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 Ca²⁺ and dipicolinic acid (DPA); the viability of these spores was ~10⁴-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.

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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, Ca²⁺-dipicolinic acid chelate; CLE, cortex-lytic enzyme; DPA, dipicolinic acid; FP, food poisoning; GUS, β -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.

C. perfringens spores initiate germination when they sense a variety of compounds termed germinants, which include L-asparagine, KCl, a 1 : 1 chelate of Ca²⁺ and pyridine-2,6-dicarboxylic acid (dipicolinic acid; DPA) (Ca-DPA) and a cationic surfactant (Paredes-Sabja *et al.*, 2008b). In spores of *Bacillus* species, nutrient germinants are sensed by specific germinant receptors located in the spore inner membrane, each encoded by a tricistronic operon of the *gerA* family (Setlow, 2003). Although there is no intact tricistronic *gerA*-type operon in *C. perfringens*, the products of the bicistronic *gerKAC* operon are essential for spore germination with either L-asparagine or Ca-DPA, and partially required for germination with KCl, and there is also a divergently transcribed *gerKB* cistron (Paredes-Sabja *et al.*, 2008b). Upon binding of the germinant to its cognate receptor, DPA is released from the spore core as a 1 : 1 chelate with divalent cations, predominantly Ca²⁺. This event, at least in *Bacillus* spores, triggers the initiation

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 (Cowan *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 pro-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* tricistronic 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* wild-type SM101 grown in TGY vegetative (Δ) and DS sporulation ($\blacktriangle$) 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 (Czeczulin *et al.*, 1996) into *C. perfringens* SM101, and erythromycin-resistant (Em^r ; 50 $\mu\text{g ml}^{-1}$) transformants were selected. Transformants carrying the *cspB-gusA* fusion plasmid pDP85 were grown in TGY vegetative medium (3% trypticase 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 OD₆₀₀ 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-SDS 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*-*gusA* 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 $\Delta cspB$ 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/PPP360 (forward and reverse primers had *KpnI* and *SpeI* cleavage sites, respectively, at the 5' ends). A 1689 bp fragment carrying 54 bp from the C-terminal coding region and 1635 bp downstream of *cspB* was PCR-amplified using primers CPP359/PPP364 (forward and reverse primers had *PstI* and *XhoI* 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 *KpnI*-*SpeI* fragment from pDP55 was cloned into pDP25 giving plasmid pDP56, and an ~1.7 kb *PstI*-*XhoI* fragment from pDP57 was cloned into pDP56, giving pDP58. Next, an ~5.0 kb *KpnI*-*XhoI* fragment from pDP58 was cloned between the *KpnI* and *Sall* sites of pMRS104, giving pDP59. Finally, a 3.2 kb *EcoRI* fragment carrying *tetM* was excised from pDP35, and the ends were filled and cloned into the *HpaI* site in *catP* of pDP59, giving plasmid pDP165, which cannot replicate in *C. perfringens*. Plasmid pDP165 was introduced into *C. perfringens* strain SM101 by electroporation (Czczulin *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).

Construction of a *cspB* mutant strain complemented with *cspB*. To construct a *cspB* strain complemented with wild-type *cspB*, a suicide-complementing plasmid targeted to the *plc* locus was constructed as follows. An ~2.4 kb DNA fragment carrying 441 bp from upstream and the coding region of *cspB* was PCR-amplified with Phusion High-Fidelity DNA polymerase (New England Biolabs) using primers CPP484/PPP485 (forward and reverse primers had *KpnI* and *Sall* sites, respectively, at their 5' ends). As shown by assays of GUS activity (Fig. 1b), the 441 bp region upstream of *cspB* contains a sporulation-specific promoter. This PCR fragment was cloned into ZeroBlunt TOPO (Invitrogen), giving plasmid pDP121. Next, a 2.4 kb *KpnI*-*Sall* fragment from pDP121 was cloned into the *KpnI*/*Sall* sites of pDP129 [a suicide plasmid containing ~1.7 and 1.3 kb upstream and downstream, respectively, of the *plc* locus (Paredes-Sabja *et al.*, 2009a)], giving plasmid pDP184, which cannot replicate in *C. perfringens*. Plasmid pDP184 was introduced into the *C. perfringens cspB* strain DPS117 by electroporation (Czczulin *et al.*, 1996) and Em^r Tet^r transformants of strain DPS117(pDP184) were selected. The presence of both plasmid pDP184 and the original *cspB* deletion in the latter strain was confirmed by PCR and Southern blot analyses (data not shown).

