SalI and PstI and cloned between SalI and PstI sites in pMRS127 to create a cspB–gusA fusion, giving plasmid pDP85. This plasmid was introduced by electroporation (Czechulin et al., 1996) into C. perfringens SM101, and erythromycin-resistant (Em; 50 μg ml⁻¹) transformants were selected. Transformants carrying the cspB–gusA fusion plasmid pDP85 were grown in TGY vegetative medium (3 % trypticae soy, 2 % glucose, 1 % yeast extract, 0.1 % L-cysteine) (Kokai-Kun et al., 1994) and in Duncan–Strong (DS) (Duncan & Strong, 1968) sporulation medium, and assayed for GUS activity as described by Zhao & Melville (1998). GUS specific activity was expressed in Miller units, which were calculated as described previously (Raju et al., 2006).
Decoating treatment of sporulating cultures and spores prior to GUS assays. Cell pellets from 1 ml of 8 h DS sporulating cultures or purified spore suspensions at OD\textsubscript{600} 1.0 were treated with 1 ml 50 mM Tris/HCl (pH 8.0), 8 M urea, 1% (w/v) SDS and 50 mM DTT for 90 min at 37 °C, and remaining spores were washed three times with 150 mM NaCl and twice with water (Popham et al., 1995). The chemically decoated samples were then extracted and assayed for GUS activity as described above. Note that this urea–DS treatment will inactivate or remove any GUS not in dormant spores, and will sensitize dormant spores to lysozyme, allowing the assay of enzymes in the dormant spore core (Mason et al., 1988; Paredes-Sabja et al., 2009b). GUS activities in these experiments were expressed relative to the amount in an equivalent volume of an untreated DS 8 h sporulating culture of the strain carrying the cspB–gus\textsubscript{A} fusion, which was set to 100%.

**Construction of C. perfringens cspB deletion mutant.** To isolate a derivative of C. perfringens SM101 with a deletion of cspB, a \textsc{Asc}sp suicide vector was constructed as follows. A 2016 bp DNA fragment carrying 112 bp from the N-terminal coding region and 1949 bp upstream of cspB was PCR-amplified using primers CPP356/CPP360 (forward and reverse primers had respectively, at the 5’ ends). An 1689 bp fragment carrying 54 bp from Kpn\textit{I} and Spe\textit{I} cleavage sites, respectively, at the 5’ ends). These PCR fragments were cloned into plasmid pCR-XL-TOPO (Invitrogen), giving plasmids pDP55 and pDP57, respectively. An ~2.0 kb Kpn\textit{I}–Spe\textit{I} fragment from pDP55 was cloned into pDP25, giving plasmid pDP56, and an ~1.7 kb Pst\textit{I}–Xho\textit{I} fragment from pDP57 was cloned into pDP56, giving plasmid pDP58. Next, an ~5.0 kb Kpn\textit{I}–Xho\textit{I} fragment from pDP58 was cloned between the Kpn\textit{I} and Sal\textit{I} sites of pMRS104, giving pDP59. Finally, a 3.2 kb Eco\textit{R}I fragment carrying tet\textit{M} was excised from pDP59, and the ends were filled and cloned into the Hpa\textit{I} site in cat\textit{P} of pDP59, giving plasmid pDP165, which cannot replicate in C. perfringens. Plasmid pDP165 was introduced into C. perfringens strain SM101 by electroporation (Czechelin et al., 1996), and the cspB deletion strain DPS117 was isolated by allelic exchange (Sarker et al., 1999). The presence of the cspB deletion in strain DPS117 was confirmed by PCR and Southern blot analyses (results not shown).

