

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 an 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. The purification of soluble SlaP from *B. anthracis*-cleared lysates did not reveal a specific ligand, and the 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- γ -glutamic acid (PDGA) tethered to the *m*-diaminopimelic acid cross bridge 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 a plasma membrane and a peptidoglycan layer, which is decorated with the secondary cell wall polysaccharide (SCWP) [\rightarrow 6)- α -GlcNAc-(1 \rightarrow 4)- β -ManNAc-(1 \rightarrow 4)- β -GlcNAc-(1 \rightarrow)_n, where α -GlcNAc is substituted with α -Gal and β -Gal at O3 and O4, respectively, and β -GlcNAc is substituted with α -Gal at O3 (8). The SCWP is tethered via GlcNAc-ManNAc linkage units to the C-6 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 the 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 noncovalent 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 interprong grooves are thought to capture the pyruvylated form of the 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). By the use of flow cytometry analyses of bacilli, mutants with inser-

tional lesions in *csaB* and *bslO* were isolated (1). BslO is an 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 (BHI) broth supplemented with 0.8% NaHCO₃ at 37°C or at 30°C when harboring the pLM4 vector (38). *Escherichia coli* strains DH5 α (22) and K1077 (*dcm dam*) (18) were cultured in Luria-Bertani broth (LB) at 30°C. Media were supplemented with 20 μ g/ml kanamycin to maintain plasmid selection in *B. anthracis* or with 50 μ g/ml kanamycin in *E. coli*. *B. anthracis* strains were sporulated in modified G (modG) medium as described previously (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 broth 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 temperature-sensitive plasmid pLM4 (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 digestion into pLM4, and transformed into *B. anthracis*. Transformants were grown for 10 h at 42°C in the presence of 20 μ g/ μ l kanamycin, diluted, and grown under the same conditions for four passages. Cultures were then diluted for another four passages into medium lacking antibiotics and grown for 10-h inter-

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TABLE 1 Bacterial strains and plasmids used in this study

| <i>B. anthracis</i> strain or plasmid | Description | Reference |
|---------------------------------------|---|------------|
| Strains | | |
| Sterne 34F2 | Wild-type (pXO1 ⁺ pXO2 ⁻) genome sequence | 49 |
| SN1 | Deletion of <i>secY2</i> (BAS2547) (nucleotides 2549507-2550730) in 34F2 | This study |
| SN2 | <i>secA2</i> ::Sp transposon insertion at nucleotide 890561 in 34F2 | |
| SN3 | <i>secA2</i> ::Sp transposon insertion at nucleotide 890732 in 34F2 | This study |
| SN4 | <i>secA2</i> ::Sp transposon insertion at nucleotide 891556 in 34F2 | This study |
| SN5 | <i>secA2</i> ::Sp transposon insertion at nucleotide 891568 in 34F2 | This study |
| SN6 | <i>secA2</i> ::Sp transposon insertion at nucleotide 891758 in 34F2 | This study |
| SN7 | <i>secA2</i> ::Sp transposon insertion at nucleotide 892627 in 34F2 | This study |
| SN8 | <i>secA2</i> ::Sp transposon insertion at nucleotide 892786 in 34F2 | This study |
| SN9 | <i>secA2</i> ::Sp transposon insertion at nucleotide 893012 in 34F2 | This study |
| SN10 | <i>secA2</i> ::Sp transposon insertion at nucleotide 893039 in 34F2 | This study |
| SN11 | Deletion of <i>sap</i> (BAS0841) (nucleotides 896758-899063) in 34F2 | This study |
| SN12 | Deletion of <i>eag</i> (BAS0842) (nucleotides 899843-902414) in 34F2 | This study |
| SN13 | Deletion of <i>sap</i> and <i>eag</i> (BAS0841 and BAS0842, respectively) (nucleotides 896758-902414) in 34F2 | This study |
| SN14 | Deletion of <i>slaP</i> (BAS0837) (nucleotides 889657-890538) in 34F2 | This study |
| SN15 | <i>bslO</i> ::Sp transposon insertion at nucleotide 1703151 in 34F2 | 16 |
| Plasmids | | |
| pLM4 | Temp-sensitive pE194 replicon; Kan ^r | 38 |
| pJK4 | P _{spac} ; <i>lacI</i> regulator; Kan ^r | 26 |
| pSN1 | pJK4 harboring <i>secA2</i> | This study |
| pSN2 | pJK4 harboring <i>secA2</i> and <i>slaP</i> | This study |
| pSN3 | pJK4 harboring <i>slaP</i> | This study |
| pSN4 | pJK4 harboring <i>slaP</i> with a C-terminal Strep tag | This study |
| pSN5 | pET15b harboring full-length <i>secA2</i> with an N-terminal His tag | This study |
| pSN6 | pET15b harboring full-length <i>slaP</i> with an N-terminal His tag | This study |

