Secretion genes as determinants of *Bacillus anthracis* chain length

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Bacillus anthracis grows in chains of rod-shaped cells, a trait that contributes to its escape from phagocytic clearance in host tissues. Using a genetic approach to search for determinants of B. anthracis chain length, we identified mutants with insertional lesions in secA2. All isolated secA2 mutants exhibited exaggerated chain length, whereas the dimensions of individual cells were not changed. Complementation studies revealed that slaP (S-layer assembly protein), a gene immediately downstream of secA2 on the B. anthracis chromosome, is also a determinant of chain length. Both secA2 and slaP are required for the efficient secretion of Sap and EA1 (Eag), the two S-layer proteins of B. anthracis, but not for the secretion of S-layer associated proteins or of other secreted products. S-layer assembly via secA2 and slaP contributes to the proper positioning of BslO, the S-layer associated protein and murein hydrolase, which cleaves septal peptidoglycan to separate chains of bacilli. SlaP was found to be both soluble in the bacterial cytoplasm and associated with the membrane. Purification of soluble SlaP from B. anthracis cleared lysates did not reveal a specific ligand, and membrane association of SlaP was not dependent on SecA2, Sap or EA1. We propose that SecA2 and SlaP promote the efficient secretion of S-layer proteins by modifying the general secretory pathway of B. anthracis to transport large amounts of Sap and EA1.
The Gram-positive microbe *Bacillus anthracis* is the causative agent of anthrax and exists in two forms - vegetative, rod-shaped bacilli and small endospores (31). Following uptake into animal or human hosts, spores germinate into vegetative forms that replicate and disseminate into all organ tissues (31). Vegetative forms are thought to evade clearance by host phagocytes through the elaboration of a thick capsule, which is composed of poly-D-\(\gamma\)-glutamic acid (PDGA) tethered to the \(m\)-diaminopimelic acid crossbridge of peptidoglycan (7, 51), and through the ability to form elongated chains of bacilli tethered end-to-end at their septal peptidoglycan (47). Chains of bacilli present a physical obstacle for engulfment by immune cells (10, 53).

The envelope of *B. anthracis* is comprised of plasma membrane and peptidoglycan layer, which is decorated with the secondary cell wall polysaccharide (SCWP): \(\rightarrow\)6\(-\alpha\)-GlcNAc-(1\(\rightarrow\)4)-\(\beta\)-ManNAc-(1\(\rightarrow\)4)-\(\beta\)-GlcNAc-(1\(\rightarrow\))\(n\), where \(\alpha\)-GlcNAc is substituted with \(\alpha\)-Gal and \(\beta\)-Gal at O3 and O4, respectively, and the \(\beta\)-GlcNAc is substituted with \(\alpha\)-Gal at O3 (8). SCWP is tethered via GlcNAc-ManNAc linkage units to the C6 position of \(N\)-acetylmuramic acid (MurNAc) in the repeating MurNAc-GlcNAc disaccharide structure of peptidoglycan (26). The *B. anthracis* S-layer is comprised of the main S-layer proteins, Sap and EA1 (14, 42, 43), which self-assemble into a paracrystalline layer of protein (41). The S-layer also harbors *B. anthracis* S-layer associated proteins (BSLs) (27), which provide for uptake of nutrients across the envelope (63), adhesion to host tissues (28) and cell separation within chains of vegetative bacilli (1). The mature forms of S-layer and S-layer associated proteins harbor SLH domains, which are responsible for the non-covalent association of these proteins with the SCWP (39, 40). SLH domains of S-layer and S-layer associated proteins assemble into three-pronged spindle structures, where three inter-
prong grooves are thought to capture the pyruvylated form of SCWP, a modification catalyzed by the csaB gene product (16, 29).

Recently developed genetic tools have been applied to the study of *B. anthracis* chain formation and S-layer assembly (1, 62). Using flow cytometry analysis of bacilli, mutants with insertional lesions in *csaB* and *bslO* were isolated (1). BslO is a S-layer associated protein that localizes to the septal portion of the *B. anthracis* S-layer where it cleaves peptidoglycan, thereby separating elongated chains of bacilli into shorter chains (1). Here we have expanded this search and report the isolation of chain length mutants with lesions in *B. anthracis* genes that are involved in the secretion of S-layer proteins across the bacterial envelope.