**Preparation of spore extracts and Western blot analysis.** For preparation of coat extracts from dormant spores, aliquots (200 μl) of spores (OD\textsubscript{600} 50) were decoated in 200 μl 50 mM Tris/HCl (pH 8.0), 8 M urea, 1% (w/v) SDS and 50 mM DTT for 90 min at 37 °C, centrifuged (13 200 r.p.m.) for 5 min, and the supernatant fluid containing coat material from dormant spores was stored at −20 °C until use. For preparation of germinated spores, aliquots (200 μl at OD\textsubscript{600} 250) of heat-activated spores were suspended at OD\textsubscript{600} 250 in 200 μl 25 mM sodium phosphate (pH 7.0), 100 mM KCl germinated for 2 h at 37 °C, and stored at −20 °C until use. Preparation of coat extracts from germinated cspB spores, aliquots (200 μl) of germinated spores (OD\textsubscript{600} 250) were decoated as described above, and the supernatant fluid was stored at −20 °C until use.

Samples (10 μl) of coat extracts or intact germinated spores were boiled in SDS-PAGE loading buffer and run on SDS-PAGE gels (12% acrylamide), and proteins were transferred to a PVDF membrane (Millipore). These Western blots were probed with a 1:10 000 dilution of goat anti-mouse IgG–horseradish peroxidase (HRP) conjugate (Promega) for 1 h at room temperature and then with a 1:10 000 dilution of goat anti-mouse IgG– horseradish peroxidase (HRP) conjugate (Promega) for 1 h at room temperature, in PBS (25 mM sodium phosphate, pH 7.4, 150 mM NaCl) with 1% BSA and 0.05% Tween. HRP activity was detected with a chemiluminescence detection system (Molecular Imager ChemiDoc XRS+ System, Bio-Rad) by using the PicoMenu sensitive chemiluminescent HRP substrate (Rockland Immunochemicals). Each Western blot also included 5 μl PageRuler Plus prestained Protein Ladder (Fermentas).

**Analytical procedures.** DPA remaining in spores during and after germination was measured as described previously (Paredes-Sabja et al., 2008a, b, 2009a). Briefly, heat-activated spore suspensions (OD\textsubscript{600} 1.5) were cooled and incubated at 40 °C in 25 mM sodium phosphate buffer (pH 7.0) or in KCl, as described above. After 1 or 24 h, aliquots (1 ml) were centrifuged in a microcentrifuge (13 200 r.p.m., 3 min), and the spore pellet was washed twice with 1 ml distilled water and suspended in 1 ml distilled water. The remaining spore preparations used in this work were >99% pure as determined by phase-contrast microscopy. Spore germination was as described previously (Paredes-Sabja et al., 2008a, b, 2009a). Briefly, spore suspensions of OD\textsubscript{600} 6 were heat-activated at 80 °C for 10 min, cooled in water at ambient temperature for 5 min, and incubated at 40 °C for 10 min. Germination of spores at 40 °C and OD\textsubscript{600} 1 with brain heart infusion (BHI) broth, KCl (100 mM KCl, 25 mM sodium phosphate buffer, pH 7.0), a mixture of 100 mM l-asparagine, 100 mM KCl and 25 mM sodium phosphate (pH 7.0) (AK) or Ca-DPA (50 mM CaCl\textsubscript{2}, 50 mM DPA, adjusted to pH 8.0 with Tris base) was routinely measured by monitoring the OD\textsubscript{600} of spore cultures (SmartSpec 3000 spectrophotometer, Bio-Rad Laboratories), which falls by ~60% upon complete germination of wild-type spores. Levels of germination were also confirmed by phase-contrast microscopy. All values reported are averages of two experiments performed on at least two independent spore preparations, and individual values varied by less than 10% from average values shown.
DPA was determined by boiling samples for 60 min, cooling on ice for 5 min, centrifuging in a microcentrifuge for 5 min, and measuring the OD270 of the supernatant fluid as described previously (Paredes-Sabja et al., 2009a). The initial DPA content of dormant spores was determined by boiling 1 ml aliquots for 60 min, centrifuging in a microcentrifuge (13 200 r.p.m., 5 min), and measuring the OD270 of the supernatant fluid as described previously (Paredes-Sabja et al., 2008a, b, 2009a). In *C. perfringens* spores, ~90% of the material absorbing at 270 nm released from spores by boiling is DPA (Paredes-Sabja et al., 2009a).

