Introduction

Inhalational, gastrointestinal and cutaneous anthrax result when a host becomes infected with *Bacillus anthracis* spores (Albrink, 1961; Mock & Fouet, 2001). Establishment of the bacterial infection requires spore germination. This morphological transition leads to the development of metabolically active vegetative cells that produce virulence factors and can initiate a fatal illness (Mock & Fouet, 2001; Setlow, 2003). Not only is the process of germination critical to infection progression but an understanding of the biological transition will allow for the development of more effective decontamination methods. Spores are highly resistant structures that can withstand extremes of pH, high temperatures, noxious chemicals, radiation, desiccation and starvation (Nicholson et al., 2000; Setlow, 2000). However, vegetative bacilli are relatively easy to eradicate. Therefore, by manipulating proteins involved in germination, the process can be constructively exploited.

Germination has been studied in an array of Gram-positive spore-forming species including *Clostridium perfringens* (Chen et al., 1997; Miyata et al., 1995), *Bacillus thuringiensis* (Hu et al., 2007), *Bacillus subtilis* (Ishikawa et al., 1998; Kodama et al., 1999; Moriyama et al., 1996a), *Bacillus megaterium* (Foster & Johnstone, 1987; Setlow et al., 2009), *Bacillus cereus* (Chen et al., 2000; Makino et al., 1994; Moriyama et al., 1996b) and *B. anthracis* (Heffron et al., 2009, 2010; Lambert & Popham, 2008; Liu et al., 2004). The *B. anthracis* germination cascade begins when nutrient germinants contact receptors within the spore. A cache of chelated Ca$^{2+}$-dipicolinic acid is released from the spore core as water influx partially rehydrates the spore. Shortly after, germination-specific lytic enzymes (GSLEs) are activated (Moir & Smith, 1990; Paidhungat et al., 2002; Popham et al., 1996a; Setlow et al., 2001). The first subset of these enzymes, termed spore cortex lytic enzymes (SCLEs), initiate the degradation of a modified layer of peptidoglycan (PG) known as the cortex, which is involved...
in the maintenance of spore core dehydration and resistance (Atrih et al., 1996; Chen et al., 2000; Gerhardt & Marquis, 1989; Makino & Moriyama, 2002; Popham et al., 1996a). B. anthracis contains three SCLEs: CwlJ1, CwlJ2 and SleB. The enzymatic activities of CwlJ1 and CwlJ2 have yet to be elucidated, but the proteins likely play the same role. SleB is a lytic transglycosylase that plays a dominant role in cortex hydrolysis (Heffron et al., 2009, 2010). A second subset of GSLEs termed cortical fragment lytic enzymes (CFLEs) advance the germination cascade (Makino & Moriyama, 2002). One such protein, SleL, further digests the cortex PG, and small muropeptide fragments are released from the spore (Heffron et al., 2010; Lambert & Popham, 2008). Cortex hydrolysis allows for complete core dehydration and loss of spore-associated resistance characteristics.

This study investigates the in vitro and in vivo activities of the CFLE SleL in an effort to further characterize the protein and its domains. SleL is an N-acetylglucosaminidase that breaks β-1,4 glycosidic bonds found in cortical PG (Chen et al., 2000; Lambert & Popham, 2008). Previous studies show that slel is expressed under the control of σB, which is active in the mother cell during early stages of sporulation (Chen et al., 2000; Kodama et al., 1999). SleL localizes to the outer layers of the developing forespore and is associated with spore coat proteins (Chen et al., 2000; Imamura et al., 2010; McKenney et al., 2010). On the basis of sequence homologies, SleL is composed of three conserved domains: two N-terminal LysM domains and a C-terminal glycosyl hydrolase family 18 domain (Terwisscha van Scheltinga et al., 1995; van Aalten et al., 2001). LysM domains are a common element in many PG-binding proteins (Buist et al., 2008). The LysM domains of several B. subtilis proteins have been shown to play a role in development by directing these proteins to the surface of the developing spore (Kodama et al., 2000). We hypothesize that the LysM domains of SleL may be involved in protein localization and/or substrate recognition (Buist et al., 2008).