**MATERIALS AND METHODS**

**Bacterial strains and culture conditions.** *B. anthracis* Sterne 34F2 (60) and its mutants (Table 1) were cultured in Brain-Heart Infusion broth (BHI) supplemented with 0.8% NaHCO₃ at 37°C, or at 30°C when harboring the pLM4 vector (38). *Escherichia coli* strains DH5α (22) and K1077 (*dcm-/dam-*) (18) were cultured in Luria-Bertani broth (LB) at 30°C. Media were supplemented with 20 µg/ml kanamycin to maintain plasmid selection in *B. anthracis* or with 50 µg/ml kanamycin in *E. coli*. *B. anthracis* strains were sporulated in modified G medium (modG) as described (30). Spore preparations were heat treated to kill any remaining vegetative bacilli and stored at 4°C. Spores were enumerated by CFU counts. Spores were germinated by inoculation into BHI and growth at 37°C.
**B. anthracis mutants and plasmids.** Plasmid DNA was purified from *E. coli* K1077 and was then used to transform *B. anthracis*. Deletion mutants were obtained by allelic replacement using the temperature-sensitive plasmid pLM4 as described (Table 1) (38). Briefly, 1 kb upstream and downstream DNA sequences flanking the gene of interest were PCR amplified with specific primers (Table 2), cloned by restriction digest into pLM4, and transformed into *B. anthracis*. Transformants were grown for 10 hours at 42°C in the presence of 20 µg/µl kanamycin, diluted and grown under the same conditions for four passages. Cultures were then diluted for another four passages into media lacking antibiotics and grown for 10 hour intervals at 30°C. Mutants were screened for growth on BHI agar and no growth on BHI/kanamycin agar. All complementation plasmids were derived from pJK4 and expression of its P$_{spac}$ promoter was induced with 0.1 mM IPTG (26). Plasmid pSN1 was generated by amplifying the open reading frame of secA2 from genomic DNA using primers smn29 and smn30. Plasmid pSN3 was constructed by amplifying the open reading frame of slaP from genomic DNA using the primers smn78 and smn79. Plasmid pSN2 was generated by amplifying both secA2 and slaP using the primers smn29 and smn79. Strep-tagged SlaP (SlaP$_{strep}$) was constructed by amplifying slaP with the primers smn96 and smn97, which contains a 24 bp insertion of the Strep-TAG coding sequence immediately adjacent to the STOP codon (tggtctcatcctcaatgtgagaag).

**Antibody production.** Recombinant SecA2 and SlaP were produced in *E. coli* BL21 (DE3) harboring plasmids pSN5 and pSN6, respectively. Overnight cultures were diluted 1:50 in LB supplemented with 100 µg/ml ampicillin at 30°C. IPTG (1 mM) was added to the cultures at A$_{600}$ 0.5 and expression of SecA2 and SlaP were induced for 3 hours. Cells were collected by
centrifugation, suspended in 50 mM Tris-HCl and 150 mM NaCl, and lysed in a French press. Cell lysates were subjected to affinity purification over a Ni-NTA column and affinity tagged proteins were eluted with 250 mM imidazole. Eluates were pooled and dialyzed in 1× PBS. 500 μg of purified protein was emulsified in complete Freund’s adjuvant (Difco) and injected subcutaneously into female New Zealand white rabbits. Antibody production was stimulated in 21 day intervals with two booster injections of antigen emulsified in incomplete Freund’s adjuvant.

Flow cytometry analysis of *Bacillus anthracis* vegetative forms. *B. anthracis* spores were germinated in 3 ml BHI at 37°C for 3 hours. Cells were fixed with 4% paraformaldehyde and subjected to flow cytometry using a LSR Fortessa instrument (BD Biosciences). FSC-A data were collected for n=10,000 events. Files were analyzed using FlowJo.

Light microscopy of bacilli. Cells were fixed with 4% paraformaldehyde and imaged. Images were captured with a CCD Camera on an Olympus IX81 microscope using 100× or 40× objectives. The chain lengths of bacilli were measured from acquired DIC images using ImageJ, and converted to lengths in micrometer using reference images of an objective micrometer.

Immunofluorescence microscopy of bacilli. *B. anthracis* strain Sterne and its mutants were germinated in 3 ml of BHI at 37°C for 3 hours. Cells were centrifuged and fixed with 4% paraformaldehyde. Samples were treated with diluted rabbit antiserum raised against purified recombinant Sap or BslO, and labeled with secondary antibody conjugated to fluorophore (Thermoscientific). Cells were counterstained with BODIPY-vancomycin (Invitrogen). A Leica SP5 Tandem Scanner Spectral 2-Photon confocal microscope was used to observe cells with a 63×
objective. Images were captured at various zooms to include the entire chain. The scale bar reflects the actual length in µm regardless of zoom function.

**S-layer fractionation.** *B. anthracis* overnight cultures were diluted 1:100 into fresh media and grown to A600 2. One ml of culture was centrifuged at 16,000 xg and separated into medium (supernatant) and pellet fraction. Proteins in the medium were precipitated with 10% trichloroacetic acid (10% TCA, vol/vol) for 30 minutes on ice and centrifuged at 16,000 xg for 10 minutes. The bacterial sediments were washed twice with 1 × PBS and boiled at 95°C for 10 minutes in 3 M urea. Cells were sedimented by centrifugation at 16,000 xg and the supernatant (S-layer fraction) was removed. The pellet was washed twice with PBS and mechanically lysed by silica bead beating for 3 minutes (MP Biomedical Fastprep-24). After sedimentation of the beads, proteins in the cell lysates were precipitated with TCA. All TCA precipitates were washed with ice-cold acetone and centrifuged at 16,000 xg for 10 minutes. Acetone was removed and protein precipitates were dried. Samples were suspended in 50 µl 0.5 M Tris-HCl (pH 7.5), 4% SDS and mixed with an equal volume of sample buffer (4% SDS, 1% β-mercaptoethanol, 10% glycerol, 50 mM Tris-HCl (pH 7.5), bromophenol blue). Proteins were separated on 10% SDS-PAGE and analyzed by Coomassie staining or electro-transferred to polyvinylidene difluoride (PVDF) membrane for immunoblot analysis. Proteins were detected with rabbit antisera raised against purified antigens. Strep-tagged proteins were detected using monoclonal antibody StrepMAB (IBA). Immunoreactive products were revealed by chemiluminescent detection after incubation with HRP-conjugated secondary antibody (Cell Signaling Technology). The percent amount of protein in subcellular fractions was calculated by averaging the ratio of immunoblot signals from one fraction to the sum of the signal in all fractions of three trials. Protective
antigen (a protein secreted into the medium), PrsA (a membrane lipoprotein) and L6 (a ribosomal protein in the cytoplasm) were used as internal controls for the proper fractionation of bacilli.

