Functional analysis of SleC from *Clostridium difficile*: an essential lytic transglycosylase involved in spore germination

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INTRODUCTION

*Clostridium difficile* is the most common cause of enteric disease and presents a major burden on healthcare systems globally due to the observed rapid rise in antibiotic resistance. The ability of *C. difficile* to form endospores is a key feature in the organism’s pathogenesis and transmission, and contributes greatly to its resilient nature. Endospores are highly resistant to disinfection, allowing them to persist on hospital surfaces. In order for the organism to cause disease, the spores must germinate and revert to a vegetative form. While spore germination in *Bacillus* spp. is well understood, very little is known about this process in *Clostridia*. Here we report the characterization of SleC (CD0551) from *C. difficile* 630. Bioinformatic analysis of SleC indicated a multi-domain protein possessing a peptidoglycan-binding (PGB) domain, a SpoIIID/LytB domain and an undefined N-terminal region. We have confirmed that SleC is an exo-acting lytic transglycosylase with the catalytic activity localized to the N-terminal region. Additionally, we have shown that both the N-terminal catalytic domain and the C-terminal PGB domain require muramyl-δ-lactam for substrate binding. As with carbohydrate-binding modules from cellulases and xylanases, the PGB domain may be responsible for increasing the processivity of SleC by concentrating the enzyme at the surface of the substrate.

Abbreviations: CBM, carbohydrate-binding module; CDAD, *Clostridium difficile*-associated disease; GlcNAcase, N-acetylglucosaminidase; LT, lytic transglycosylase; PG, peptidoglycan; PGB, peptidoglycan-binding; RP-HPLC, reversed phase HPLC; SCLE, spore cortex lytic enzyme.

A supplementary table and a supplementary figure are available with the online version of this paper.
of a normal vegetative cell, allowing the core to expand and ultimately facilitate enzyme activity, metabolism and spore outgrowth (Setlow, 2003).

Our understanding of spore cortex lytic enzymes (SCLEs) from Clostridium spp. is limited, but homologues of some of these enzymes have been studied in Bacillus spp. In Bacillus, it has been established that during spore germination, the cortex PG is degraded by members of three different classes of enzymes: N-acetylglucosaminidase (GlcNAcase), lytic transglycosylase (LT) and amidase (Fig. 1). CwlJ and SleB are two SCLEs involved in Bacillus cortex hydrolysis. These SCLEs break down PG containing muramic-δ-lactam (Popham et al., 1996). SleB is a LT, while CwlJ is responsible for calcium dipicolinate-induced germination in Bacillus subtilis. Inactivation of either of these enzymes results in germination-incompetent spores (Ishikawa et al., 1998). Studies on spore germination in Clostridium perfringens have identified two SCLEs, SleC and SleM, of which SleC is required during germination (Paredes-Sabja et al., 2009; Paredes-Sabja & Sarker, 2011) for complete cortex hydrolysis. In C. perfringens, SleC has been shown to be a bifunctional enzyme possessing both LT and amidase activity (Kumazawa et al., 2007). Additionally, the germination-specific serine protease CspB is essential for cortex hydrolysis and converts inactive pre-pro-SleC to an active enzyme (Shimamoto et al., 2001). Bioinformatic analysis of the C. difficile genome revealed the presence of homologues of cwlJ and sleB from B. subtilis (Burns et al., 2010a, b) and sleC from C. perfringens (CD0551) (Paredes-Sabja et al., 2009). Work by Burns et al. (2010a) has demonstrated that SleC is essential for germination of C. difficile under nutrient-rich conditions.

