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

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Clostridium difficile is the most common cause of enteric disease and presents a major burden on healthcare systems globally due in part 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-domained protein possessing a peptidoglycan-binding (PGB) domain, a SpoIID/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.

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INTRODUCTION

Clostridium difficile is a Gram-positive, spore-forming obligate anaerobe that is the cause of enteric disease in many animal species including humans. It is a leading cause of nosocomial diarrhoea and pseudomembranous colitis worldwide (Carroll & Bartlett, 2011; Kelly & LaMont, 2008; Rupnik *et al.*, 2009) with symptoms of *C. difficile*-associated disease (CDAD) ranging from mild diarrhoea to pseudomembranous colitis and potentially death. The emergence of hypervirulent NAP1/027 strains in hospitals globally has resulted in a sharp increase in mortality rates (Tillotson & Tillotson, 2011). Traditionally, *C. difficile* epidemics were restricted to single institutions or wards. However, recent reports suggest a wider distribution of outbreaks that are accompanied by increased severity of disease and number of fatalities (Kee, 2012; Pépin *et al.*, 2005; Walk *et al.*, 2013). The high cost and problematic treatment of CDAD is predominantly due to

its ability to form endospores (Ghantaji *et al.*, 2010; Leffler & Lamont, 2009). While prevention through antibiotic stewardship and optimal management of the disease is the strategy currently used, there is a dire need for alternate methods of treatment.

Endospores are metabolically dormant and resistant to physical insult (Lawley *et al.*, 2010; Leggett *et al.*, 2012; Rupnik *et al.*, 2009). This property allows *C. difficile* to resist antibiotic treatment and persist in healthcare settings. Because of this, spores are the primary vectors for transmission (Deakin *et al.*, 2012) and the cause of recurrent infections. These recurrent CDAD infections occur in 25% of all cases and can lead to severe CDAD (Leffler & Lamont, 2009; Maroo & Lamont, 2006). In order to initiate infection in the host, ingested *C. difficile* spores must germinate into vegetative cells in the intestinal tract (Carroll & Bartlett, 2011). As with many spore-forming bacteria, *C. difficile* spores germinate in response to chemical signals (germinants) such as bile salts (Burns *et al.*, 2010a; Sarker & Paredes-Sabja, 2012). Upon activation, there is a release of monovalent cations including H⁺, K⁺ and Na⁺ as well as calcium dipicolinate. This is immediately followed by the hydrolysis of the spore peptidoglycan (PG) cortex. It is during hydrolysis that the spore's low water content is restored to the levels

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 (SCLs) 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 SCLs involved in *Bacillus* cortex hydrolysis. These SCLs 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 SCLs, 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 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 SCL 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 (SleC_{FL}; 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: SleC_{1–292}, SleC_{293–423} and SleC_{342–423} (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* DH5 α and BL21[λ DE3], screened for correct insert size, and sequenced to confirm lack of PCR-induced errors.

Overexpression and purification of SleC derivatives. *E. coli* BL21[λ DE3] containing each plasmid was grown in Super Broth supplemented with kanamycin (30 μ g 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 SCLs GlcNAcase, LT and amidase indicated by arrows.

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.

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 MgSO₄ 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 MgSO₄ 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 OD₆₀₀ loss min^{−1} of a reaction mixture containing either spore cortex (0.2 OD₆₀₀ unit ml^{−1}) (Heffron *et al.*, 2011) or vegetative (1.33 μg ml^{−1}) *B. subtilis* PG. Reactions (200 μl total volume) contained between 0.05 to 0.158 nmol SleC_{FL}, 0.76 to 2.28 nmol SleC₁₋₂₉₂, 1.72 to 5.71 nmol SleC₂₉₃₋₄₂₃ or 2.5 to 7.5 nmol SleC₃₄₂₋₄₂₃. 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 OD₆₀₀ 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; Ursinus *et al.*, 2004). 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_{FL}, SleC₁₋₂₉₂) or 15% (SleC₂₉₃₋₄₂₃, SleC₃₄₂₋₄₂₃) 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 mucopeptides. Soluble mucopeptides 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 mucopeptides were reduced with NaBH₄ for 5 min followed by adjustment of pH to 2 using H₃PO₄ (Dowd *et al.*, 2008). Reduced mucopeptides 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. Mucopeptides were eluted for 5 min with 10 mM NH₄H₂PO₄, pH 5.6, followed by a 120 min linear gradient of methanol (0–20%). Separated mucopeptides were analysed by MALDI-MS using either 2,5-dihydroxybenzoic acid or α-cyano-4-hydroxycinnamic acid.