**Affinity purification of SlaPStrep.** *B. anthracis* vegetative forms were diluted 1:50 from an overnight culture in BHI supplemented with 0.8% sodium carbonate and 20 µg/ml kanamycin, induced with 1 mM IPTG and grown to $A_{600}$ 1.5. Cells were sedimented by centrifugation, suspended in column buffer (100 mM Tris-HCl (pH 8.0), 150 mM NaCl), and lysed by bead beating. Lysates were centrifuged for 5 minutes at 1,000 ×g to remove beads and unbroken cells. Lysates were then subjected to ultracentrifugation at 100,000 ×g for 30 min. Cleared lysates were loaded on Strep-tactin sepharose pre-equilibrated with column buffer and the column was washed extensively with column buffer (IBA). Bound proteins were eluted with 2.5 mM desthiobiotin in column buffer, separated by 10% SDS-PAGE, and analyzed by Coomassie staining or immunoblotting.

**Extraction of proteins from *B. anthracis* membranes with sodium carbonate.** *B. anthracis* overnight cultures were diluted 1:50 into fresh media, expression of SlaPStrep induced with 1 mM IPTG and bacilli grown to $A_{600}$ 1.5. Vegetative bacilli were sedimented by centrifugation, suspended in cytosol buffer (50 mM HEPES, pH 7.5, 10 mM MgOAc, 66 mM KOAc), and lysed by bead beating. Lysates were centrifuged for 5 minutes at 1,000 ×g to remove beads and unbroken cells, and the supernatant was subjected to ultracentrifugation at 100,000 ×g for 30 min. Proteins in the supernatant were precipitated with 10% TCA. Membrane sediments were suspended in 0.1 M sodium carbonate (pH 11.4) for 30 minutes on ice and then subjected to ultracentrifugation 100,000 ×g for 30 min. Proteins in the supernatant and pellet
RESULTS

Isolation of *B. anthracis* secA2 mutants. Random mutagenesis with the *bursa aurealis* transposon was used to generate mutants of *B. anthracis* Sterne (62), an attenuated strain that harbors the virulence plasmid pXO1, which contains the genes for anthrax toxins [protective antigen (PA), lethal factor (LF) and edema factor (EF)], but lacks pXO2 and the PDGA capsule genes (45, 60). Flow cytometry forward scatter (FSC) and side scatter (SSC) parameters were used to assess *B. anthracis* chain length and refractivity (1). By comparing mean FSC-A and SSC-A signals (n=10,000 events per strain), we identified nine mutants that produced increased forward scattering relative to an age matched wild-type control (Fig. 1B). Inverse PCR mapped the transposon insertions to BAS0838, a gene specifying a 788 residue polypeptide with homology (43% sequence identity) to the SecA ATPase of *E. coli* (46)(Fig. 1A). The genome of *B. anthracis* encodes a second gene, BAS5038, whose 835 amino acid residue product displays 51% sequence identity with *E. coli* SecA (901 amino acids). We assigned BAS5038 to be the secA homologue of *B. anthracis* and BAS0838 to be secA2, an accessory secretion gene whose function is required for the efficient secretion of the S-layer proteins of this microbe (see below).

*B. anthracis* secA2 variants display a chain length phenotype. *B. anthracis* spores were suspended in fresh media and monitored for changes in optical density ($A_{600}$) over time. The
growth curves of *B. anthracis* Sterne and its secA2 mutants were super-imposable, indicating that secA2 is not required for growth (data not shown). Three hours after the inoculation, *i.e.* during early exponential phase, culture aliquots were analyzed by light microscopy (Fig. 2). *B. anthracis* Sterne formed chains that were on average 34 (±22.68) µm in length (Fig. 2A). As previously reported, the chain lengths of *B. anthracis* Sterne or its mutants are variable when analyzing many different chains (*n*=100)(1) (Fig. 2A). The average chain length of bacilli increases during early exponential phase and gradually decreases as cultures enter late exponential phase (1). At the three hour growth interval, the average chain length of the secA2 mutant was increased to 83 (±62.05) µm (WT vs. secA2, *P*<0.001). Similar increases in chain lengths (secA2 mutants vs. WT) were observed when cultures were sampled at later time intervals (data not shown). The chain length phenotype of the secA2 mutant was reduced when secA2 bacilli were transformed with plasmid pSN2, encoding wild-type secA2 and *slaP* [51 (±31.66) µm; secA2 vs. secA2 (pSN2), *P*<0.001]. As a control, a mutant with a *bursa aurealis* insertion in the *bslO* gene formed chains with greatly exaggerated length 298 (±290.46) µm (Fig. 2)(1). Furthermore, a mutant lacking *sap*, the gene encoding the surface array protein, also formed chains that were increased in length [79 (±47.52) µm; WT vs. *sap*, *P*<0.001]. The chain length phenotype of the *sap* mutant appears to be due to its inability to properly position BslO in the vicinity of septal peptidoglycan (see below); BslO cleaves septal peptidoglycan and controls the chain length of *B. anthracis* (1).