The process of spore germination in C. difficile is beginning to be unravelled with recent reports of genetic analysis of sleC (Burns et al., 2010a), and structural and functional studies on the spore-specific protease CspB (Adams et al., 2013). In order to better understand the germination process in C. difficile and in Clostridia in general we have undertaken a biochemical study of SleC (CD0551). Based on protein length and sequence similarity to its C. perfringens homologue, C. difficile SleC appears to be produced in a pre-pro form. However, it is not known if in C. difficile the full-length pre-pro form requires proteolytic cleavage for activity. We have engineered both full-length and truncated forms of SleC, and investigated the role of various domains in substrate binding and PG degradation. Armed with a better understanding of SCLE function, these enzymes can serve as potential targets for inhibitors of spore germination and prevention of disease progression.

**METHODS**

Construction of plasmids for overexpression of SleC. The full-length sleC ORF encoding the pre-pro form of the protein (SleCFL; CD0551) and its truncations were amplified from C. difficile 630 genomic DNA with primers containing NdeI and XhoI restriction sites (Table S1, available in Microbiology Online). Three truncated forms were generated to examine the functions of the predicted domains: SleC1-292, SleC293-423 and SleC424-423 (Fig. 2). The PCR amplicons were digested and cloned into the NdeI–XhoI sites of the pET-28a(+) expression vector (Novagen), which carries an N-terminal His6-tag. The resulting plasmids were used to transform Escherichia coli DH5α and BL21(DE3), screened for correct insert size, and sequenced to confirm lack of PCR-induced errors.

Overexpression and purification of SleC derivatives. E. coli BL21(DE3) containing each plasmid was grown in Super Broth supplemented with kanamycin (30 μg ml⁻¹) at 37 °C with shaking. At early exponential phase (OD600 ~0.6) the cultures were cooled to room temperature and IPTG was added to a final concentration of 0.1 mM. Expression was induced overnight at 20 °C with shaking and cells were harvested by centrifugation (5000 g, 10 min, 4 °C). The cell pellets were resuspended in 20 ml ice-cold lysis buffer [50 mM Na₂HPO₄ pH 8.0, 400 mM NaCl, 0.1% (v/v) Triton X-100, 10% (v/v) glycerol] containing DNase I, RNase A and lysozyme. Cells were ruptured by sonication (5 min total processing time, 60 % amplitude, 10 s intervals) and cell debris removed by centrifugation (15900 g, 10 min, 4 °C). Soluble protein was purified from the cell lysate.

![Fig. 1. Structure of spore PG with the C. difficile stem peptide shown. (a) Schematic representation of spore PG. (b) Chemical structure of spore PG with the cleavage sites of the major SCLEs GlcNAcase, LT and amidase indicated by arrows.](image-url)
Functional analysis of SleC from C. difficile

**RESULTS**

**Bioinformatic analysis, overexpression and purification of SleC**

CD0551 encodes the predicted pre-pro form of SleC (SleC\textsubscript{FL}), a 423 amino acid protein with no detectable supernatant by immobilized metal affinity chromatography using a Bio-Rad Econo gradient pump employing a pH gradient from pH 8.0 to 4.5. Fractions testing positive for protein by the Bradford assay were analysed by SDS-PAGE stained with Coomassie blue to verify the presence of the desired protein and purity of the fractions. Fractions containing the protein of interest were pooled, concentrated and buffer exchanged into 30 mM NaOAc pH 4.0 using an Amicon Ultra-15 centrifugal filter device (Millipore).

**Preparation of PG substrates.** Vegetative cell PG substrate was prepared from cultures of *B. subtilis* as described elsewhere (Kopp *et al.*, 1996). Briefly, harvested cells were resuspended in 1× PBS and sonicated for 5 min (60 % amplitude, 10 s intervals). The cell suspension was boiled in 8 % (w/v) SDS, the insoluble PG was collected by centrifugation and washed extensively with distilled water until free of SDS. The resulting pellet was rotated in 1× PBS containing 8U DNase I, 40U RNase A and 20 mM MgSO\textsubscript{4} at room temperature for 2 h. Covalently attached proteins were removed by treatment with proteinase K at room temperature overnight. The PG suspension was boiled in an equal volume of 8 % SDS for 1 h under reflux and the SDS was removed by repeated washes with distilled water. The recovered PG was resuspended in hydrofluoric acid (HF) (48 %) at 4 °C overnight to remove wall teichoic acids. Insoluble PG was washed five times with distilled water, lyophilized and stored at −20 °C.