By studying SleL derivatives containing both, one or no LysM domains in vitro and in vivo, we were able to determine that in vitro SleL does have N-acetylglucosaminidase activity, and the protein recognizes and digests partially fragmented spore PG containing the muramic-δ-lactam recognition determinant. LysM domains not only play a role in substrate recognition and binding but are also required in directing the SleL protein to the developing forespore.

**METHODS**

**Bacterial strains and plasmids.** B. anthracis and Escherichia coli strains and plasmid vectors used in this study are summarized in Table 1. B. anthracis strains were cultured in brain heart infusion (BHI; Difco) containing 5 μg erythromycin ml⁻¹ to select for resistance markers when necessary. Endospores were prepared by culturing strains in Modified G broth (Kim & Goepfert, 1974) containing erythromycin for selection when necessary. Sporulating cultures were incubated at 37 °C for 3 days, at which point spores were harvested and purified by water washing and centrifugation through 50% (w/v) sodium diatrizoate as described by Nicholson & Setlow (1990). E. coli strains were cultured at 37 °C in LB containing 50 μg ampicillin ml⁻¹ and 30 μg chloramphenicol ml⁻¹ (Jersey Lab Supply).

**Overexpression of His₆-MBP-SleL in E. coli.** Overexpression vectors encoding SleL and its truncated derivatives were constructed using the Invitrogen Gateway Cloning system and restriction-free cloning as described previously (Nallamsetty & Waugh, 2007; van den Ent & Löwe, 2006). Briefly, regions of slel including base pairs 4–1290, 151–1290 or 295–1290 were amplified by PCR using primers Slel-A + Slel-D, Slel-B + Slel-D or Slel-C + Slel-D, respectively (primer sequences available on request). The amplicons were introduced into the entry vector pDONR201 (Invitrogen) and then transferred to the destination vector pDest-HisMBP (Austin et al., 2009). The resulting plasmids encoded an N-terminal His₆-tagged maltose binding protein (MBP) fused to a tobacco etch virus (TEV) cleavage site and an SleL derivative. Because the full-length SleL fusion protein could not be successfully cleaved using TEV, restriction free cloning (van den Ent & Löwe, 2006) using primer Slel-A2 + Slel-D was used to integrate three additional glycine residues at the N terminus of SleL. Plasmid constructs pDPV405, pDPV381 and pDPV382 were verified by restriction enzyme digestion and sequencing. Overexpression vectors were transformed into DBPE13 ([BL21 (DE3) pLysS]). E. coli strains were cultured at 37 °C until OD₆₀₀ was 0.8, at which point isopropyl-β-D-thiogalactopyranoside was added to a final concentration of 0.7 mM. The culture temperature was reduced to 10 °C and incubation continued for 16 h.