* *B. anthracis* secY2 variants do not display a chain length phenotype.* The genome of *B. anthracis* harbors two copies of the secY gene, which in *E. coli* encodes one of the three components of pre-protein translocase (SecYEG) (20) and represents the site for SecA-mediated
protein secretion (12, 13). The predicted product of BAS0130 (433 residues) displays 43% sequence identity with *E. coli* SecY (443 residues)(24), whereas BAS2547 (434 residues) is 42% identical. As BAS0130 is located in the *B. anthracis* operon for ribosomal protein synthesis, similar to the *E. coli* operon expressing secY (23), the gene was assigned secY. To test whether BAS2547 functions as an accessory secretion gene in the same pathway as *B. anthracis* secA2, we deleted its open reading frame. Mutants lacking BAS2547 were viable and without general defect in protein secretion (see below). We therefore designated BAS2547 as secY2. The secY2 mutant did not display a *B. anthracis* chain length phenotype [32 (±20.44) µm; P>0.5], suggesting that secY2 and secA2 may not function in the same accessory secretion pathway (Fig. 1B and Fig. 2).

**B. anthracis secA2** variants display reduced secretion of the S-layer proteins Sap and EA1. Cultures of *B. anthracis* Sterne or its secA2 and secY2 mutants were grown to $A_{600}$ 2 and centrifuged to separate the extracellular medium (M) from the bacterial sediment. Bacilli were suspended in 3 M urea and boiled, thereby extracting S-layer proteins from *B. anthracis* cells. Extracts were again centrifuged to separate extracted S-layer proteins in the supernatant (S) from *B. anthracis* cells in the sediment. The peptidoglycan of bacilli was broken with glass beads to release cellular proteins (C). Proteins in the medium (M) and cellular (C) fractions were precipitated with TCA, washed in acetone and solubilized in sample buffer prior to SDS-PAGE and immunoblot analysis (Fig. 3). Similar amounts of Sap were detected in the medium (47±8.48%) and in the S-layer [42% (±13.48)] of *B. anthracis* Sterne cultures (Fig. 3), however only small amounts of Sap [11% (±5.11)] were found within bacilli. PrsA, a membrane associated lipoprotein involved in the folding of secreted polypeptides (67), was detected
predominantly in the cellular fraction (Fig. 3B). Protective antigen (PA), a secreted toxin (64, 68), was found in the medium (Fig. 3). Most of the EA1 protein was detected in the medium of *B. anthracis* Sterne cultures [53% (±15.51), while the remainder was found in the S-layer (Fig. 3). The S-layer associated protein BslO was only detected in the S-layer (1); BslA was found in the extracellular medium (42.62%) and in the S-layer (57.38%) (27). These results suggest that *B. anthracis* secretes significant amounts of Sap and EA1 into the medium and that not all of these S-layer proteins are permanently retained in the bacterial envelope through their binding to the SCWP (26).

Mutants with an insertional lesion in the secA2 gene secreted reduced amounts of Sap [7% (±4.50)] and EA1 [13% (±12.52)] into the culture medium, however significant amounts of these polypeptides were retained in the S-layer (Fig. 3). The defect in Sap and EA1 secretion could be complemented by plasmid pSN2 [33% (±0.41) secreted Sap and 48% (±14.32) secreted EA1], which expresses the secA2 and slaP genes (*vide infra*) from the IPTG inducible Pspac promoter (Fig. 1A and Fig. 3). The subcellular locations of PA, BslA, BslO and PrsA were not affected in the *B. anthracis* secA2 mutant (Fig. 3B). Deletion of the secY2 gene did not have an effect on S-layer protein secretion, as 48% (±14.62) Sap and 57% (±23.80) EA1 were detected in the medium of *B. anthracis* secY2 mutant cultures (Fig. 3B).

**B. anthracis slaP mutants display a chain length phenotype and partial defect for Sap and EA1 secretion.** Plasmid pSN1 harbors wild-type secA2 under the control of an IPTG inducible promoter; transformation of pSN1 into *B. anthracis* secA2 mutants did not completely restore Sap and EA1 secretion to wild-type levels (Fig. 4B), suggesting that the insertional lesion resulted in a polar effect on the neighboring gene BAS0837 (Fig. 4B). To explore this further,
we generated a mutant with a deletion in the 294 codon open reading frame of BAS0837 (Fig. 2A). Compared to *B. anthracis* Sterne, the mutant was defective for the efficient secretion of Sap [10\% (±3.00\%)] and EA1 [15\% (±15.28\%)], whereas the secretion of PA and BslA was not altered (Fig. 4A). We therefore designated BAS0837 as *slaP* (*B. anthracis* S-layer assembly protein). Similar to *B. anthracis* *secA2* mutants, the variant lacking *slaP* displayed an increased chain length [76 (±46.97) µm; WT vs. *slaP*, *P*<0.001] (Fig. 4C). Transformation of the *slaP* mutant with pSN3, a plasmid encoding wild-type *slaP* under control of the IPTG-inducible *Pspac* promoter, restored the efficient secretion of Sap [32\% (±9.91\%)] and EA1 [55\% (±8.64\%), Fig. 4A] and reduced the increased chain length phenotype of the *slaP* mutant (Fig. 4CD). In contrast, transformation of the *slaP* mutant with pSN1 (*secA2*) did not lead to a complete restoration of S-layer protein secretion (Fig. 4A) or to a reduction in chain length (data not shown).