vals 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 the expression of its P_{spac} promoter was induced with 0.1 mM isopropyl-β-D-thiogalactopyranoside (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 primers smn78 and smn79. Plasmid pSN2 was generated by amplifying both *secA2* and *slaP* using primers smn29 and smn79. Strep-tagged SlaP (SlaP_{Strep}) was constructed by amplifying *slaP* with primers smn96 and smn97, which contains a 24-bp insertion of the Strep tag coding sequence immediately adjacent to the stop codon (TGGTCTCATCCTCAATTGAGAAG).

Antibody production. Recombinant SecA2 and SlaP were produced in *E. coli* BL21(DE3) cells harboring plasmids pSN5 and pSN6, respectively. Cultures grown overnight were diluted 1:50 in LB supplemented with 100 μg/ml ampicillin at 30°C. IPTG (1 mM) was added to the cultures at an A₆₀₀ of 0.5, and the expressions of SecA2 and SlaP were induced for 3 h. 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-nitrilotriacetic acid (NTA) column, and affinity-tagged proteins were eluted with 250 mM imidazole. Eluates were pooled and dialyzed in 1× phosphate-buffered saline (PBS). Five hundred micrograms 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 broth at 37°C for 3 h. Cells were fixed with 4% paraformaldehyde and subjected to flow cytometry using an LSR Fortessa instrument (BD Biosciences). Forward-scatter A (FSC-A) data were collected for 10,000 events. Files were analyzed by using FlowJo software.

Light microscopy of bacilli. Cells were fixed with 4% paraformaldehyde and imaged. Images were captured with a charge-coupled-device (CCD) camera on an Olympus IX81 microscope using a 100× or 40× objective. The chain lengths of bacilli were measured from acquired differential interference contrast (DIC) images by using ImageJ and converted to lengths in micrometers by 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 broth at 37°C for 3 h. 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 a secondary antibody conjugated to a fluorophore (Thermoscientific). Cells were counterstained with boron dipyrromethene (BODIPY)-vancomycin (Invitrogen). A Leica SP5 Tandem Scanner Spectral 2-Photon confocal microscope with a 63× objective was used to observe cells. Images were captured at various zooms to include the entire chain. The scale bar reflects the actual length in micrometers regardless of the zoom function.

S-layer fractionation. *B. anthracis* cultures grown overnight were diluted 1:100 into fresh medium and grown to an A₆₀₀ of 2. One milliliter of culture was centrifuged at 16,000 × g and separated into medium (supernatant) and pellet fractions. Proteins in the medium were precipitated with 10% (vol/vol) trichloroacetic acid (TCA) for 30 min on ice and centrifuged at 16,000 × g for 10 min. The bacterial sediments were washed twice with 1× PBS and boiled at 95°C for 10 min in 3 M urea. Cells were sedimented by centrifugation at 16,000 × g, and the supernatant (S-layer fraction) was removed. The pellet was washed twice with PBS and mechanically lysed by silica bead beating for 3 min (Fastprep-24; MP Biomedical). After the 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 × g for 10 min. Acetone was removed, and protein precipitates were dried. Samples were suspended in 50 μl of 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 gels and analyzed by Coomassie staining or electrotransferred onto a polyvinylidene difluoride (PVDF) membrane for immunoblot analysis. Proteins were detected with rabbit antisera raised against purified antigens. Strep-tagged proteins were detected by using monoclonal antibody StrepMAB (IBA). Immunoreactive products were revealed by chemiluminescent detection after incubation with a horseradish peroxidase (HRP)-conjugated secondary antibody (Cell Signaling Technology). The percentage of protein in subcellular fractions was calcu-