**Purification of SleL derivatives.** Cells were harvested by centrifugation, resuspended in 50 mM Tris/HCl pH 7.5, 5% (v/v) glycerol, 25 mM imidazole, 50 mM NaCl (Slel-2–429) or 125 mM NaCl (Slel-429 and Slel-99–429) and lysed by two passes through a French press at 19,000 p.s.i. Soluble and insoluble protein fractions were separated by centrifugation at 117,000 g for 1 h. Soluble His₆-MBP-SleL protein fusions were purified using a nickel-charged HisTrap HP column (GE Healthcare) equilibrated with 50 mM Tris/HCl pH 7.5, 5% (v/v) glycerol, 25 mM imidazole and 50 mM NaCl (Slel-429) or 125 mM NaCl (Slel-429 and Slel-99–429). Proteins were eluted with 50 mM Tris/HCl pH 7.5, 50 mM NaCl, 5% (v/v) glycerol and 250 mM imidazole, and fractions were dialysed in 50 mM Tris/HCl pH 7.5, 50 mM NaCl, 5% (v/v) glycerol. Proteins at ~1.5 mg ml⁻¹ were incubated with 0.5 mg His-tagged TEV (S219V) protease ml⁻¹ (Kapust et al., 2001) and allowed to digest at 15 °C for 16 h. Cleavage was verified by SDS-PAGE analysis. Digested proteins were separated using a HiTrap SP-HP cation exchange column (GE Healthcare) equilibrated with 50 mM Tris/HCl pH 7.5, 5% (v/v) glycerol. Proteins were eluted with a linear gradient to 1 M NaCl in the same buffer. Fractions containing SleL proteins were concentrated to 200 μl using an Amicon Ultra Centrifugal filter with a 30 kDa molecular mass cut off (Millipore) and then dialysed in 25 mM Tris/HCl pH 7.5, 250 mM NaCl, 5% (v/v) glycerol before further purification using a Superdex 200 gel filtration column equilibrated with the same buffer. After elution, the concentration of each pure protein was determined by measuring A₃₄₀ and using the Beer–Lambert law: A = εlC, where ε is the molar absorption coefficient (M⁻¹ cm⁻¹), l is the path length (cm) and C is the protein concentration (M) (Page et al., 1995). Purified SleB was prepared as described previously (Heffron et al., 2011).

**Antibody preparation and Western blot analysis.** Polyclonal anti-SleL antibodies were raised by injecting rabbits (Open Biosystems) with purified SleL₉₉–429. SleL derivatives were identified after Western blotting using colorimetric detection. Primary anti-SleL and secondary
and pBKJ236SeqA purified sacculi were partially digested by combining sacculi at OD600 0.2 in 30 mM NaPO₄ pH 7.0, 1 mM EDTA, 1 mM DTT and 0.1 % (v/v) Triton with 0.01, 0.05 or 0.25 µg purified SleB ml⁻¹ or 0.1 µg lysozyme ml⁻¹ (Sigma). To prepare cortex PG fragments, sacculi at OD₆₀₀ 0.5 were resuspended in 500 µl 50 mM Tris/HCl pH 8.0 and physically broken with 250 mg 0.1 mm glass beads using a Wig-L-Bug bead beater. Sacculi were pulsed ten times at 3800 r.p.m. for 30 s and were incubated on ice for 1 min between pulses. Coat protein fragments were extracted from cortex fragments by mixing the suspension with chloroform and collecting the aqueous phase. Residual chloroform was removed by rinsing the pellets repeatedly with dH₂O.

**PG binding assay.** SleL binding affinity was analysed by combining wild-type or Δcwld B. subtilis spore cortex PG fragments with the various SleL proteins. Fragmented spore PG totalling ~0.3 OD units was centrifuged and resuspended in a final volume of 15 µl containing 30 mM NaPO₄ pH 7.0, 1 mM EDTA, 1 mM DTT, 0.1 % (v/v) Triton and 5 µg of each SleL protein. Protein suspensions were allowed to incubate on ice for 10 min. Supernatants and pellets were separated by centrifugation and pellets were resuspended in the buffer solution. Samples were combined with SDS-PAGE sample buffer, boiled, centrifuged briefly and separated using a 12 % (w/v) SDS-PAGE. The acrylamide gels were stained by using Sypro Ruby (Lonza) as per the manufacturer’s instructions. Stained proteins were detected using a Typhoon Trio Imager (GE Healthcare), and banding was quantified using ImageQuant TL software (GE Healthcare).