**SlaP is located in the cytoplasm of *B. anthracis***. When examined with the Kyte-Doolittle hydrophobicity plot (33) or a signal peptide algorithm (65), the *in silico* translated product of *slaP*, a polypeptide of 293 amino acids, was not predicted to harbor an N-terminal signal peptide or a hydrophobic transmembrane domain. Homologues of SlaP were identified in the genome sequences of *B. anthracis*, *B. cereus* and *B. thuringiensis* strains, but not in the genome of *Bacillus subtilis* or *Clostridium difficile* (data not shown). To detect a *slaP* gene product, we generated specific rabbit antisera against full length recombinant SlaP. Immunoblotting with rabbit antisera detected SlaP in the cellular fraction of *B. anthracis* Sterne (WT), but not in the cellular fraction of the *slaP* mutant (Fig. 4A). Increased amounts of SlaP were detected in the cytoplasm of the *slaP* mutant harboring plasmid pSN3, but not in *slaP* (pSN1) cells (Fig. 4A). To identify SlaP binding partners, a plasmid encoding a *slaP* variant with a 3′ extension of its open reading frame was used.
reading frame for eight codons (WSHPQFEK) specifying a Strep-tag (pSN4, Fig. 1) was constructed. Similar to pSN3, transformation of the slaP mutant with pSN4 restored the efficient secretion of S-layer proteins Sap and EA1 (Fig. 5A). Cleared lysates derived from the vegetative forms of B. anthracis slaP (pSN3) and B. anthracis slaP (pSN4) were subjected to affinity chromatography on Strep-tactin sepharose, eluted with desthiobiotin and analyzed by Coomassie-stained SDS-PAGE (55). The data in Fig. 5B reveal the affinity purification of a 32 kDa polypeptide (white arrowhead) from the cleared lysate of B. anthracis slaP (pSN4) cells, but not from B. anthracis slaP (pSN3). Mass spectrometry experiments with tryptic peptides derived from the 32 kDa polypeptide confirmed its identity as SlaPStrep (data not shown). The eluates of affinity chromatography samples were subjected to immunoblotting with antibodies specific for the Strep-tag as well as Sap and EA1. As expected, only the eluate of B. anthracis slaP (pSN4) cells, not B. anthracis slaP (pSN3), harbored SlaPStrep (Fig. 5B). S-layer proteins Sap and EA1 were not detected in the eluate of B. anthracis slaP (pSN4) cells (Fig. 5B). As a control, the medium fraction (M) of B. anthracis Sterne cultures was subjected to SDS-PAGE in order to calibrate immunoblotting experiments for Sap and EA1 (Fig. 5B). Thus, the soluble form of SlaPStrep does not co-purify with Sap or EA1 precursors, i.e. the substrates for an accessory secretion pathway that is defined by its unique requirement for slaP and secA2 function.

SlaP associates with the plasma membrane of B. anthracis. To examine the membrane association of SlaP, lysate derived from the vegetative forms of B. anthracis (obtained by mechanically lysing bacilli with glass beads), was subjected to slow-speed centrifugation to remove unbroken cells. The lysate was then subjected to ultra-centrifugation to separate soluble cytoplasmic components in the supernatant (S) from integral membrane proteins and
membrane associated proteins in the sediment (P, pellet). Membrane samples were extracted
with 0.1 M sodium carbonate (Na₂CO₃, pH >11), a perturbant that displaces proteins
peripherally associated with membranes (59), and again centrifuged to separate extracted
proteins in the supernatant (S) from integral membrane proteins in the sediment (P, pellet).
Proteins in all fractions were precipitated with trichloroacetic acid (TCA) and analyzed by
Coomassie-stained SDS-PAGE and immunoblotting (Fig. 6). Following fractionation, most of SlaP
sedimented with the membranes of \textit{B. anthracis} cells, however a small portion of SlaP was
extracted from the membranes by treatment with sodium carbonate. These data suggest a
peripheral membrane association for SlaP (Fig. 6).

\textbf{Membrane association of SlaP does not require S-layer proteins or SecA2.} We
wondered whether the membrane association of SlaP is caused by engagement of the
precursors of the Sap and EA1 S-layer proteins with the SecA2 secretory pathway. If so, mutants
that are unable to express the secretion substrates (Sap or EA1) or the accessory secretion
component (SecA2) would be expected to release SlaP\textsubscript{Strep} from the bacterial membrane (Fig.
7). This was tested, however mutants lacking \textit{secY2}, \textit{secA2}, \textit{sap}, \textit{eag} or both S-layer protein
genes (\textit{sap}, \textit{eag}) displayed similar patterns of SlaP\textsubscript{Strep} association with the membranes of
bacilli, \textit{i.e.} the protein was found to be associated with bacterial membranes in a manner that
could be perturbed by treatment with sodium carbonate (Fig. 7). Thus, neither the accessory
secretion genes (\textit{secA2} and \textit{secY2}) nor the genes for the S-layer protein substrates of SlaP- and
SecA2-mediated secretion are required for the association of SlaP\textsubscript{Strep} with the bacterial
membrane.
BslO is mislocalized in *B. anthracis* secA2 and slaP mutants. Spores of *B. anthracis* strain Sterne and its mutants in secA2 and slaP were germinated for three hours. Cells were fixed in 4% paraformaldehyde and stained for Sap and BslO using specific rabbit antisera and secondary antibody conjugated to fluorophore. BODIPY-vancomycin was used to stain peptidoglycan lipid II, which accumulates in the septum between adjacent vegetative cells (1).