RESULTS

Bioinformatic analysis, overexpression and purification of SleC

CD0551 encodes the predicted pre-pro form of SleC (SleC_{FL}), a 423 amino acid protein with no detectable

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 the spore-specific PG-acting enzymes SleB from *Bacillus anthracis*, SleC from *C. perfringens* and in a number of other PG-acting enzymes (Dideberg *et al.*, 1982; Foster, 1991; Hizukuri *et al.*, 2009). Using the NCBI BLASTP algorithm, a SpoIID/LytB domain was identified on the N-terminal side of the PGB domain, which 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 and PGB domains (SleC_{293–423}), or the PGB domain (SleC_{342–423}). All constructs were cloned into pET28a(+) on an *NdeI*–*XhoI* fragment to produce N-terminal His₆-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 His₆-tagged protein was purified to 85 % or greater as determined by densitometry (Fig. 3).

Fig. 3. SDS-PAGE analysis of purified SleC constructs stained with Coomassie blue. SleC_{FL} and SleC_{1–292} were separated in 12 % polyacrylamide gels, while 15 % polyacrylamide gels were used to resolve SleC_{293–423} and SleC_{342–423}. All constructs migrated to their predicted molecular masses.

SleC_{FL} and SleC_{1–292} degrade spore cortex PG

In vitro conditions were established using SleC_{FL} acting on spore cortex fragments using a turbidometric assay. SleC_{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_{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_{FL}. Both SleC_{FL} and SleC_{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_{293–423} and SleC_{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_{FL} and SleC_{1–292} as 11.01 and 2.61 % change in OD₆₀₀ min^{–1} nmol^{–1}, respectively. The removal of the PGB domain (SleC_{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_{FL} and SleC_{1–292} from turbidity assays were retained and analysed

Fig. 4. Action of SleC_{FL} and SleC_{1–292} on spore PG. Spore sacculi were prepared from *B. subtilis* spores. Spore sacculi (OD₆₀₀ 0.2) were incubated with either 1.6 nmol SleC_{FL} (○), 2.3 nmol SleC_{1–292} (Δ) or buffer control (□) at 45 °C in a final volume of 200 μl 50 mM sodium acetate buffer pH 4.5. The OD₆₀₀ was monitored every minute with mixing between measurements. The data presented are the mean of three independent protein preparations. Error bars represent the SD of the mean. SleC_{293–423} and SleC_{342–423} were excluded due to overlap with the buffer control.

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.

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.

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

Sample	Ion	<i>m/z</i> observed (calc.)	$\Delta m/z$	Error (%)	Muropeptide composition (GlcNAc)
SleC_{FL}					
1	[M + H] ⁺	904.76 (904.56)	−0.2	0.02	GlcNAcAnhMurNAc-tetrapeptide
	[M + Na] ⁺	860.56 (855.53)	5.0	0.59	GlcNAcAnhMurNAc-tripeptide
2	[M + Na] ⁺	772.97 (771.51)	1.5	0.19	δ-Mur(red)GlcNAcAnhMurNAc-Ala
3	[M + H] ⁺	659.36 (661.33)	1.0	0.14	GlcNAcAnhMurNAc-dipeptide
SleC_{1–292}					
4	[M + H] ⁺	949.54 (952.49)	3.0	0.31	GlcNAc ₂ δMur ₁ AnhMurNAc ₁ -Ala
	[M + Na] ⁺	859.99 (855.53)	4.5	0.52	GlcNAcAnhMurNAc-tripeptide
5	[M + H] ⁺	660.91 (661.33)	0.42	0.063	GlcNAcAnhMurNAc-dipeptide
	[M + H] ⁺	905.56 (904.56)	1	0.11	GlcNAc-AnhMurNAc-tetrapeptide

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

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 mucopeptide profiles of both SleC_{FL} and SleC_{1–292} 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 mucopeptides characteristic of an endo-acting enzyme, such as hexasaccharide and octasaccharide products. Activity analysis of both SleC_{FL} and SleC_{1–292} demonstrated a 76 % drop in specific activity for SleC_{1–292} compared to SleC_{FL}, 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; Boraston *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 SleC_{FL} is active *in vitro*, CspB may be required to control activity *in vivo*. Cleavage of SleC_{FL} (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 SleC_{1–292} 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 SleC_{FL} 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 (SleC_{1–292}) and PGB domain (SleC_{293–423}) 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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