**Peptidoglycan substrate preparation.** A variety of PG substrates were prepared from B. subtilis PS832 (wild-type) and DPBV19 (Δcwld::Sp) spores. B. subtilis was cultured in 2 x SG medium (Leighton & Doi, 1971) for 3 days at which point spores were purified by water washing as described by Nicholson & Setlow (1990). Decoated spores were described as previously (Popham et al., 1996b) and then stored at 4 °C. PG sacculi were purified as described previously (Popham & Setlow, 1993). When indicated, purified sacculi were partially digested by combining sacculi at OD₆₀₀ 0.2 in 30 mM NaPO₄ pH 7.0, 1 mM EDTA, 1 mM DTT and 0.1 % (v/v) Triton with 0.01, 0.05 or 0.25 µg purified SleB ml⁻¹ or 0.1 µg lysozyme ml⁻¹ (Sigma). To prepare cortex PG fragments, sacculi at OD₆₀₀ 0.5 were resuspended in 500 µl 50 mM Tris/HCl pH 8.0 and physically broken with 250 mg 0.1 mm glass beads using a Wig-L-Bug bead beater. Sacculi were pulsed ten times at 3800 r.p.m. for 30 s and were incubated on ice for 1 min between pulses. Coat protein fragments were extracted from cortex fragments by mixing the suspension with chloroform and collecting the aqueous phase. Residual chloroform was removed by rinsing the pellets repeatedly with dH₂O.

**PG hydrolysis assays.** Decorated spores or chemically and physically prepared PG fractions were incubated with 20 or 40 nM purified SleL derivatives, and in some cases with purified SleB (Heffron et al., 2011). PG hydrolysis was measured as a decrease in OD₆₀₀. Pellet and soluble samples were separated by centrifugation and analysed by HPLC for N-acetylmuramic acid (NAM) release and for muropeptide composition and abundance (Dowd et al., 2008).
Sporangia and spore fractionation. Sporangia samples were collected between t4 and t6 of sporulation. Free spores were collected and purified after 3 days of incubation, as described above. Sporangia culture samples (10 ml) and purified spore samples were pelleted by centrifugation at 10 000 g for 10 min, resuspended in 8 mM NaPO4 pH 7.0, centrifuged at 13 000 g for 1 min, decanted and frozen at −80 °C. Frozen pellets were lyophilized and then broken using the bead beater as described above at 4200 r.p.m. for ten 1 min intervals. Broken samples were then resuspended in 2.5 % (v/v) 2-mercaptoethanol, 1 % (w/v) SDS, boiled and lyophilized. Proteins were quantified using the Lowry assay and 50 μg was analysed by Western blotting. Additional 1 ml sporangia culture samples were pelleted and resuspended in a lysis solution containing 10 mM glucose, 2 mM EDTA, 5 mM Tris/HCl pH 8.0, and 6 mg lysozyme ml−1. After incubating at 37 °C for 20 min, Sarkosyl was added to a final concentration of 2 % (v/v) and incubation continued for 20 min. Insoluble, forespore-associated material was separated from soluble proteins by centrifugation and then analysed by Western blotting.

Purified spores were further evaluated by suspension in 500 μl 50 mM Tris/HCl pH 8.0 and physical breakage with 250 mg 0.1 mm glass beads using a Wig-L-Bug bead beater followed by centrifugation at 13 000 g for 3 min. The soluble supernatant sample was collected, and the insoluble pellet was allowed to incubate in 50 mM Tris/HCl pH 8.0 at 37 °C for 1 h. Proteins that had become soluble during this incubation were then separated from insoluble coat proteins by centrifugation. All samples were combined with protein sample loading buffer and boiled for 5 min before being separated by 12 % (w/v) SDS-PAGE. Proteins were evaluated by Western blot analysis using the polyclonal anti-SleL antibody.

To evaluate the localization of SleL after germination, purified spores at OD600 0.4 were germinated in either a buffered solution of 8 mM NaPO4 pH 7, 10 mM L-alanine (Fisher) and 1 mM inosine (Sigma) or 50 mM Ca2+ –DPA pH 7.5 for 45 min. Spore- and exudate-associated SleL was separated by centrifugation. Samples were lyophilized, resuspended in sample loading buffer, boiled and analysed by Western blotting using polyclonal anti-SleL antibody.