TABLE 2 Oligonucleotides used in this study

| Primer | Sequence ^a | Use(s) |
|---------|--|------------|
| smn11 F | <u>TTTCCCGGGTGGT</u> GATTGGACAGCTGAAA | sap |
| smn12 R | TTTGGT <u>ACCGCTAGCC</u> CAGGAACGTCTGGGAATGTT | sap |
| smn13 F | TTTGGT <u>ACCGCTAGC</u> GAAAGAAAACCTGCAACAAA | sap |
| smn14 R | TTTGAGCTCTACAGCTGCAGAAGCACGAT | sap |
| smn17 F | TTTCCCGGGCTGCGACTACAGCAAAAAGCA | eag |
| smn18 R | TTTGGT <u>ACCGCTAGC</u> TGCTGCTGTCATTGTACCTG | eag |
| smn19 F | TTTGGT <u>ACCGCTAGC</u> TGAAAAATCAGTGGGGATTG | eag |
| smn20 R | TTT <u>GAGCTCG</u> ACCAATCCATGCCTGCTAT | eag |
| smn23 F | TTTCCCGGGTGGCACAATAAAATCAACCTTT | secY2 |
| smn24 R | TTTGGT <u>ACCGCTAGC</u> TGTCGCCTCCTATATTTTATCA | secY2 |
| smn25 F | TTTGGT <u>ACCGCTAGC</u> TGTCGACTTGAACAATGAA | secY2 |
| smn26 R | TTT <u>GAGCTCCAATT</u> TGTAATTTGCACGTGACTGGT | secY2 |
| smn29 F | TTTTCTAGAAATGCTGAATTCGGTAAAAAAGC | pSN1, pSN2 |
| smn30 R | TTTGGT <u>ACCTT</u> ATTGTACGTTTTTCAGGAACACC | pSN1 |
| smn78 F | TTTTCTAGAAATGTTATCATTCTAAAAAAGCTAAGAA | pSN3 |
| smn79 R | TTTGGT <u>ACCTT</u> AGTCTTGTGGTACAAGTGC | pSN2, pSN3 |
| smn86 F | TTTTCCCGGGGAGCAAGAAGCTGATTTAATCG | slaP |
| smn87 R | CGAGCCTCTCTATGACTCGAGACTAGTAATCTTTCACCTCGTTATTGTACGTTTTTC | slaP |
| smn90 F | ACGAGGTGAAAGATTACTAGTCTCGAGTCATAGAGAGGCTCGCCTC | slaP |
| smn91 R | TTTTGAATTCGTGCAAAGTGTAGGTGGTGAT | slaP |
| smn96 F | TTTTCCATGGCAGCAGCATGTTATCATTCTAAAAAAGCTAAGAAAAACG | pSN4 |
| smn97 R | TTTTGGTCTCAGATTACTTCTCAAATGAGGATGAGACCAGTCTTGTGGTACAAAGTGCTTTC | pSN4 |
| smn137 | TTTTCTCGAGATGCTGAATTCGGTAAAAAAGC | pSN5 |
| smn138 | TTTTGGATCCTTATTGTACGTTTTTCAGGAACACC | pSN5 |
| smn122 | TTTTCATATGTTATCATTCTAAAAAAGCTAAGAAAAACG | pSN6 |
| smn95 | TTTTGGATCCTCAGATTAGTCTTGTGGTACAAGTGCTTTC | pSN6 |

^a Primer sequences encompassing restriction enzyme cleavage sites are underlined.