*B. subtilis* spores were prepared, harvested and purified as described elsewhere (Popham *et al.*, 1996). Briefly, *B. subtilis* was cultured in Wilson’s Broth at 30 °C for at least 48 h and sporulation was confirmed by microscopy. Spores were harvested by centrifugation and resuspended in 50 mM Tris/HCl pH 7.5, 1 % SDS and 50 mM DTT. After boiling for 20 min, SDS was removed by warm water washes and the pellet containing cortex PG was stored in water at 4 °C overnight. Nucleic acids were removed by incubating in 100 mM Tris/HCl pH 7.5, 20 mM MgSO\textsubscript{4} and 100 U DNase I, and RNase A each at 37 °C for 2 h, followed by an overnight treatment with proteinase K. Digestion was stopped by boiling in 1 % SDS for 20 min. The resulting spore sacculi were washed with warm water to remove all detectable SDS and stored at 4 °C. Spore cortex fragments were prepared by sonication (30 % amplitude, 10 s intervals, total processing 2 min).

**Enzyme activity.** SleC activity was measured by monitoring the percentage OD\textsubscript{600} loss min\textsuperscript{-1} of a reaction mixture containing either spore cortex (0.2 OD\textsubscript{600} unit ml\textsuperscript{-1}) (Heffron *et al.*, 2011) or vegetative (1.33 µg ml\textsuperscript{-1}) *B. subtilis* PG. Reactions (200 µl total volume) contained between 0.05 to 0.158 nmol SleC\textsubscript{FL}, 0.76 to 2.28 nmol Sle\textsubscript{C1–292}, 1.72 to 5.71 nmol Sle\textsubscript{C293–423} or 2.5 to 7.5 nmol Sle\textsubscript{C342–423}. Enzyme activity was measured over a pH range of 3.5 to 7.0 and a temperature range of 23 to 50 °C. Reactions were monitored in 96-well plates using a Molecular Devices SpectraMax 190 plate reader in kinetic mode. Reaction products were retained, separated by reversed phase HPLC (RP-HPLC) and analysed by MALDI-MS (see below).

**PG-binding assay.** The peptidoglycan-binding (PGB) affinity of SleC full-length and truncated forms was analysed by mixing 5 µg purified protein with 300 µg *B. subtilis* vegetative PG or 0.3 OD\textsubscript{600} unit spore cortex fragments in a total volume of 50 µl containing 5 % (v/v) glycerol and 30 mM NaOAc pH 4.0, as described elsewhere (Heffron *et al.*, 2011; Reid *et al.*, 2006). Control reactions contained protein in buffer without PG (no substrate), and each PG substrate alone in buffer (no enzyme). BSA was used as a negative control for binding affinity and to account for non-specific binding. The suspensions were incubated on ice for 1 h to inhibit PG hydrolysis activity. Insoluble substrate was collected by centrifugation and the supernatant containing unbound protein was retained. Pellets were washed twice with 50 µl buffer solution (above) to remove residual unbound or weakly bound material. Pellets and lyophilized supernatants were resuspended in Laemmli sample buffer (50 µl) (Bio-Rad) containing DTT (5 mM), boiled, briefly centrifuged (16873 g, 10 min) and separated by 12 % (SleC\textsubscript{FL},SleC\textsubscript{C1–292}) or 15 % (SleC\textsubscript{293–423},SleC\textsubscript{C342–423}) SDS-PAGE. Proteins were visualized by silver staining of the acrylamide gels and Western blotting using anti-SleC antibody (A. Shen, University of Vermont, Burlington, VT, USA).