The release of fragments of cortex PG containing hexosamine during germination was measured by germinating heat-activated spores at OD600 25 in 100 mM KCl and 10 mM Tris/HCl (pH 7.4). After 2 h incubation at 40 °C, samples (1 ml) were centrifuged (13 200 r.p.m., 5 min), and analyses of hexosamine in the supernatant fluid were carried out as described previously (Ghuysen et al., 1966; Paredes-Sabja et al., 2009a). Analysis of total hexosamine-containing material in dormant spores was also carried out as described previously (Ghuysen et al., 1966; Paredes-Sabja et al., 2009a).

**RESULTS**

Arrangement of the csp–sleC locus and expression of cspB in *C. perfringens* SM101

Earlier studies (Masayama et al., 2006; Shimamoto et al., 2001) with *C. perfringens* S40 indicate that three Csp proteases (CspA, B and C) belonging to the subtilisin family of serine proteases are encoded by a tricistronic operon upstream of the *sleC* gene (Paredes-Sabja et al., 2009a). However, in the FP isolate SM101, there is only one csp, cspB, upstream of *sleC* (Fig. 1a). Amino acid sequence alignments indicate that CspBSM101 shares high homology [92% identity (I) and 96% similarity (S)] to CspBS40, with much lower homology to CspAS40 (32% I, 50% S) and CspCS40 (31% I, 51% S) (Supplementary Fig. S1).

To determine whether cspB is expressed during sporulation in *C. perfringens* SM101, upstream DNA from cspB, including the intergenic region between cspB and the preceding gene (Fig. 1a), which most likely contains the cspB gene promoter, was fused to *E. coli* gusA, and GUS activity was measured after introducing the fusion into *C. perfringens* SM101. Vegetative cultures of SM101 carrying the cspB–gusA fusion had no significant GUS activity (Fig. 1b), and there was also no detectable GUS activity in sporulating cultures of strain SM101 without the cspB–gusA fusion (data not shown). However, there was significant GUS activity in sporulating cultures carrying the cspB–gusA fusion (Fig. 1b), indicating that a sporulation-specific promoter is located upstream of cspB. GUS expression from this putative cspB promoter appeared ~4 h after the start of sporulation, and reached a maximum after 10 h (Fig. 1b). To evaluate the site of expression of cspB, sporulating cultures and purified spores of strain SM101(pDP85) (wild-type carrying the cspB–gusA fusion) were treated with a decoating regime (Popham et al., 1995) that inactivates mother cell enzyme activity but has no effect on enzymes within the spore core. Although significant GUS activity was detected in untreated sporulating cultures of the strain carrying cspB–gusA as expected, no significant GUS activity was detected in decoated sporulating cultures and either untreated or decoated purified spores (data not shown). These results clearly indicate that cspB is expressed uniquely in the mother cell compartment of the sporulating cell.

Effect of cspB deletion on SM101 spore germination and colony formation with BHI broth

Since cspB is expressed during sporulation, we hypothesized that cspB is essential for pro-SleC processing and therefore for cortex hydrolysis during germination of spores of *C. perfringens* SM101. Consequently, we constructed a cspB deletion (strain DPS117) (Fig. 2a). As expected, wild-type spores germinated well in BHI broth as measured by changes in the OD600 of the germinating culture (Fig. 2b). However, cspB spores exhibited little if any germination in BHI broth, and their germination was even less than that of wild-type spores in phosphate buffer as measured by the fall in OD600 (Fig. 2b). Phase-contrast microscopy also showed that >90% of wild-type spores had become phase dark after 60 min in BHI broth, indicating that the great majority of these spores had completed germination, while cspB spores remained phase bright (data not shown), indicative of no germination. The germination defect of cspB spores was complemented by ectopic insertion of a wild-type cspB gene in the cspB strain, indicating that the germination defect in cspB spores is due to specific inactivation of cspB (Fig. 2b). These results suggest that cspB is essential for germination of spores of *C. perfringens* FP isolates.