lated by averaging the ratio of immunoblot signals from one fraction to the sum of the signal in all fractions of three trials. Protective antigen (PA) (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 SlaP_{Strep}. *B. anthracis* vegetative forms were diluted 1:50 from a culture grown overnight in BHI broth supplemented with 0.8% sodium carbonate and 20 µg/ml kanamycin, induced with 1 mM IPTG, and grown to an A_{600} of 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 min at $1,000 \times g$ to remove beads and unbroken cells. Lysates were then subjected to ultracentrifugation at $100,000 \times g$ for 30 min. Cleared lysates were loaded onto Strep-tactin Sepharose preequilibrated 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* cultures grown overnight were diluted 1:50 into fresh medium, the expression of SlaP_{Strep} was induced with 1 mM IPTG, and bacilli were grown to an A_{600} of 1.5. Vegetative bacilli were sedimented by centrifugation, suspended in cytosol buffer (50 mM HEPES [pH 7.5], 10 mM magnesium acetate (MgOAc), 66 mM potassium acetate [KOAc]), and lysed by bead beating. Lysates were centrifuged for 5 min at $1,000 \times g$ to remove beads and unbroken cells, and the supernatant was subjected to ultracentrifugation at $100,000 \times g$ 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 min on ice and then subjected to ultracentrifugation at $100,000 \times g$ for 30 min. Proteins in the supernatant and pellet were precipitated with TCA, separated on 10% SDS-PAGE gels, and analyzed by Coomassie staining or immunoblotting.

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 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 that of an age-matched wild-type (WT) 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* contains a second gene, BAS5038, whose 835-amino-acid product displays 51% sequence identity to *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 medium and monitored for changes in optical density (A_{600}) over time. The growth curves of *B. anthracis* Sterne and its *secA2* mutants were superimposable, indicating that *secA2* is not required for growth (data not shown). Three hours after the inoculation, i.e., during the early exponential phase, culture aliquots were analyzed by light micros-

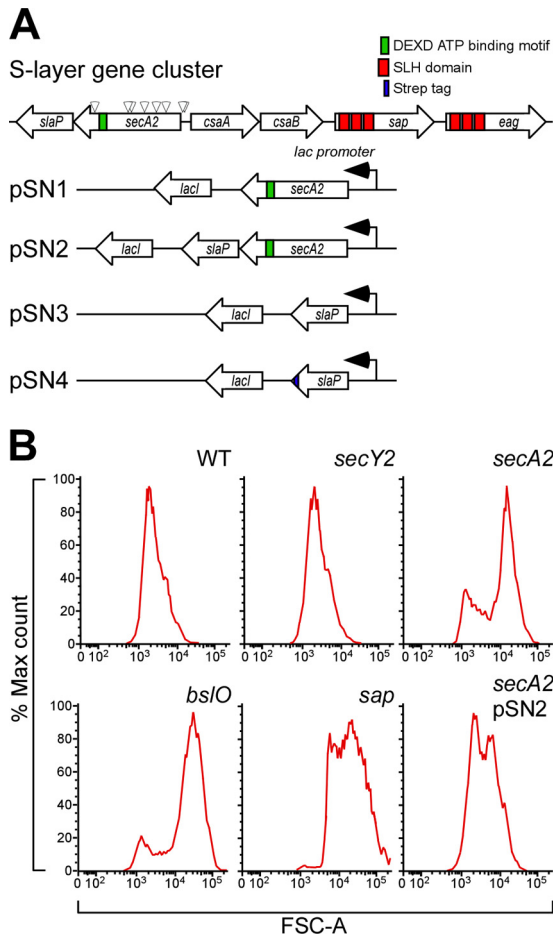


FIG 1 Isolation of *Bacillus anthracis* mutants that display chain length phenotypes. (A) Diagram illustrating the *B. anthracis* chromosomal gene cluster for the expression of S-layer proteins (*sap* and *eag* encoding signal peptide-bearing precursors with three SLH domains), the pyruvylation of the *B. anthracis* secondary cell wall polysaccharide (*csaB*), as well as the secretion of S-layer proteins, including the *secA2* gene product, a predicted ATPase with a DEXD binding motif, and the *slaP* gene product. Arrowheads positioned above the *secA2* gene identify nine different *bursa aurealis* insertional lesions that confer a chain length phenotype to 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 gene products via the *lacI*-controlled, IPTG-inducible P_{spac} promoter. (B) The chain length of *B. anthracis* strain Sterne (WT) 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 (harboring *secA2* and *slaP*) to analyze the complementation of the chain length phenotype of the *secA2* insertional lesion.