**Separation and analysis of muropeptides.** Soluble muropeptides generated by SleC and associated constructs were separated by RP-HPLC following the method described by Courtin and coworkers (Courtin *et al.*, 2006). Briefly, soluble muropeptides were reduced with NaBH\textsubscript{4} for 5 min followed by adjustment of pH to 2 using H\textsubscript{3}PO\textsubscript{4} (Dowd *et al.*, 2008). Reduced muropeptides were separated on a Phenomenex kinetix C-18 column (50 × 4.6 mm) at 50 °C on a Shimadzu Prominence uHPLC system with detection at 205 nm. Muropeptides were eluted for 5 min with 10 mM NH\textsubscript{4}H\textsubscript{2}PO\textsubscript{4} pH 5.6, followed by a 120 min linear gradient of methanol (0–20 %). Separated muropeptides were analysed by MALDI-MS using either 2,5-dihydroxybenzoic acid or α-cyano-4-hydroxycinnamic acid.

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**Fig. 2.** Schematic of CD0551 (SleC) domains and constructs generated in this study. Two domains were identified using Pfam and NCBI BLAST analysis of the CD0551 protein sequence.
signal sequence (SignalP) and two predicted domains (NCBI BLAST, Pfam) (Fig. 2). The effective database for prediction of secreted bacterial proteins (Jehl et al., 2011) indicates that SleC is most likely a type III secreted protein (score 0.9994) and possesses a C-terminal PGB domain-1 (Pfam PF01471, CaZy CBM 50). The C-terminal PGB domain is a member of the carbohydrate active enzymes (CaZy) database carbohydrate-binding module (CBM) family 50. This is a module of approximately 50 amino acids found attached to various enzymes that cleave either chitin or PG. This domain is found in amidase enhancer proteins and predicted acids found attached to various enzymes that cleave either chitin or PG. This domain is found in amidase enhancer proteins and predicted to be involved in membrane migration during sporulation. The N-terminal region, comprising amino acids 1–292, does not possess any conserved domains based on NCBI BLAST results. SleC_{FL} shares 67% sequence similarity to its counterpart in C. perfringens, with the majority of the sequence differences residing in the N-terminal region (aa 1–150). Based on the above bioinformatic analysis, primers were designed (Table S1) to amplify the full-length protein (SleC_{FL}), the N-terminal domain (SleC_{1–292}), the SpoIID/LytB domain and PGB domains (SleC_{293–423}), or the PGB domain (SleC_{342–423}). All constructs were cloned into pET28a(+) on an Ndel–Xhol fragment to produce N-terminal His_{6}-tagged constructs, which were expressed in E. coli BL21(DE3) at 18 °C to maximize protein solubility. All constructs were purified by immobilized metal affinity chromatography using a pH gradient (pH 8 to 4.5). Protein purity was determined by SDS-PAGE with Coomassie blue staining and in all cases, the His_{6}-tagged protein was purified to 85% or greater as determined by densitometry (Fig. 3).

SleC\textsubscript{FL} and SleC\textsubscript{1–292} degrade spore cortex PG

In vitro conditions were established using SleC\textsubscript{FL} acting on spore cortex fragments using a turbidometric assay. SleC\textsubscript{FL} activity was initially compared on spore cortex fragments from either B. subtilis or C. difficile with no appreciable difference noted between the substrates. In order to avoid growing large cultures of a biosafety level 2 pathogen for spore isolation, all subsequent data were collected using spore cortex isolated from non-pathogenic B. subtilis. No activity was detected for SleC\textsubscript{FL} in turbidity assays when incubated with vegetative PG from B. subtilis (data not shown). Optimal in vitro conditions were found to be 30 mM sodium acetate pH 4.5 at 45 °C (data not shown). Spore cortex degradation was monitored for all four constructs using the conditions set for SleC\textsubscript{FL}. Both SleC\textsubscript{FL} and SleC\textsubscript{1–292} demonstrated activity on spore cortex fragments as evidenced by decreased turbidity at 600 nm, confirming that PG-degrading activity resides in the N-terminal region of the protein (Fig. 4). Both C-terminal constructs (SleC\textsubscript{293–423} and SleC\textsubscript{342–423}) demonstrated no detectable activity on spore cortex fragments, consistent with the predicted role of this domain in PGB (data not shown). Specific activity was calculated for SleC\textsubscript{FL} and SleC\textsubscript{1–292} as 11.01 and 2.61 % change in OD\textsubscript{600} min\textsuperscript{-1} mmol\textsuperscript{-1}, respectively. The removal of the PGB domain (SleC\textsubscript{1–292}) resulted in a 76% decrease in specific activity, indicating a decrease in processivity of the SleC catalytic domain.