The severity of the germination defect of cspB spores suggested that, as found for sleC spores (Paredes-Sabja et al., 2009a), the colony forming efficiency of cspB spores is lower than that of wild-type spores, since Csp proteins appear to be required for activation of the major *C. perfringens* CLE, SleC (Paredes-Sabja et al., 2009a; Shimamoto et al., 2001). Indeed, the colony forming efficiency of cspB spores was ~10^4-fold lower than that of wild-type spores (Table 1). However, this defect was eliminated when cspB spores were decoated and plated on BHI agar plates containing lysozyme (Table 1), indicating that cspB spores were viable, but probably incapable of degrading their PG cortex, as noted previously for sleC spores (Paredes-Sabja et al., 2009a). The latter result also suggests that active SleC is not generated during germination of cspB spores, since SleC alone is essential for cortex degradation during spore germination, and thus full spore viability (Paredes-Sabja et al., 2009a). The absence of active SleC in cspB spores could be due to lack of conversion of pro-SleC to SleC during germination (see below). However, since the low viability of cspB spores on plates without lysozyme was complemented by wild-type cspB inserted in the plc locus (Table 1), it appears most likely that pro-SleC is present in cspB spores but cannot be converted to active SleC during germination. Interestingly, incubation of cspB spores on BHI plates without lysozyme
for long periods of time did slowly give rise to colonies in a manner similar to that previously reported for sleC spores (Paredes-Sabja et al., 2009a), i.e. some colonies started to appear only surrounding those colonies that appeared within the first 24 h, suggesting that a cell wall hydrolase released from growing or sporulating cells was diffusing through the medium and germinating a small percentage of cspB spores that have a defective coat.

In Bacillus subtilis and Bacillus megaterium, Ca-DPA triggers germination by activation of the CLE CwlJ (Paidhungat et al., 2001; Setlow et al., 2009). In contrast, recent work (Paredes-Sabja et al., 2008b, 2009a) suggests that Ca-DPA triggers C. perfringens spore germination through the GerKA-KC receptor and not by activating either pro-SleC or SleC. Since cspB spores possess pro-SleC (see below), we sought to provide definitive evidence for this suggestion by examining the germination of wild-type, cspB and DPS117(pDP184) (cspB mutant complemented with wild-type cspB) spores with exogenous Ca-DPA. As expected, wild-type spores germinated well with exogenous Ca-DPA, while cspB spores exhibited little if any germination, as assessed either by the decrease in OD_{600} of germinating cultures or by phase-contrast microscopy (Fig. 2c and data not shown). However, the Ca-DPA germination defect in cspB spores was complemented by an ectopic copy of cspB (Fig. 2c and data not shown). These results suggest that Ca-DPA does not initiate germination by activating pro-SleC, but rather presumably through the GerKA-KC receptor, as suggested previously (Paredes-Sabja et al., 2008b).

CspB is essential for processing of pro-SleC into mature SleC during spore germination

To provide definitive evidence that the germination defect of cspB spores is due to lack of processing of inactive pro-SleC into active SleC, spore extracts were analysed by Western blotting with anti-SleC antibody (Fig. 3a). As expected (Miyata et al., 1997), coat extracts of dormant wild-type spores had pro-SleC but not SleC, while wild-type germinated spores had pro-SleC and a fraction processed into the smaller SleC (Fig. 3a). Coat extracts from dormant cspB spores also had pro-SleC, but there was no detectable processing of pro-SleC into SleC either in intact germinated cspB spores or in coat extracts from these spores (Fig. 3a). This defect was complemented by an ectopic copy of cspB (Fig. 3a). These results indicate that CspB is essential for processing of inactive pro-SleC into mature SleC.