copy (Fig. 2). *B. anthracis* Sterne formed chains that were on average $34 (\pm 22.68) \mu\text{m}$ in length (Fig. 2A). As previously reported, the chain lengths of *B. anthracis* Sterne or its mutants are variable when many different chains are analyzed ($n = 100$) (1) (Fig. 2A). The average chain length of bacilli increases during the early exponential phase and gradually decreases as cultures enter the late exponential phase (1). At a 3-h growth interval, the average chain length of the *secA2* mutant was increased to $83 (\pm 62.05) \mu\text{m}$ ($P < 0.001$ for the WT versus the *secA2* mutant). Similar increases in chain lengths (*secA2* mutants versus the WT) were observed when

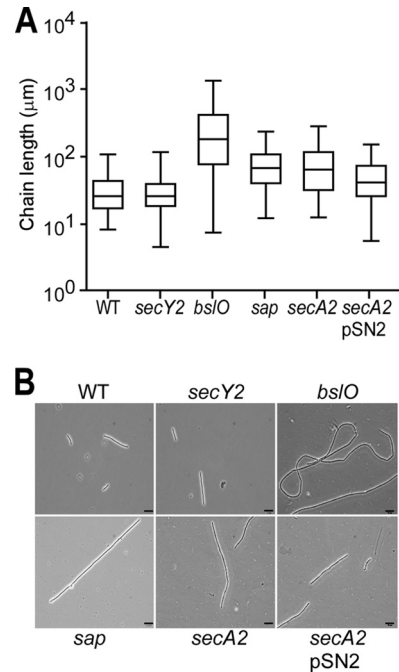


FIG 2 Chain lengths of *B. anthracis secA2* mutants. (A) Box-and-whisker plot of the chain length of *B. anthracis* Sterne (WT) and its mutants with mutational lesions in *secY2*, *bslO*, *sap*, or *secA2* or a *secA2* mutant harboring pSN2 at 3 h postgermination in BHI medium. (B) Chain lengths were measured from DIC micrographs of vegetative bacilli ($n = 100$). Scale bars represent $10 \mu\text{m}$. The statistical significance of differences in *B. anthracis* chain lengths were examined with the two-tailed Student *t* test [$P > 0.5$ for the WT versus the *secY2* mutant, $P < 0.001$ for the WT versus the *bslO* mutant, $P < 0.001$ for the WT versus the *sap* mutant, $P < 0.001$ for the WT versus the *secA2* mutant, and $P < 0.001$ for the *secA2* versus the *secA2*(pSN2) mutant].

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, carrying wild-type *secA2* and *slaP* [$51 (\pm 31.66) \mu\text{m}$; $P < 0.001$ for the *secA2* versus the *secA2*(pSN2) mutant]. As a control, a mutant with a *bursa aurealis* insertion in the *bslO* gene formed chains with a greatly exaggerated length of $298 (\pm 290.46) \mu\text{m}$ (Fig. 2) (1). Furthermore, a mutant lacking *sap*, the gene encoding the surface array protein, also formed chains that were increased in length ($79 [\pm 47.52] \mu\text{m}$; $P < 0.001$ for the WT versus the *sap* mutant). 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 preprotein 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 to *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 the *secY* gene product (23), the gene was designated *secY*. To test whether BAS2547 functions as an accessory secretion gene in the same pathway as *B. anthracis secA2*, we deleted its open reading

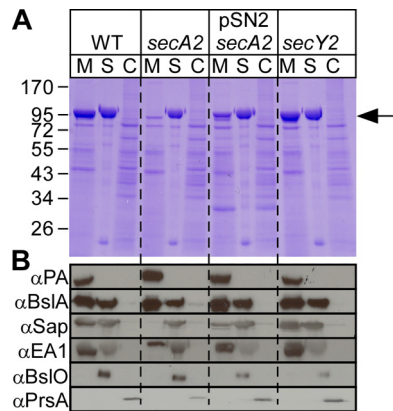


FIG 3 *B. anthracis* *secA2* mutants are defective for the secretion of the S-layer proteins Sap and EA1. (A) Vegetative forms of *B. anthracis* Sterne (WT), its *secA2* or *secY2* mutant, or a *secA2* mutant harboring pSN2 were grown to the mid-log phase. Cultures were centrifuged to separate the extracellular 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 with Coomassie-stained 10% SDS-PAGE gels. The position of Sap/EA1 is indicated by a black arrow. (B) *B. anthracis* cultures fractionated as described above for panel A were subjected to immunoblotting with rabbit antisera raised against purified Sap, EA1, PA, BslA, BslO, PrsA, SecA2, or SlaP.