SleC is a LT

Reaction supernatants and pellets from SleC\textsubscript{FL} and SleC\textsubscript{1–292} from turbidity assays were retained and analysed.
by RP-HPLC (Fig. 5). Reaction supernatants either were run directly, or were subjected to a second digestion by lysozyme. As lysozyme cleaves the same glycosidic linkage as LTs, a secondary digest by lysozyme could liberate unique SleC fragments that were too large to separate by RP-HPLC. Analysis of chromatograms of SleC FL and SleC₁₋₂₉₂ reaction supernatants revealed several unique peaks that were not found in the control and lysozyme-digested spore cortex samples. Unique peaks were collected and analysed by MALDI-MS, which identified several muropeptides that are diagnostic of LT enzymes (Table 1). In both SleC FL (peaks 1–3) and SleC₁₋₂₉₂ (peaks 4 and 5), 1,6-anhydromuramoyl-containing disaccharides were identified. Unlike SleC from *C. perfringens*, a proposed bifunctional SCLE, no reaction products characteristic of an amidase were identified (e.g. disaccharide-tetrapeptide-tetrapeptide) (Kumazawa *et al.*, 2007). Subsequent lysozyme digestion of SleC reaction supernatants and pellets did not reveal any unique SleC/lysozyme muropeptides compared to control reactions (data not shown). This suggests that SleC is a mono-functional processive LT producing small anhydromuramyl containing disaccharide products (*exo*-acting), in contrast to its *C. perfringens* homologue.

![Figure 5](http://mic.sgmjournals.org)  
**Fig. 5.** RP-HPLC separation of muropeptides released from spore PG digested with either SleC FL or SleC₁₋₂₉₂. Spore PG was incubated with no enzyme (a), 1.6 nmol SleC FL (b) or 2.3 nmol SleC₁₋₂₉₂ (c). Reaction progress was monitored by turbidometric analysis as described in Methods. Soluble muropeptides were reduced with sodium borohydride prior to separation by RP-HPLC. Numbered peaks were collected and analysed by MALDI-MS.
The PGB domain confers specificity to spore cortex PG

All four SleC constructs were examined for their ability to bind to spore cortex and vegetative PG using a PG pull-down assay as described elsewhere (Heffron et al., 2011; Reid et al., 2006; Ursinus et al., 2004). BSA was used as a control for non-specific binding to both spore and vegetative PG (Fig. 6, top panel). BSA showed significant non-specific binding to both the microcentrifuge tube and PG substrates. Binding studies using SleC FL and truncations with spore PG demonstrated strong substrate binding (Fig. 6) with minimal recognition of vegetative PG. The lack of binding to vegetative PG supports observations from the turbidometric assays indicating inactivity of SleC on this substrate. This suggests that both the N-terminal catalytic domain and the PGB domain have a requirement for muramyl δ-lactam, a feature specific to spore PG.

