Location and stoichiometry of the protease CspB and the cortex-lytic enzyme SleC in Clostridium perfringens spores

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A B S T R A C T
The protease CspB and the cortex-lytic enzyme SleC are essential for peptoglycan cortex hydrolysis during germination of spores of the Clostridium perfringens food poisoning isolate SM101. In this study, Western blot analyses were used to demonstrate that CspB and SleC are present exclusively in the C. perfringens SM101 spore coat layer fraction and absent in the lysate from decoated spores and from the purified inner spore membrane. These results indicate why decoating treatments greatly reduce both germination and apparent viability of C. perfringens spores in the absence of an exogenous lytic enzyme. In addition, quantitative Western blot analyses showed that there are approximately 2000 and 130,000 molecules of CspB and pro-SleC, respectively, per C. perfringens SM101 spore, consistent with CspB’s role in acting catalytically on pro-SleC to convert this zymogen to the active enzyme.

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1. Introduction

Clostridium perfringens is an anaerobic, gram-positive, and spore-forming bacterium that can cause gastrointestinal (GI) diseases in humans and animals (McClane et al., 2004; McClane, 2007). Depending on the production of various major toxins (alpha, beta, epsilon, and iota), C. perfringens can be classified into five types, A through E (McDonnell, 1986). However, less than 5% of C. perfringens type A isolates produce C. perfringens enterotoxin, the major virulence factor responsible for C. perfringens type A food poisoning (FP) and non-food borne (NFB) GI diseases (McClane, 2007; Sarker et al., 1999). Spores of C. perfringens may remain in a dormant state for long periods of time due to their innate resistance to many environment stresses such as heat, radiation, and toxic chemicals (Paredes-Sabja et al., 2008a, 2008b; Raju et al., 2006; Raju et al., 2007). Under favorable conditions in the environment, these spores can return to active growth through the processes of spore germination and outgrowth that lead to the synthesis and release of toxins causing diseases (Paredes-Sabja et al., 2008c; Setlow, 2013).

In Bacillus and Clostridium species, spore germination is initiated when spores sense compounds called germinants, such as nutrients (i.e., amino acids and salts), a 1:1 chelate of Ca2+ and pyridine-2,6-dicarboxylic acid (dipicolinic acid [DPA]) (Ca-DPA) and cationic surfactants (Paidhungat and Setlow, 2000; Paredes-Sabja et al., 2008c; Setlow, 2013). In Bacillus subtilis, two spore-specific cortex-lytic enzymes (CLEs), CwlJ and SleB, are responsible for degradation of spore cortex peptidoglycan. Loss of either CwlJ or SleB does not abolish cortex degradation, but spores lacking both enzymes do not degrade their cortex and cannot complete germination (Ishikawa et al., 1998; Paidhungat et al., 2001; Setlow, 2013). CwlJ and SleB are both synthesized in a mature form during sporulation; CwlJ is activated by Ca-DPA released from the spore core in the initial stage of germination, signaling activation of cortex hydrolysis, while SleB is perhaps activated when the spore cortex undergoes structural...
deformation during Ca-DPA release (Ishikawa et al., 1998; Olguin-Araneda et al., 2014; Paidhungat et al., 2000, 2001; Setlow et al., 2009).

Two CLEs have been identified in \textit{C. perfringens} spores, SleM and SleC, and these enzymes appear to differ significantly from CWj and SleB in \textit{Bacilli} (Paredes-Sabja et al., 2009b). SleM seems to be an N-acetylmuramidase, and SleC exhibits transglycosylase and N-acetylmuramoyl-Lalanine amidase activity (Kumazawa et al., 2007). However, only SleC is required for cortex hydrolysis during \textit{C. perfringens} spore germination, and Ca-DPA release does not act as a signaling molecule to trigger cortex hydrolysis. Rather SleC is present in dormant \textit{C. perfringens} spores as an inactive zymogen, pro-SleC, and early in germination the pro-sequence is removed, generating mature active SleC (Paredes-Sabja et al., 2009b, 2011; Xiao et al., 2011). Pro-SleC is activated by the Csp serum proteases that are members of the subtilisin family of proteases, and the \textit{C. perfringens} 540 genome encodes three Csp proteases, CspA, B, and C, any of which can convert pro-SleC into mature SleC \textit{in vitro} (Masayama et al., 2006; Shimamoto et al., 2001). The Csp proteases are also made aszymogens (pro-CspA, B or C) that most likely autoprocess to the mature active enzymes during sporulation. However, the \textit{C. perfringens} FP strain SM101 has only one Csp protease, CspB, which is essential for cortex hydrolysis and rapid Ca-DPA release during spore germination (Paredes-Sabja et al., 2009a).