Bacilli were imaged on a Leica SP5 Tandem Scanner Spectral 2-Photon confocal microscope. The S-layer protein Sap was found to be distributed throughout the envelope of *B. anthracis* Sterne vegetative cells (Fig. 8A). BslO localized to the septal portion of the *B. anthracis* envelope, as demonstrated by the superimposable fluorescence signal for BslO and BODIPY-vancomycin (Fig. 8B)(1). The distribution of Sap in the envelope of secA2 and slaP mutants appeared uneven, with patches of strong fluorescent intensity (Fig. 8A). Unlike its physiological septal localization in wild-type bacilli, BslO was found deposited in patches throughout the envelope of *B. anthracis* secA2 and slaP mutant vegetative forms. Localization of BslO in the secA2 and slaP mutants was restored to the septal position when expressing the wild-type alleles of secA2 or slaP from complementing plasmids (Fig. 8B). These data suggest that the secretion defect of secA2 and slaP mutants for the S-layer proteins (Sap and EA1) perturbs the distribution of the S-layer associated protein BslO, whose localization to the septal portion of the envelope is required for the physiological control of *B. anthracis* chain length (1).

**DISCUSSION**
The secretion of signal peptide bearing precursor proteins in *B. anthracis* has not yet been studied. Assuming that the protein secretory pathway, as defined in *E. coli*, is operational in *B. anthracis*, signal peptide bearing precursors would likely use the ATPase SecA and the membrane translocon SecY/SecE/SecG as well as SecD/SecF/YajC complex for their travels across the plasma membrane (54). Removal of the signal peptide by signal peptidase (9) would release mature proteins into the extracellular medium of *B. anthracis*, unless polypeptides are endowed with S-layer homology domains (39), which retain S-layer proteins and S-layer associated proteins in the envelope by binding to the SCWP (26, 27, 29) or with sorting signals for sortase-mediated anchoring to peptidoglycan (19, 36, 37). *E. coli* also employs the signal recognition particle (SRP), a ribonucleoprotein complex comprised of Ffh polypeptide and 4.5S RNA, which interacts with the nascent precursors of membrane proteins to regulate translation and deliver the ribosome to the SRP receptor (FtsY) and eventually to the SecY/SecE/SecG translocon for co-translational secretion (21, 44). Several chaperones, including SecB (48), heat shock proteins (DnaK/DnaJ/GrpE)(66) as well as trigger factor, a peptidyl-prolyl isomerase (61), contribute to secretion by maintaining specific substrate proteins of *E. coli* in a secretion competent state (3, 11). The genome of *B. anthracis* harbors *secA*, *secD*, *secE*, *secF*, *secG*, *secY*, *ffh*, *ftsY* and *yajC* genes (50). A notable difference from *E. coli* is that *B. anthracis* lacks the *secB* gene but expresses three homologues of *prsA* (67), a lipoprotein peptidyl-prolyl isomerase involved in the folding of secreted polypeptides (32, 57).

Some Gram-positive bacteria express accessory secretion genes designated *secA2* or *secY2* (52). In *Streptococcus gordonii* and *Staphylococcus aureus*, the *secA2* and *secY2* genes are essential for the secretion of the large glycoprotein GspB and SraP, respectively (4).
post-translational glycosylation, GspB cannot be secreted via the canonical SecA pathway (4, 58). A 90 residue N-terminal signal peptide as well as the first twenty amino acids of mature GspB are required for its initiation into an accessory secretion pathway requiring SecA2 and SecY2 as well as the accessory secretion proteins Asp1-5 (5, 56). Several Gram-positive microbes also use accessory secretion genes, secA2 and secY2 (either alone or together), for the selective transport of specific substrates (52). For example, *Listeria monocytogenes* employs a SecA2 dependent pathway for the secretion of the p60 murein hydrolase, a protein whose gene is located immediately adjacent to the secA2 gene (34, 35). In contrast to the narrow substrate specificity of SecA2/SecY2 in streptococci and staphylococci, both listeria and mycobacteria, i.e. *Mycobacterium smegmatis* and *Mycobacterium tuberculosis*, appear to transport a larger spectrum of proteins via SecA2; this includes some proteins that lack an N-terminal signal peptide (2, 6). The biochemical details for SecA2 function in any one of these bacteria, i.e. the selection of specific substrate by SecA2 or its presumed interaction with other components of the secretory pathway, are still unknown (52).

Several spore forming Firmicutes including *B. cereus*, *B. anthracis*, *B. thuringiensis*, *C. difficile* as well as other clostridial species elaborate a proteinaceous S-layer from precursor proteins harboring an N-terminal signal peptide (15, 17). *C. difficile*, an anaerobic microbe, encodes two secA genes of which the non-canonical secretion gene, secA2, is required for the assembly of its S-layer proteins and its cell wall protein CwpV (15). In contrast to *C. difficile*, members of the *B. cereus sensu lato group* (25) - *B. cereus*, *B. anthracis*, *B. thuringiensis* – harbor the slaP gene and require SLH domains within S-layer proteins for their envelope deposition via a non-covalent association with the SCWP (26, 29). Here we demonstrate that
the secA2 and slaP genes of B. anthracis are both required for the efficient secretion of the S-
layer proteins Sap and EA1 and for the physiological control of the chain length of the
bacterium’s vegetative forms. The latter phenotype is explained as a result of the reduced
deposition of S-layer proteins in the B. anthracis envelope. S-layer proteins, for example Sap,
are required for the proper positioning of the BslO murein hydrolase in the bacterial envelope
(1). Further, we show that SlaP can be isolated as a soluble protein from the bacterial
cytoplasm; this species does not appear to interact with other proteins of B. anthracis. SlaP is
associated with bacterial membranes in a manner that can be perturbed with sodium
carbonate, which is indicative of a peripheral membrane association. We surmise, but do not
know, that SlaP interacts with components of the secretion pathway to promote the efficient
secretion of Sap and EA1. This interaction does not seem to involve SecA2, SecY2, Sap or EA1,
as mutants lacking any one of these components still retain SlaP in the bacterial membrane. To
characterize the SecA2/SlaP-mediated secretion of Sap and EA1 in greater detail, future work
must focus on characterizing the interaction of these proteins with components of the B.
anthracis secretion pathway.