DISCUSSION

In this study, the C. difficile 630 SleC (CD0551) protein was overexpressed in E. coli BL21(DE3) and purified in four forms: as the full-length protein and as three truncated forms harbouring different combinations of the three identified domains (Fig. 2). In vitro analyses of all protein constructs confirmed that SleC binds specifically to cortex PG and functions as a SCLE with LT activity. In vitro analysis of SleC FL indicated that unlike its homologue from

Table 1. Identified muropeptides from B. subtilis spore cortex PG digested with either SleC FL or SleC1–292 and separated by RP-HPLC

<table>
<thead>
<tr>
<th>Sample</th>
<th>Ion</th>
<th>m/z observed (calc.)</th>
<th>Δm/z</th>
<th>Error (%)</th>
<th>Muropeptide composition (GlcNAc)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SleC FL</td>
<td>[M + H]^+</td>
<td>904.76 (904.56)</td>
<td>-0.2</td>
<td>0.02</td>
<td>GlcNAcAnhMurNAc-tetrapeptide</td>
</tr>
<tr>
<td></td>
<td>[M + Na]^+</td>
<td>860.56 (855.53)</td>
<td>5.0</td>
<td>0.59</td>
<td>GlcNAcAnhMurNAc-tripeptide</td>
</tr>
<tr>
<td></td>
<td>[M + Na]^+</td>
<td>772.97 (771.51)</td>
<td>1.5</td>
<td>0.19</td>
<td>δ-Mur(red)GlcNAcAnhMurNAc-Ala</td>
</tr>
<tr>
<td></td>
<td>[M + H]^+</td>
<td>659.36 (661.33)</td>
<td>1.0</td>
<td>0.14</td>
<td>GlcNAcAnhMurNAc-dipeptide</td>
</tr>
<tr>
<td>SleC1–292</td>
<td>[M + H]^+</td>
<td>949.54 (952.49)</td>
<td>3.0</td>
<td>0.31</td>
<td>GlcNAc,δMur1AnhMurNAc,1-Ala</td>
</tr>
<tr>
<td></td>
<td>[M + Na]^+</td>
<td>859.99 (855.53)</td>
<td>4.5</td>
<td>0.52</td>
<td>GlcNAcAnhMurNAc-tripeptide</td>
</tr>
<tr>
<td></td>
<td>[M + H]^+</td>
<td>660.91 (661.33)</td>
<td>0.42</td>
<td>0.063</td>
<td>GlcNAcAnhMurNAc-dipeptide</td>
</tr>
<tr>
<td></td>
<td>[M + H]^+</td>
<td>905.56 (904.56)</td>
<td>1</td>
<td>0.11</td>
<td>GlcNAc-AnhMurNAc-tetrapeptide</td>
</tr>
</tbody>
</table>

The PGB affinity of SleC and its truncations. Full-length SleC and its truncations were incubated with B. subtilis spore cortex (left) or vegetative cell PG (right). Supernatants (unbound protein, U) and pellets (bound protein, B) were separated by centrifugation and analysed by SDS-PAGE followed by silver staining for protein detection. Substrate alone (PG control) was used to control for proteins extracted from the PG by the sample buffer. Purified protein without substrate (protein control) was used as a negative control for binding. In order to account for non-specific binding in the assay, BSA was employed as a control. M, Protein marker; W, supernatant from the first pellet wash.