Previous work with \textit{B. subtilis} spores showed that CWj is in the spore’s outer layers, most likely the spore coats, as this protein is removed by coat extraction with detergents plus a reducing agent at high pH, and also does not assemble properly on spores of a \textit{cotE} mutant that have very defective coats (Bagyan and Setlow, 2002; Chirakkal et al., 2002). Some SleB is also in the outer layers of \textit{B. subtilis} spores, but most SleB as well as its partner protein YpeB are found associated with the inner spore membrane that surrounds the spore core, and this latter SleB is not removed by decoating treatments (Chirakkal et al., 2002). In \textit{C. perfringens} S40 spores, the localization of CLEs was determined using immunoelectron microscopy, and pro-SleC was found on the outside of the cortex but was not detected in germinated spores and chemically decoated spores (Miyata et al., 1997). However, pro-SleC’s antigenicity might have been destroyed by the chemical decoating procedure used in the latter work. The Csp proteases that activate pro-SleC are made in the mother cell compartment of the sporulating cell, and can also be extracted by mild detergents from germinated \textit{C. perfringens} spores (Masayama et al., 2006; Shimamoto et al., 2001). The latter results suggest that Csp proteases are also in \textit{C. perfringens} spores’ outer layers, but this has not been shown definitively. Consequently, in this work we have examined the location of both pro-SleC and the Csp protease CspB in spores of the \textit{C. perfringens} FP strain SM101 that contains only one Csp protease. In addition, the numbers of pro-SleC and CspB molecules in spores have been determined.

2. Materials and methods

2.1. Bacterial strains and spore preparation and purification

The \textit{C. perfringens} strains used in this work were the FP strain SM101 (wild-type) (Collie and McClane, 1998; Zhao and Melville, 1998), and its derivatives DPS107 lacking sleC (Paredes-Sabja et al., 2009b), and DPS117 lacking cspB (Paredes-Sabja et al., 2009a). Spores of these strains were prepared and purified as previously described (Duncan and Strong, 1968; Kokai-Kun and McClane, 1994; Paredes-Sabja et al., 2008c), and stored at –20 °C until used. All spores used in this work were free (>98%) from growing or sporulating cells and germinated spores as assessed by phase contrast microscopy.

2.2. Preparation of various extracts from dormant spores

Coat extracts, lysesates from decoated spores and the spores’ inner membrane were prepared from dormant \textit{C. perfringens} spores essentially as described previously (Banawas et al., 2013; Korza and Setlow, 2013; Paidhungat and Setlow, 2001; Stewart and Setlow, 2013). Briefly, purified spores of \textit{C. perfringens} strains, ~3 ml at an optical density at 600 nm (OD$_{600}$) of 25 (~3.2 × 10$^7$ spores/ml), were first decoated by incubation at 37 °C for 1.5 h in 8 M urea – 1% SDS – 0.05 M dithiothreitol – 0.05 M Tris–HCl buffer (pH 8.0), centrifuged at 22 °C in a microcentrifuge for 5 min at 14,000 × g, the supernatant fluid saved as the coat extract, and the decoated spores washed 10 times with water as described (Bagyan et al., 1998). The washed decoated spores were suspended at an OD$_{600}$ of 50–70 in 0.5 ml TEP buffer (50 mM Tris–HCl buffer (pH 7.4) – 5 mM EDTA – 1 mM phenylmethylsulfonylfluoride plus 1 mg of lysozyme, 1 μg of RNase, 1 μg DNase I, and 20 μg of MgCl$_2$. This mixture was incubated for 5 min at 37 °C, and then kept on ice for 20 min. Glass beads (100 mg) were added to each sample and spores were gavien four bursts of sonication (15 s pulses with 30 s pauses on ice in between pulses) to complete spore disruption as assessed by microscopic analysis, and to reduce the viscosity of the extract. Following the final sonication pulse, samples were allowed to settle for 15 s and 100 μL of fluid was withdrawn and saved as the total decoated spore lysate. The rest of the decoated spore lysate that contained the glass beads was used for preparation of the spore inner membrane fraction. The various spore fractions were stored at –20 °C until use.