RESULTS

SleL domain definition and protein purification

The NCBI Conserved Domains Database (Marchler-Bauer et al., 2005) was used to predict domains of SleL. Three domains were identified: two N-terminal LysM domains and a C-terminal glycosyl hydrolase family 18 domain (Fig. 1a). The secondary structure of SleL was predicted using Protein (Lasergene). Results suggest that there are loop regions at residues 51–53 and 98 of SleL. Therefore, SleL derivatives were designed in a way that the ends of deletions removing each LysM domain occurred in these loop regions in order to maintain the integrity of the remaining protein (Fig. 1a). SleL derivatives were purified using affinity, cation exchange and gel filtration chromatography. The resulting SleL2–429, SleL51–429 and SleL99–429 proteins have predicted molecular masses of 48.2, 43.0 and 37.6 kDa, respectively. Purified SleL derivatives are shown in Fig. 1(b). Gel filtration analyses of SleL derivatives suggest that the purified proteins exist as monomers when loaded at a concentration of ~0.6 mg ml−1 (data not shown).

SleL is a CFLE that digests partially hydrolysed cortex PG

SleL was combined with an array of PG constituents in order to determine what types of substrates the protein can recognize and cleave. When combined with decoated B. anthracis spores, SleL2–429 was unable to digest the cortical PG as no reduction in OD was observed, while a purified SCLE, SleB, was able to digest the cortex of this substrate (Heffron et al., 2011). To study the enzymic activity of the SleL derivative on intact, purified cortical PG, B. subtilis spores were chemically and enzymically treated to extract the cortex sacculi. B. subtilis cortical PG has been shown to have a nearly identical structure to that of B. anthracis (Dowd et al., 2008) but B. subtilis spores are much easier to purify. SleL2–429 could only weakly digest intact cortical PG isolated from wild-type B. subtilis spores, as indicated by the release of only 16 % of the NAM (Fig. 2). This limited release may indicate that the integrity of the cortical PG was slightly disrupted during purification. Most likely, there were not substantial flaws in the sacculi since there was no reduction in OD after 1 h when SleL was used as the sole lytic enzyme (data not shown). Increasing concentrations of purified SCLE SleB were used to partially hydrolyse the cortical sacculi. NAM release was dependent on the concentration of SleB (Fig. 2). Partial digestion with SleB produced a substrate that SleL2–429 was able to recognize and actively digest. Maximum NAM release was not achieved until both proteins were combined. Significantly more NAM (P<0.0001 as determined by a one-way analysis of variance) was released to the supernatant when SleL and SleB were combined as compared with no protein, SleL alone or SleB alone. The extent of this release became independent of the SleB concentration because it approached 100 % of the NAM available.

Soluble fractions resulting from SleB and/or SleL2–429 digestion were analysed for muropeptide content. HPLC analysis indicated that there are no detectable muropeptides present following digestion with excess SleL or with limiting SleB. Yet, digestion using both proteins resulted in PG breakdown and the appearance of peaks G6 and G7 (Fig. 3). These muropeptides are tetrasaccharide–tetrapeptide and tetrasaccharide–alanine products, respectively, that result from N-acetylg glucosaminidase digestion (Dowd et al., 2008; Lambert & Popham, 2008). Digestion with excess SleB produces different muropeptide products (Heffron et al., 2011).

To characterize the role of LysM domains in SleL PG degradation, spore PG was fragmented using glass beads and digested using 40 mM SleL2–429, SleL51–429 or SleL99–429 (Fig. 4). The PG was physically fragmented rather than SleB-digested in order to produce a stable substrate that could reveal slow SleL activity in the absence of continued slow SleB digestion. Samples were compared using longitudinal data analysis over a given time frame (10–40 min) in order to compare trends. Treatment of fragmented wild-type B. subtilis sacculi with SleL99–429 produced no discernible difference from the undigested control sample. However, when one LysM domain was present, the SleL51–429
A derivative recognized the substrate and digested it, thus significantly ($P<0.001$) increasing the rate of OD reduction compared with the undigested control. With both LysM domains present, the SleL2–429 protein caused significantly more rapid OD reduction ($P<0.001$) than observed for the undigested control and for PG digested by SleL99–429 or SleL51–429.