Why does B. anthracis require an alternative secretion pathway for the assembly of its
S-layer and S-layer associated functions? Currently available data cannot provide a conclusive
answer, however we have entertained two possibilities. First, the S-layer proteins Sap and EA1
are perhaps the most abundantly synthesized proteins of B. anthracis (27). Because protein
translocation across the plasma membrane is a catalytic process, the considerable abundance
of S-layer precursors may affect the secretion of other substrates. To prevent a traffic jam at
the plasma membrane, B. anthracis may have evolved another catalyst (SecA2/SlaP). Second, S-
layer protein secretion and assembly may be confined to discrete locations in the *B. anthracis* envelope of which SecA2/SlaP may represent the secretion machinery components. Such dedicated S-layer secretion/assembly pathway could further involve folding catalysts enabling transfer of proteins into the envelope and formation of the paracrystalline lattice. The latter model implies that S-layer proteins are transferred directly (without prior secretion into the medium) to their final destination; the former model implies a pathway whereby S-layer proteins are first secreted into the medium and subsequently seated by binding to the SCWP in the bacterial envelope. Although a mechanism of direct transfer seems favorable to us, we also appreciate that the experimental tools presented here cannot distinguish between the aforementioned models for S-layer protein secretion and assembly.

**ACKNOWLEDGEMENTS**

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12. Economou, A., J. A. Pogliano, J. Beckwith, D. B. Oliver, and W. Wickner. 1995. SecA membrane cycling at SecYEG is driven by distinct ATP binding and hydrolysis events and is regulated by SecD and SecF. Cell 83:1171-1181.


conserved mechanism involving wall polysaccharide pyruvylation. EMBO J. 19:4473-4484.


FIG. 1. Isolation of Bacillus anthracis mutants that display chain length phenotypes. (A) Diagram illustrating the B. anthracis chromosomal gene cluster for the expression of S-layer proteins (sap and eag encoding signal peptide bearing precursors with three SLH domains), the pyruvylation of the B. anthracis secondary cell wall polysaccharide (csaB) as well as the secretion of S-layer proteins, including secA2, a predicted ATPase with DEXD binding motif, and slaP. Arrowheads positioned above the secA2 gene identify nine different bursa aurealis insertional lesions that confer a chain length phenotype onto mutant strains. Plasmids used in this study harbor wild-type secA2 (pSN1), wild-type secA2 and slaP (pSN2), wild-type slaP (pSN3) or slaP with a 3’ extension of its open reading frame specifying a Strep-TAG peptide for affinity-chromatography of the encoded product (pSN4). All plasmids are recombinants of pJK4 and express inserted secretion genes via the lacI controlled, IPTG-inducible Psac promoter. (B) The chain length of B. anthracis strain Sterne (WT, wild-type) or its mutants with mutational lesions in the secY2, secA2, bslO and sap genes was assessed by flow cytometry. B. anthracis secA2 mutants were transformed with pSN2 (encoding secA2 and slaP) to analyze the complementation of the chain length phenotype of the secA2 insertional lesion.

FIG. 2. Chain length of B. anthracis secA2 mutants. (A) Box and whisker plot of the chain length of B. anthracis Sterne (WT) and its mutants with mutational lesions in secY2, bslO, sap, secA2 or a secA2 mutant harboring pSN2 at 3 hours post-germination in BHI medium. (B) Chain lengths were measured from DIC micrographs of vegetative bacilli (n=100). Scale bars represent 10 μm in length. Statistical significance of differences in B. anthracis chain lengths were
examined with the two-tailed Student’s t-test: WT vs. secY2, P>0.5; WT vs. bslO, P<0.001; WT vs. sap, P<0.001; WT vs. secA2, P<0.001; secA2 vs. secA2 (pSN2), P<0.001.

FIG. 3. *B. anthracis* secA2 mutants are defective for the secretion of S-layer proteins Sap and EA1. (A) Vegetative forms of *B. anthracis* Sterne (WT) and its secA2, secY2 or a secA2 mutant harboring pSN2 were grown to mid-log phase. Cultures were centrifuged to separate the extracellular medium (M) from the bacterial sediment. The S-layer (S) of bacilli was extracted by boiling in 3 M urea. Extracted cells (C) were broken in a bead beater. Proteins in all fractions were precipitated with TCA, washed in acetone and analyzed by Coomassie stained 10% SDS-PAGE. The position of Sap/EA1 is indicated by the black arrow. (B) *B. anthracis* cultures fractionated as described in panel (A) were subjected to immunoblotting with rabbit antisera raised against purified Sap, EA1, protective antigen (PA), BslA, BslO, PrsA, SecA2, or SlAP.