2.3. CspB and SleC expression and purification and preparation of antisera

For CspB and SleC expression the \textit{cspB} and \textit{slec} genes were PCR amplified from \textit{C. perfringens} strain SM101 DNA and cloned into a modified PET15b expression vector fusing a His$_6$-tag plus a tobacco etch virus (TEV) protease cleavage site to the N-termini of CspB and SleC protein. In these constructs, the N-terminus of the full length cspB coding sequence was fused to the His-tag giving the CspB zymogen pro-CspB, and the \textit{slec} sequence encoding SleC residues 115-413, equivalent to pro-SleC, was fused to the His-tag. The protein fusions were overexpressed in \textit{Escherichia coli} BL21 cells and purified by Ni$^{2+}$-nitrioltriacetic acid affinity chromatography. Following TEV protease cleavage to remove the His$_6$-tag, the proteins were further purified by anion (CspB) or cation (SleC) exchange and gel filtration (SD200; GE Healthcare, Piscataway, NJ) chromatography. Purified protein (2 mg) was used to raise polyclonal antibodies in two New Zealand White female rabbits (Pacific Immunology Group, Ramona, CA). Anti-CspB and -SleC antibodies were detected by Western blotting in a blood sample collected 2 months after the initial injection, at which time the animals were exsanguinated. The specificity of the antiserum was confirmed by Western blotting using purified proteins and various spore extracts (see Results).

2.4. Western blot analysis

Western blot analysis was carried out following separation of proteins by SDS-polyacrylamide (12%) gel electrophoresis (SDS-PAGE) and transferring the proteins to polyvinylidene difluoride (PVDF) membranes as described previously (Stewart and Setlow, 2013). The CspB and SleC proteins were detected on the PVDF membranes essentially as described previously (Banawas et al., 2013).
by incubation with 1:1000 dilutions of the polyclonal rabbit antisera against CspB or SleC for 1 h at room temperature, followed by incubation with a 1:10,000 dilution of goat anti-rabbit IgG-horseradish peroxidase (HRP) conjugate (Milipore Corporation, Billerica, MA, USA) for 45 min at room temperature. The binding of the secondary antibody was detected by incubation with a chemiluminescent HRP substrate (Pierce SuperSignal; Thermo Scientific, Rockford, IL, USA) using X-ray film.

2.5. Determination of molecules of CspB and SleC per spore

The average number of CspB and SleC molecules per spore was determined by comparing band intensities given by wild-type spore coat extracts with intensities given by known amounts of purified CspB and SleC antigens on the same Western blots. The concentrations of the purified proteins were determined by their absorbance and molecular extinction coefficient at 280 nm. The intensities of all bands on Western blots were quantitated using ImageJ software (National Institutes of Health, Bethesda, MD USA) and the average numbers of CspB and SleC protein per spore were calculated (Korza and Setlow, 2013; Stewart and Setlow, 2013). Briefly, the amount of the purified protein equivalent to the amount of the corresponding protein in the spores' coat extract was divided by this protein's molecular weight giving the moles of the protein, and this value was multiplied by Avogadro’s number. The latter number was then divided by the number of spores in the amount of coat extract analyzed to give the number of molecules of the protein per spore. The simplified formula for determining the number of protein molecules per spore:

\[
\left(\frac{1}{\text{MW} \times \text{g}^{-1}}\right) \times \left(\frac{\text{antigen} \times \text{mol}^{-1}}{\text{g}^{-1}}\right) \times \left(6.023 \times 10^{23}\right)
\]

(3. Results

3.1. Localization of CspB and SleC in spores

Using Western blot analysis, CspB and SleC were readily detected in spores using polyclonal rabbit antisera raised against recombinant C. perfringens CspB and SleC (Fig. 1). However, these proteins were found only in the coat extract of wild-type dormant spores, as no immunoreactive CspB and SleC species were detected in lysates from decoated spores (<1% of the levels in coat extracts). In addition, Western blot analysis of purified spore inner membrane also detected no CspB or SleC antigen (data not shown). The sizes of the antigens detected in coat extracts, ~50 kDa with anti-CspB and ~34 kDa with anti-SleC, were in agreement with the expected molecular weights of CspB and pro-SleC. Furthermore, while these antigens were detected in coat extracts of dormant wild-type spores, the appropriate antigens were absent from spores of strains with deletions in either cspB or sleC (Fig. 1). These latter results indicate that the antisera used in these Western blots were highly specific.

The results of Western blot analysis of various spore fractions strongly indicated that CspB and SleC are located exclusively in coat extracts, consistent with these proteins being located outside the spore cortex. It was possible that the coat extraction regimen inactivates spores, and thus extracts proteins from more interior spore layers. However, this was not the case, as the decoated spores retained >80% of the viability of intact spores (as shown previously), although required addition of lysozyme to plating media in order to get efficient colony formation (Paredes-Sabja et al., 2009a, 2009b).