To evaluate the enzymic activity of SleL on cortical peptidoglycan lacking muramic-$\delta$-lactam, a substrate recognition determinant (Atri et al., 1996; Chen et al., 2000; Popham et al., 1996a; Sekiguchi et al., 1995), protein derivatives were combined with purified AcwLD $B. subtilis$ sacculi that had been partially digested with a limiting concentration of lysozyme. Lysozyme was used to partially digest the sacculi since SleB is unable to hydrolyse the substrate in the absence of muramic-$\delta$-lactam (Heffron et al., 2011). Although lysozyme loosened the cortical PG resulting in a reduction in OD, there was no difference in digestion of the muramic-$\delta$-lactam-deficient PG in the presence of any SleL derivative (data not shown). The same combination of lysozyme and SleL2–429 was able to digest wild-type $B. subtilis$ sacculi (data not shown).

### LysM domains are involved in substrate recognition and bind cortical PG

To test the hypothesis that LysM domains are involved in substrate recognition and binding we analysed the percentage of SleL derivatives that associated with purified spore PG. Quantification of SleL in the bound and

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**Fig. 1.** Purified SleL proteins. SleL proteins with and without LysM domains were purified using affinity chromatography and gel filtration. (a) Putative SleL domains and derivative constructs. SleL51–429 and SleL99–429 have single glycine (G) residues at their N termini, resulting from TEV digestion. SleL2–429 has an N terminal 4-glycine linker required to achieve efficient TEV cleavage. (b) SDS-PAGE analysis shows standard protein markers (M; kDa), SleL2–429, SleL51–429 and SleL99–429.

**Fig. 2.** NAM release as a result of SleL and/or SleB digestion. After the enzymic activity assay, samples were collected and centrifuged to separate sacculi-associated NAM (pellets) and released NAM (supernatants). When either SleL2–429 or SleB is used as the sole lytic enzyme, little NAM is released. When both proteins cooperatively digest the wild-type $B. subtilis$ sacculi nearly all of the cortical NAM is released. Data shown are the mean±SD of three independent assays. Asterisk indicates a significant difference ($P<0.0001$ as determined by a one-way analysis of variance) relative to no protein, SleL or SleB digestion alone.
unbound fractions following incubation with purified wild-type *B. subtilis* cortical PG showed that the highest percentage (~77%) of SleL bound its substrate when both LysM domains were intact (Fig. 5). There was a significant decrease ($P = 0.0001$, as determined by a one-way analysis of variance) in the amount of SleL associated with the bound fraction when one or both LysM domains were removed. Only ~35% of SleL$_{51–429}$ and ~8% of SleL$_{99–429}$ associated with the wild-type substrate. The presence or absence of SleL LysM domains did not affect the ability of the derivatives to associate equally ($P > 0.72$) with *B. subtilis* cwlD$^-$ PG lacking muramic-δ-lactam. However, substantially less ($P < 0.0004$) SleL$_{2–429}$ and SleL$_{51–429}$ bound cortical PG lacking this recognition determinant compared with wild-type PG. Conversely, when both SleL LysM domains were removed, more ($P < 0.05$) SleL$_{99–429}$ bound the muramic-δ-lactam-deficient cortical PG than bound the wild-type PG.