Table 1. Colony formation by spores of C. perfringens strains

Heat-activated spores of various strains were plated on BHI agar with or without lysozyme, and colonies were counted after incubation at 37 °C for 24 h.

<table>
<thead>
<tr>
<th>Strain/genotype</th>
<th>Spore titre (c.f.u. ml⁻¹ per OD_{600} unit)*</th>
<th>BHI</th>
<th>BHI + Lyz†</th>
</tr>
</thead>
<tbody>
<tr>
<td>SM101/wild-type</td>
<td>4.1 × 10⁷</td>
<td>6.6 × 10⁷</td>
<td></td>
</tr>
<tr>
<td>DPS117/cspB</td>
<td>3.8 × 10⁴</td>
<td>6.0 × 10⁴</td>
<td></td>
</tr>
<tr>
<td>DPS117(pDP184)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>cspB carrying</td>
<td>5.3 × 10⁷</td>
<td>ND‡</td>
<td></td>
</tr>
<tr>
<td>wild-type cspB</td>
<td></td>
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</tr>
</tbody>
</table>

*Titres are the mean c.f.u. ml⁻¹ per OD_{600} unit determined in three experiments and the variance was less than 25%.
†Spores were decoated and plated on BHI plates containing 1 μg lysozyme ml⁻¹.
‡ND, Not determined.
active SleC early during germination, and that pro-SleC is present in cspB spores.

It was somewhat surprising that only a fraction of pro-SleC was processed into SleC by CspB during 2 h of germination of C. perfringens SM101 as well as DPS117(pDP184) (cspB complemented with an ectopic copy of cspB) with KCl, since ~99% of spores of both strains became phase dark after 2 h of germination (data not shown). To evaluate whether further incubation was required to complete pro-SleC processing, wild-type spores were incubated for 2, 4 and 8 h with KCl. However, the fraction of pro-SleC converted to active SleC increased only slightly upon extended incubation (Fig. 3b). Similar results were observed using the germinant AK (data not shown). These results suggest that only a fraction of inactive pro-SleC needs to be converted into active SleC by CspB to trigger full germination of spores of C. perfringens FP isolates.

**CspB is essential for initiation of cortex hydrolysis during germination of C. perfringens spores**

Previously we reported that during C. perfringens spore germination, sleC spores release their DPA slower than wild-type spores and are unable to degrade their PG cortex (Paredes-Sabja et al., 2009a). To gain more insight into the role of CspB in C. perfringens spore germination, wild-type, cspB and DPS117(pDP184) spores were germinated with KCl (Paredes-Sabja et al., 2008b). Wild-type spores germinated completely with KCl, while cspB spores showed a minimal fall in OD600 upon incubation with KCl. However, this defect was eliminated in the cspB strain complemented with an ectopic copy of cspB (Fig. 4a).

**DISCUSSION**

Results presented in this paper provide strong support for the hypothesis that Csp proteases are essential in germination of C. perfringens spores in order to activate pro-SleC and thus allow cortex PG degradation. This has been suggested previously based on results from in vitro studies of the effects of Csp proteins on pro-SleC and the activity of pro-SleC and SleC on cortex PG in decoated spores (Okamura et al., 2000; Urakami et al., 1999). The current work shows that C. perfringens spores lacking their sole Csp protein, CspB, cannot germinate with all germinants tested. In addition, these cspB spores had extremely low viability, most likely because of their poor
germination. The fact that the low viability of cspB spores could be corrected if these spores were decoated and applied to plates containing lysozyme is consistent with the defect in the germination of cspB spores being in cortex degradation, and thus that the function of CspB is to convert inactive pro-SleC into active SleC that can degrade cortex PG. When this work was begun, it was considered possible that a cspB deletion somehow prevents sleC expression, and that cspB spores lack even pro-SleC; since cspB is just upstream of sleC, a cspB deletion might interfere with sleC transcription somehow, perhaps even transcription from a promoter upstream of cspB. However, this possibility seemed unlikely for a variety of reasons including: (i) an ectopic sleC with only upstream DNA from the intergenic region between cspB and sleC can completely complement the spore germination defects of a sleC deletion strain (Paredes-Sabja et al., 2009a); (ii) there is a very stable stem–loop structure encoded in the intergenic region between cspB and sleC, and it appears likely that this is a strong terminator of cspB transcription with a predicted ΔG of −6.3 kcal mol$^{-1}$ (−26.4 kJ mol$^{-1}$) [determined using mfold (http://mfold.bioinfo.rpi.edu/cgi-bin/rna-form1.cgi)]; and (iii) there is a strong promoter in the intergenic region between cspB and sleC that drives sporulation-specific sleC expression (Paredes-Sabja et al., 2009a). In addition, as shown in the current work: (iv) ectopic expression of cspB completely complemented the germination and viability defects in cspB spores; and (v) the presence of pro-SleC in cspB spores, and at levels similar to those in wild-type spores, was shown in this work by Western blot analysis.