3.2. Levels of CspB and SleC in spores

Work with B. subtilis has indicated that levels of spore proteins involved in initial steps of germination are significantly lower than levels of proteins involved in late germination steps (Bagyan and Setlow, 2002; Stewart and Setlow, 2013). To establish if this is also the case with C. perfringens SM101 spores, the average numbers of CspB and SleC molecules per spore were determined by comparing intensities given by coat extracts from several amounts of spores to the intensities from known amounts of purified CspB and SleC antigen on the same Western blot (Fig. 2). These comparisons allowed calculation that there are ~2000 molecules of CspB per spore and ~130,000 molecules of SleC per spore (Fig. 2), indicating that CspB levels are ~65-fold lower than its substrate pro-SleC.
4. Discussion

With *B. subtilis* spores, the signaling process in germination is likely amplified by increasing the levels of the proteins involved in sequential germination steps. Cortext peptidoglycan hydrolysis is the culmination of the germination process and in *B. subtilis* spores the level of the CLE CwIj (the SleB level is not known) is ~8 × 10³ molecules per spore (Bagyan and Setlow, 2002) – ~8-fold higher than the level of all germinant receptors (GRs) that sense nutrient germinants (Stewart and Setlow, 2013). This increase in levels of germination proteins further along in the germination process was also seen with *C. perfringens* spores, as numbers of CspB and SleC molecules in spores were both much higher than the 250 molecules per spore of the GR protein GerKc found previously (Banawas et al., 2013). In addition, in *C. perfringens* spores, levels of CspB were ~65-fold lower than its substrate pro-SleC, consistent with signal amplification from CspB to pro-SleC, with CspB catalytic activity generating the active SleC that is responsible for the major final event in spore germination.

The major conclusion from the work in this communication concerns the localization of the Csp protease, CspB, as well as the single CLE, SleC, in *C. perfringens* spores. In contrast to the situation in *B. subtilis* spores where most SleB is associated with the spore’s inner membrane (Chirakkal et al., 2002; Korza and Setlow, 2013; Stewart and Setlow, 2013), neither pro-SleC nor CspB were associated with the inner membrane of *C. perfringens* spores. This latter finding plus the extraction of essentially all SleC and CspB antigen from dormant *C. perfringens* spores by regimens designed to extract coat and outer membrane proteins strongly suggest that pro-SleC and CspB are on the outer surface of the spore cortex. This further suggests that these two proteins may physically interact in this location in the dormant spore, facilitating CspB action on pro-SleC when CspB enzyme activity is stimulated. However, at present, the mechanism whereby Csp protease is activated during germination of *C. perfringens* spores is not known.

The absence of CspB and SleC from *C. perfringens* spores’ inner membrane which is close to the inner edge of the spore cortex, and the likely concentration of CspB and SleC at the cortex’s outer edge also suggests that in germination of *C. perfringens* spores, cortex hydrolysis may start from the outer edge of the cortex and proceed inward. In contrast, in spores of *B. subtilis*, where CLEs are located at both the outer and inner edges of the cortex (Bagyan et al., 1998; Chirakkal et al., 2002), the hydrolysis of the cortex during spore germination could proceed from both the inner and the outer edges simultaneously. Indeed, loss of CwlJ from spores of *Bacillus* species greatly slows rates of Ca-DPA release during germination of individual spores while loss of SleB has no effect (Peng et al., 2009; Setlow et al., 2009), suggesting that there is something different in the cortex hydrolysis catalyzed by these two CLEs perhaps due to where the cortex hydrolysis is taking place. It would be interesting to determine exactly where cortex hydrolysis takes place during germination of spores of *C. perfringens* and *B. subtilis*, and of both cwlJ and sleB *B. subtilis* spores.

Finally, the extraction of all pro-SleC and CspB from *C. perfringens* SM101 spores by a decoating regimen explains why such treatments greatly decrease the apparent viability of *C. perfringens* spores, as these spores simply cannot carry out cortex degradation and complete spore germination. However, as shown in several studies, these decoated spores are not really dead, but can readily be revived by inclusion of low concentrations of a lytic enzyme such as lysozyme in plating media (Paredes-Sabja et al., 2009a, 2009b). The results in this manuscript on CspB and pro-SleC location in spores thus provide new information indicating that: 1) these essential germination proteins are in spores’ outer layers, which may be inactivated by chemical or physical treatments rather readily, probably more readily than irreversible spore inactivation; 2) as a consequence, *C. perfringens* spores may appear inactivated only because they cannot complete spore germination due to CspB or SleC inactivation; but 3) these apparently inactivated spores can be rescued in foods that retain lytic enzymes that could substitute for SleC and germinate the apparently dead *C. perfringens* spores, which then grow and produce toxins in foods.

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References