**LysM domains are involved in directing protein localization**

A second predicted function of the LysM domains of SleL is to direct the protein to the developing forespore during sporulation. To test this hypothesis, several forms of *sleL* were inserted into the pBKJ236 vector and then recombinated into the chromosome of an *sleL* deletion mutant strain. Sporangia samples were collected from these strains throughout sporulation, and whole sporangia and forespore-associated proteins were separated and analyzed by Western blotting using anti-SleL antiserum (Fig. 6). Full-length SleL from wild-type or DPBa96 (SleL$_{2–429}$) spores was first detected at $T_3$ of sporulation. The protein appeared in the forespore fraction as early as $T_5$. Each full-length protein was present in purified spores. Both SleL$_{51–429}$ and SleL$_{99–429}$ were detectable by $T_3$, but the amount of each protein gradually decreased and was barely detectable by $T_7$. Neither derivative was found in purified forespores or free spores.

Mature wild-type and DPBa96 (SleL$_{2–429}$) spores were fractionated in an effort to characterize the final location of SleL (Fig. 6). When spores of each strain were physically broken down with SleB and then further digested with SleL, *N*-acetylglucosaminidase products G6 (tetrasaccharide-tetrapeptide) and G7 (tetrasaccharide-alanine) (Dowd et al., 2008; Lambert & Popham, 2008) were evident.
DISCUSSION

The *B. anthracis* SleL protein, which is thought to be an *N*-acetylglucosaminidase involved in spore cortex hydrolysis (Lambert & Popham, 2008), was characterized in *vitro* and in *vivo* in order to substantiate its enzymic activity, characterize its substrate preference and evaluate the role(s) of its LysM domains in enzymic activity, PG binding and protein localization. Purified *B. anthracis* SleL is incapable of digesting intact cortical PG in the form of decoated spores or purified spore sacculi. However, SleL is capable of digesting cortical PG that has been partially fragmented. The resulting muropeptides, G6 and G7, have been shown to be *N*-acetylglucosaminidase products (Dowd *et al.*, 2008; Lambert & Popham, 2008). This supports previously published data that suggest the protein is a CFLE that recognizes partially degraded cortical PG as a substrate (Chen *et al.*, 2000).

During germination, CwlJ1 and SleB play the major roles in initiating cortex degradation, thus producing identifiable substrates for SleL. Both in *vivo* and in *vitro*, in the absence of SleL the majority of SleB lytic transglycosidase products are quite large (Heffron *et al.*, 2010, 2011). We have now demonstrated that these large products are further digested by SleL in *vitro*. This digestion produces the G6 and G7 *N*-acetylglucosamine-terminated tetrasaccharide products of SleL *N*-acetylglucosaminidase activity, indicating that SleL can cleave an SleB product multiple times. This SleL action also presumably produces small amounts of anhydro-trisaccharides containing the terminal anhydro-muramic acid product of SleB, but we have not yet been able to clearly demonstrate this muropeptide’s structure due to its low abundance.

LysM domains have been identified in more than 4000 prokaryotic and eukaryotic proteins. Non-covalent binding of *N*-acyethylglucosamine moieties of cell wall PG is one characteristic of the LysM domains (Buist *et al.*, 2008). Proteins seem to possess a particular number of LysM domains for optimum PG binding and biological function. Both the addition and removal of LysM domains have resulted in decreases in PG-binding and enzymic activity (Shao *et al.*, 2009; Steen *et al.*, 2005). Loss of one or both LysM domains from SleL decreased the rate of PG hydrolysis as well as the affinity for cortex PG. We suspect that the differences in digestion rates are likely indirect effects of decreases in the abilities of the protein derivatives to bind the substrate efficiently. In bacterial hydrolases, evaluated for the presence of SleL. When germination was triggered with DPA, only 2–5% of SleL was released from wild-type, DPBa74 and DPBa85 spores. However, when spores were triggered to germinate using Ca^{2+}.DPA there was no SleL released from the spores. Spores that were physically disrupted, digested with lysozyme and then treated with Ca^{2+}.DPA did not release any SleL from the coat proteins.

At that point, ~15% of the spores’ wild-type or SleL2–429 cache had become soluble. However, the remaining >80% of the protein remained associated with the insoluble material.