FIG. 4. *B. anthracis* slaP mutants are defective for the secretion of S-layer proteins Sap and EA1. (A) Vegetative forms of *B. anthracis* Sterne (WT) and its slaP mutant without plasmid or with pSN1 or pSN3 were grown to mid-log phase. Cultures were centrifuged to separate the extracellular medium (M) from the bacterial sediment. Bacilli were extracted with hot urea to remove the S-layer (S) and again centrifuged to sediment cell extracts (C). Proteins were precipitated with TCA, washed in acetone and analyzed by Coomassie-stained 10% SDS-PAGE (top panel). The position of Sap/EA1 is indicated by an arrow. Bottom panel, samples from the upper panel were subjected to immunoblotting with rabbit antisera raised against purified Sap, EA1, protective antigen (PA), BslA, BslO, PrsA, SecA2, and SlAP. (B) Vegetative forms of *B.*
*B. anthracis* Sterne (WT) and its *secA2* mutant without plasmid or with pSN1 or pSN3 were grown to mid-log phase and analyzed as described for panel A. (C) Box and whisker plot of the chain length of *B. anthracis* Sterne (WT) and its mutants with mutational lesions in *secA2*, *slaP*, or a *slaP* mutant harboring pSN3 at 3 hours post-germination. (D) Chain lengths were measured from DIC micrographs of vegetative bacilli (n=100). Scale bars represent 10 μm in length.

Statistical significance of differences in *B. anthracis* chain lengths were examined with the two-tailed Student’s t-test: WT vs. *slaP*, *P*<0.001; *slaP* vs. *slaP* (pSN3), *P*<0.001.

**FIG. 5.** *B. anthracis slaP* mutants can be complemented by affinity tagged *slaP*, which encodes a cytoplasmic protein. (A) Vegetative forms of *B. anthracis* Sterne (WT) and its *slaP* mutant without plasmid or with pSN4, harboring *slaP* with a 3′ extension of its open reading frame specifying a Strep-TAG peptide, were grown to mid-log phase. Cultures were centrifuged to separate the extracellular medium (M) from the bacterial sediment. Bacilli were extracted with hot urea to remove the S-layer (S) and again centrifuged to sediment cells, which were subsequently broken in a bead beater (C). Proteins in all fractions were precipitated with TCA, washed in acetone and analyzed by Coomassie stained 10% SDS-PAGE (top panel). The position of Sap/EA1 is indicated by the black arrow. Bottom panel, samples were subjected to immunoblotting with Strep-TAG specific monoclonal antibody, which identified Slap<sub>Strep</sub> in *B. anthracis slaP* (pSN4) cells. (B) *B. anthracis slaP* (pSN3) and *slaP* (pSN4) cells were broken in a bead beater instrument and cleared lysates subjected to affinity chromatography on Strep-tactin sepharose, eluted with desthiobiotin and analyzed by Coomassie-stained SDS-PAGE. A medium (M) sample from *B. anthracis* Sterne cultures was added as a control. The black arrow
identifies the position of Sap/EA1, the white arrowhead that of SlaP<sub>Strep</sub>. The grey arrowhead identifies the position of a <i>B. anthracis</i> protein in <i>slaP</i> (pSN3) and <i>slaP</i> (pSN4) cells that co-purified during chromatography on Strep-tactin sepharose but that did not react with Strep-TAG specific monoclonal antibody (<i>vide infra</i>). Bottom panels, samples were subjected to immunoblotting with Strep-TAG specific monoclonal antibody or with rabbit antisera raised against purified Sap or EA1.

**FIG. 6.** SlaP occurs as a soluble and a membrane associated species in <i>B. anthracis</i>. Vegetative forms of <i>B. anthracis</i> Sterne and the <i>slaP</i> mutant were grown to mid-log phase. Cultures were centrifuged to sediment vegetative forms, which were subsequently lysed in a bead beater. Beads and unbroken cells were removed by slow speed centrifugation and crude extracts were subjected to ultracentrifugation at 100,000 ×<i>g</i>, separating soluble cytoplasmic proteins in the supernatant (S) from membrane proteins in the pellet (P). Membranes were extracted on ice with 0.1 M Na<sub>2</sub>CO<sub>3</sub> and again subjected to ultracentrifugation at 100,000 ×<i>g</i>, separating peripheral membrane proteins in the supernatant (S) from integral membrane proteins in the pellet (P). All samples were analyzed by Coomassie stained 10% SDS-PAGE and immunoblotting with SlaP-specific rabbit antiserum.

**FIG. 7.** SlaP<sub>Strep</sub> association with the membranes of <i>B. anthracis</i> does not require S-layer proteins (Sap/EA1) or SecA2. <i>B. anthracis</i> mutants with mutational lesions in <i>secY2</i>, <i>secA2</i>, <i>sap</i>, <i>eag</i> or <i>sap/eag</i> were transformed with pSN4. Lysates of vegetative forms that had been broken in a bead beater instrument were subjected to membrane co-sedimentation analysis of SlaP<sub>Strep</sub>
as described in the legend to Fig. 6. All samples were analyzed by Coomassie stained 10% SDS-PAGE and immunoblotting with rabbit antiserum against Sap, EA1, L6, SecA2, SlaP, and PrsA.

FIG. 8. Localization of Sap and BsIO in the envelope of *B. anthracis* secA2 and slaP mutants. *B. anthracis* strain Sterne and its mutants with mutational lesions in *secA2* and *slaP* were fixed in 4% buffered formalin 3 hours post germination. Localization of (A) Sap and (B) BsIO were observed with specific rabbit antisera as well as secondary antibody-conjugates and counterstained with BODIPY-vancomycin to reveal the septal peptidoglycan. Images were obtained with a Leica SP5 Tandem Scanner Spectral 2-Photon confocal microscope (100× objective). Scale bars represent 2 μm.
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**Plasmids**

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This study
Table 2. Oligonucleotides used in this study

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smn122 TTTTCATATTGTATCATTCTCTAAAAAGCTAAAGAAAAACG  pSN6

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S-layer gene cluster

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FSC-A