Spore preparation and germination. Spores of various *C. perfringens* strains were prepared as previously described (Paredes-Sabja *et al.*, 2008a, b, 2009a) and stored at -20 °C until use. All 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 OD₆₀₀ 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 OD₆₀₀ 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 CaCl₂, 50 mM DPA, adjusted to pH 8.0 with Tris base) was routinely measured by monitoring the OD₆₀₀ 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.

Spore decoating treatment and assessment of colony forming ability. Spores at OD₆₀₀ 20 were decoated in 1 ml 0.1 M sodium borate (pH 10), 2 % 2-mercaptoethanol for 60 min at 37 °C (Miyata *et al.*, 1997; Paredes-Sabja *et al.*, 2008a), and the decoated spores were washed 10 times with distilled water before use.

To assess the colony forming efficiency of the spores, untreated and decoated spores at OD₆₀₀ 1 were heat-activated (80 °C, 10 min), aliquots of various dilutions were plated on BHI agar with or without lysozyme (1 µg ml⁻¹), the plates were incubated at 37 °C anaerobically for 24 h, and colonies were counted.

Preparation of spore extracts and Western blot analysis. For preparation of coat extracts from dormant spores, aliquots (200 µl) of spores (OD₆₀₀ 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 OD₆₀₀ 250) of heat-activated spores were suspended at OD₆₀₀ 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. For preparation of coat extracts from germinated *cspB* spores, aliquots (200 µl) of germinated spores (OD₆₀₀ 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 anti-SleC polyclonal antibody (Miyata *et al.*, 1995) 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 PicoMax 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 (OD₆₀₀ 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

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 CspB^{SM101} shares high homology [92 % identity (I) and 96 % similarity (S)] to CspB^{S40}, with much lower homology to CspA^{S40} (32 % I, 50 % S) and CspC^{S40} (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 spor-

ulating 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 ~10⁴-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

Fig. 2. (a) Schematic representation of *cspB* deletion mutant in *C. perfringens* SM101 and (b, c) germination of mutant spores in BHI broth. (a) Arrangement of *cspB* in the *C. perfringens* wild-type and *cspB* mutant strains. (b, c) Heat-activated spores of *C. perfringens* strains SM101 (wild-type) (■), DPS117 (*cspB*) (□) and DPS117 (pDP184) (*cspB* mutant complemented with wild-type *cspB*) (△) were germinated at 40 °C in (b) BHI broth or (c) Ca-DPA, and the OD₆₀₀ was measured as described in Methods. Heat-activated spores of SM101 (wild-type) (▲) were germinated at 40 °C in 25 mM sodium phosphate (pH 7.0) (b).

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 OD₆₀₀ 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).

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.

Strain/genotype	Spore titre (c.f.u. ml ⁻¹ per OD ₆₀₀ unit)*	
	BHI	BHI + Lyz†
SM101/wild-type	4.1 × 10 ⁷	6.6 × 10 ⁷
DPS117/ <i>cspB</i>	3.8 × 10 ³	6.0 × 10 ⁷
DPS117(pDP184) <i>cspB</i> carrying wild-type <i>cspB</i>	5.3 × 10 ⁷	ND‡

*Titres are the mean c.f.u. ml⁻¹ per OD₆₀₀ 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.