Finally, the location of SleL after treatment with nutrient and non-nutrient germinants was assessed (data not shown). Wild-type, DPBa35 (ΔsleL), DPBa74 (ΔcwlJ1 ΔcwlJ2) and DPBa85 (ΔcwlJ1 ΔcwlJ2 ΔsleB) spores were germinated, and then pellet and exudate samples were

![Fig. 5.](http://mic.sgmjournals.org)
LysM domains may be required to properly position the active site of a catalytic domain towards its substrate (Steen et al., 2005). Thus, in the absence of one or both LysM domains, digestion could still occur but would be solely dependent on substrate binding by the enzymic active site.

GSLEs are thought to recognize muramic-δ-lactam residues that are found in cortical PG but are absent in the germ cell wall, the PG layer that remains as the initial cell wall of the germinated spore (Attri et al., 1998; Meador-Parton & Popham, 2000). This allows the enzymes to selectively digest the cortex without compromising the integrity of the germ cell wall. Indeed, all of our SleL derivatives were unable to hydrolyse PG lacking muramic-δ-lactam. Binding of SleL derivatives to spore PG that lacks the muramic-δ-lactam recognition determinant is significantly altered. Binding of SleL containing one or two LysM domains to muramic-δ-lactam-free PG is greatly reduced relative to binding to wild-type PG, while binding of SleL lacking LysM domains is slightly increased. However, all SleL derivatives bound PG lacking muramic-δ-lactam poorly and to similar degrees. This suggests that the LysM domains are unable to contribute to binding in the absence of muramic-δ-lactam. The low residual binding of the SleL derivatives to lactam-free PG may be a reflection of the affinity of the active site alone for the PG, and the increased binding by SleL51–429 may be due to the failure of the hydrolysis reaction on this substrate.

LysM domains are involved in localization of several proteins to the developing forespore (Buist et al., 2008; Kodama et al., 2000; Wang et al., 2009). When the signal sequence of β-lactamase was replaced with two LysM domains from the B. subtilis SleL homologue, YaaH, the fusion protein was detectable by T2 of sporulation, and it localized to the developing forespore (Kodama et al., 2000). Fusion protein lacking the LysM domains was also detectable from T2, but the protein was gradually degraded and undetectable in purified spores. Loss of B. anthracis SleL LysM domains adversely affected protein assembly into the spore. Strains expressing wild-type SleL and SleL2–429 produced the protein between T3 and T7, and it was incorporated into developing forespores by T5. Proteins without one or both LysM domains, SleL51–429 and SleL99–429, were expressed but did not localize to the forespore and were apparently degraded. It is not yet clear if degradation was the result of a failure to localize or if localization was impossible due to degradation. However, the SleL51–429 protein was stable and enzymatically active in vitro.

Two other spore-associated proteins that contain LysM domains are SafA and SpoVID. These two proteins interact with one another at the cortex–coat interface adjacent to the outer forespore membrane (Costa et al., 2006; Ozin et al., 2000), and then promote attachment of additional spore coat proteins including SleL (YaaH) (McKenney et al., 2010). It is not yet clear if the LysM domains play roles in localization of these proteins to the spore via interaction with cortex PG. Such an interaction would require part or all of the proteins to traverse the outer forespore membrane during spore formation, and protein secretion signals involved in such an event are not evident. Alternatively, these proteins may localize to this site via interactions with proteins or with the membrane in some manner dependent on LysM motifs, or perhaps the LysM domains interact with lipid-linked PG precursors on the cytoplasmic side of the membrane. The LysM domains of SleL would then contribute to cortex interaction and hydrolysis only after the outer forespore membrane becomes permeable during spore dormancy and germination.

The data presented here have given us insights into how SleL is assembled into a spore, and how it actively degrades cortical peptidoglycan during germination. Further analysis of SleL localization and possible protein–protein interaction studies may reveal why the protein is associated with not only the cortex but also the coat proteins. This information will be useful in designing methods to
externally activate GSLEs in an effort to initiate germination. This strategy will be helpful in developing simpler spore decontamination methods.

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