**Fig. 6.** PGB affinity of SleC and its truncations. Full-length SleC and its truncations were incubated with B. subtilis spore cortex (left) or vegetative cell PG (right). Supernatants (unbound protein, U) and pellets (bound protein, B) were separated by centrifugation and analysed by SDS-PAGE followed by silver staining for protein detection. Substrate alone (PG control) was used to control for proteins extracted from the PG by the sample buffer. Purified protein without substrate (protein control) was used as a negative control for binding. In order to account for non-specific binding in the assay, BSA was employed as a control. M, Protein marker; W, supernatant from the first pellet wash.
C. perfringens (Kumazawa et al., 2007), SleC is not produced in an inactive pre-pro form that requires proteolytic activation by a germination-specific protease. Characterization of SleC activity for all four constructs prepared herein has localized LT activity to the N-terminal region of the protein (Fig. 4). We demonstrate for what is believed to be the first time that full-length SleC is active on spore cortex PG. Previous studies on homologues of SleC did not demonstrate cortex lytic activity in the full-length ‘precursor’ form of the protein (Kumazawa et al., 2007). The muropeptide profiles of both SleCFL and SleC1–292 were analysed by HPLC and MALDI-MS (Fig. 5, Table 1). Results indicated that SleC produced small disaccharide products consistent with an exo-acting LT. Additionally, C. difficile SleC did not display bifunctional LT/amidase activity like its counterpart in C. perfringens. Double digestion of SleC-treated spore cortex supernatants and pellets did not reveal novel muropeptides characteristic of an endo-acting enzyme, such as hexasaccharide and octasaccharide products. Activity analysis of both SleCFL and SleC1–292 demonstrated a 76% drop in specific activity for SleC1–292 compared to SleCFL, which is suggestive of a requirement for the PGB domain for strong substrate binding and enzyme processivity. The decrease in activity is likely not due to misfolding of the protein as all truncations studied retained their ability to bind spore PG to a degree comparable to the full-length version. These results are consistent with what has been observed in other enzymes with CBMs. CBMs have three general roles in relation to their cognate catalytic modules; these are: (i) proximity effects, (ii) targeting functions and (iii) disruptive functions (Boraston et al., 2004). Based on our results the PGB domain of SleC appears to serve a proximity role. Through the sugar-binding activity of the PGB domain, SleC is concentrated on the PG substrate. By maintaining SleC in proximity to the PG, and thereby increasing the concentration of the enzyme on the substrate surface, more rapid degradation of the cortex PG occurs (Bolam et al., 1998). Our results with SleC correlate with other examples in which removal of the CBM domain results in significant decrease in the activity of the enzyme on insoluble polysaccharides (Ali et al., 2001; Bolam et al., 1998; Bolam et al., 2003; Tomme et al., 1988). Recent work by Adams and coworkers reports the characterization of CspB, a spore-specific protease required for C. difficile germination (Adams et al., 2013). In the study, it was shown that CspB is essential for SleC cleavage to produce active SleC enzyme. While we have shown that SleCFL is active in vitro, CspB may be required to control activity in vivo. Cleavage of SleCFL (47 kDa) by CspB produces a truncated protein that migrates in SDS-PAGE with a molecular mass of ~28 kDa although the site of cleavage is unknown at this time (Adams et al., 2013). The current study provides evidence that SleC LT activity resides within the N-terminal region of the molecule (aa 1–292).

While the mechanism of action of LTs is not fully understood, cleavage of the scissile glycosidic linkage involves a catalytic glutamate. To date, the only SCLE for which the catalytic acid has been identified is SleB from B. anthracis (Jing et al., 2012). Sequence alignment of SleC1–292 with the catalytic domain of SleB identified a potential candidate for the catalytic acid, E176 (Fig. S1). In the context of our results, it would suggest that CspB cleavage of SleCFL may produce an active SleC possessing the putative catalytic acid E176 from the N-terminal domain. Processing of SleC by CspB could be required to augment protein–protein interactions between SleC and other members of the germination machinery, much like the N-terminal region of PBP1b in E. coli (Chalut et al., 2001). Finally, our binding data demonstrate that both the catalytic domain (SleC1–292) and PGB domain (SleC293–423) require muramyl δ-lactam for substrate binding. This correlates with in vivo studies demonstrating that muramyl δ-lactam is the structural determinant for cortex recognition by SCLEs (Popham et al., 1996).

Future studies examining SleC will focus on mapping amino acid residues involved in catalysis, and defining how the PGB domain recognizes and binds the cortex PG. The characterization of the PGB domain could lead to improved understanding of PG–protein interactions. Knowledge of how the PGB domain binds and recognizes cortex PG can be extrapolated to other PG hydrolases and lyases, and could ultimately help in the design of inhibitors that interfere with spore PG degradation. This knowledge could also be exploited in biotechnology applications such as the use of PGB domains for the detection and removal of foodborne pathogens (Callewaert et al., 2011).

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