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

Fig. 3. Detection of SleC in *C. perfringens* spores. (a) Aliquots of coat extracts from dormant (C) and germinated spores (GS), and intact germinated spores (GS) were separated by SDS-PAGE, proteins were transferred to a PVDF membrane, and SleC was detected with anti-SleC antibody as described in Methods. Samples run in various lanes were from spores of strains SM101 (wild-type), DPS117 (*cspB* mutant) and DPS117(pDP184) (*cspB* complemented with wild-type *cspB*), and the arrows on the right indicate the predicted migration positions of pro-SleC (34 kDa) and SleC (31 kDa) (Miyata *et al.*, 1995). (b) Spores of strain SM101 (wild-type) were germinated with 100 mM KCl for 2, 4 and 8 h, the intact spore was extracted, aliquots were run on SDS-PAGE, and SleC was detected by Western blot analysis as described for (a).

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, although this defect was eliminated in the *cspB* strain complemented with an ectopic copy of *cspB* (Fig. 4a). Phase-contrast microscopy further showed that >99% of wild-type and DPS117(pDP184) spores had become phase dark after 1 h of KCl germination, while the *cspB* spores remained phase bright (data not shown). The phase-contrast microscopy results and the lack of fall in the OD₆₀₀ of *cspB* spores suggested that little if any DPA was released during KCl germination of these spores. To directly confirm this conclusion, we measured DPA remaining in KCl-germinated wild-type, *cspB* and DPS117(pDP184) spores. After 1 h of incubation in KCl, wild-type spores had released most of their DPA (Fig. 4b). However, the amount of DPA released from *cspB* spores after 1 h of incubation with KCl was minimal (Fig. 4b), and similar to that released from wild-type spores incubated for 1 h at 40 °C in sodium phosphate buffer alone (data not shown), in which *C. perfringens* spores germinate minimally if at all. Incubation of *cspB* spores in KCl for 24 h led to only an insignificant increase in the amount of DPA released (Fig. 4b). However, DPA release during KCl germination was restored in *cspB* spores upon their complementation with wild-type *cspB* (Fig. 4b).

The culminating event in spore germination is the hydrolysis of the spore PG cortex and release of PG fragments into the medium. Therefore, we monitored cortex hydrolysis by assaying the release of hexosamine-containing material into the medium (Ghuysen *et al.*, 1966; Paredes-Sabja *et al.*, 2009a). As expected, wild-type spores released significant amounts of hexosamine-containing material into the medium after 2 h of germination with KCl (Fig. 4c). In contrast, hexosamine-containing material was not released by *cspB* spores during KCl germination, although this release was restored in *cspB* spores complemented with wild-type *cspB* (Fig. 4c). These results clearly indicate that CspB is essential for cortex hydrolysis during *C. perfringens* spore germination through the processing of pro-SleC into mature SleC.

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

Fig. 4. Germination of spores of *C. perfringens* strains with KCl. (a) Heat-activated *C. perfringens* spores of strains SM101 (wild-type), DPS117 (*cspB*) and DPS117(pDP184) (*cspB* mutant complemented with *cspB*) were germinated with KCl, and the OD₆₀₀ was measured after 1 h as described in Methods. (b) DPA release during *C. perfringens* spore germination with KCl. Heat-activated spores of *C. perfringens* strains were germinated with KCl and after 1 h (grey bars) and 18 h (black bars) the DPA content of spores was measured as described in Methods. DPA release from spores of *C. perfringens* strains incubated at 40 °C in 25 mM sodium phosphate buffer (pH 7.0) was less than 20 %. (c) Release of hexosamine-containing material during *C. perfringens* spore germination with KCl. Heat-activated spores of *C. perfringens* strains were germinated with KCl for 2 h and the hexosamine-containing material released into the medium was measured as described in Methods. Values of hexosamine-containing material released are expressed relative to the amount of hexosamine in dormant spores, which was defined as 100 %. Data represent the mean of three independent experiments with two different spore preparations; error bars, SD.

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 $-6.3 \text{ kcal mol}^{-1}$ ($-26.4 \text{ kJ mol}^{-1}$) [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 this zymogen 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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