Since cspB spores have normal levels of pro-SleC, as noted above, it seems likely that it is CspB that processes thiszymogen and thus triggers cortex hydrolysis during spore germination. Interestingly, only a fraction of total pro-SleC is processed to active SleC by CspB during spore germination, but this fractional amount of active SleC is sufficient for complete germination of C. perfringens spores. The obvious question is then what triggers CspB activity during spore germination, as Csp proteins appear to be present in spores in a potentially active form. Unfortunately, there is no information available on the regulation of the activity of CspB, or Csp proteases in general, and this is clearly an important matter for further study.

While it appears clear that CspB is essential for spore germination by activating pro-SleC, it is possible that CspB has some other function and/or substrate. The evidence for this is that the sole function of CspB is to activate pro-SleC, then the germination phenotype of cspB spores should be identical to that of sleC spores. Indeed, the viability of sleC spores is 10$^4$-fold lower than that of wild-type spores and sleC spores germinate very poorly with all germinants tested (Paredes-Sabja et al., 2009a), as is also the case for cspB spores. However, while sleC spores cannot complete germination very efficiently because they cannot degrade their PG cortex, they slowly release much of their Ca-DPA...
during germination with a number of germinants (Paredes-Sabja et al., 2009a). In contrast, cspB spores do not. Thus, cspB spores have a more severe germination phenotype than sleC spores, consistent with CspB having some germination-associated function in addition to processing pro-SleC. However, the nature of this additional function as well as that of other possible CspB substrates in spores is not known, and these topics are also matters for further work.

The involvement of CspB in pro-SleC activation and thus cortex hydrolysis is a further example of the differences in the signalling pathways in the germination of spores of *Clostridium* and *Bacillus* species. In *Bacillus* species, spores have two redundant CLEs, CwlJ and SleB, and both are present in spores in a mature form that is activated during germination either by Ca-DPA released from the spore core (CwlJ), or perhaps by some drastic change in strain on the cortical PG following Ca-DPA release and its replacement by water in the spore core (SleB) (Paidhungat et al., 2001; Setlow et al., 2009; Setlow, 2003). In further contrast to the situation in *Clostridium* spores, there is no known involvement of Csp proteases, or indeed of any protease at all in the regulation of CLE activity in spores of *Bacillus* species.

The final conclusion from the current work concerns the difference in the number of Csp proteases in the FP and non-FP strains, one in the former and three in the latter. While it is formally possible that three Csp proteases are needed for full SleC processing, including removal of the N- and C-terminal pre-sequences (Urakami et al., 1999), clearly a single Csp is sufficient to generate enough active SleC in strain SM101 to allow complete germination. Thus there appears to be no clear necessity for three Csp proteases for the germination of *C. perfringens* spores. However, it is certainly possible that the additional two Csp proteases in non-FP isolates have some additional roles in spore germination, and this too is a matter for further study.

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