frame. Mutants lacking BAS2547 were viable and without general defects in protein secretion (see below). We therefore designated BAS2547 *secY2*. The *secY2* mutant did not display a *B. anthracis* chain length phenotype ($32 [\pm 20.44] \mu\text{m}$; $P > 0.5$), suggesting that *secY2* and *secA2* may not function in the same accessory secretion pathway (Fig. 1B and 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 an A_{600} of 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 from *B. anthracis* cells in the sediment. The peptidoglycan of bacilli was broken with glass beads to release cellular proteins. Proteins in the medium and cellular 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 ($47\% \pm 8.48\%$) and in the S layer ($42\% [\pm 13.48\%]$) of *B. anthracis* Sterne cultures (Fig. 3); however, only small amounts of Sap ($11\% [\pm 5.11\%]$) 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). PA, a secreted toxin component (64, 68), was found in the medium (Fig. 3). Most of the EA1 protein was detected in the medium of *B. anthracis* Sterne cultures ($53\% [\pm 15.51\%]$), while the remainder was found in the S layer (Fig. 3). The S-layer-associated protein BslO was detected only 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 ($7\% [\pm 4.50\%]$) and EA1 ($13\% [\pm 12.52\%]$) 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 ($33\% [\pm 0.41\%]$ secreted Sap and $48\% [\pm 14.32\%]$ secreted EA1), which harbors the *secA2* and *slaP* genes (see below) from the IPTG-inducible P_{spac} promoter (Fig. 1A and 3). The subcellular locations of PA, BslA, BslO, and PrsA were not affected in the *B. anthracis* *secA2* mutant (Fig. 3B). The deletion of the *secY2* gene did not have an effect on S-layer protein secretion, as $48\% (\pm 14.62\%)$ Sap and $57\% (\pm 23.80\%)$ EA1 were detected in the medium of *B. anthracis* *secY2* mutant cultures (Fig. 3B).

***B. anthracis* *slaP* mutants display a chain length phenotype and a partial defect in Sap and EA1 secretion.** Plasmid pSN1 harbors wild-type *secA2* under the control of an IPTG-inducible promoter; the 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. 1A). Compared to *B. anthracis* Sterne, the mutant was defective for the efficient secretion of Sap ($10\% [\pm 3.00\%]$) and EA1 ($15\% [\pm 15.28\%]$), whereas the secretion of PA and BslA was not altered (Fig. 4A). We therefore designated BAS0837 *slaP* (*B. anthracis* S-layer assembly protein). Similar to *B. anthracis* *secA2* mutants, the variant lacking *slaP* displayed an increased chain length ($76 [\pm 46.97] \mu\text{m}$; $P < 0.001$ for the WT versus the *slaP* mutant) (Fig. 4C). The transformation of the *slaP* mutant with pSN3, a plasmid carrying wild-type *slaP* under the control of the IPTG-inducible P_{spac} promoter, restored the efficient secretion of Sap ($32\% [\pm 9.91\%]$) and EA1 ($55\% [\pm 8.64\%]$) (Fig. 4A) and reduced the increased-chain-length phenotype of the *slaP* mutant (Fig. 4C and D). In contrast, the 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*, *Bacillus cereus*, and *Bacillus 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 harboring a *slaP* variant with a 3' extension of its open reading frame for eight codons (WSHPQFEK) specifying a Strep tag (pSN4) (Fig. 1) was constructed. Similar to pSN3, the transformation of the *slaP* mutant with pSN4 restored the efficient secretion of the 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) cells were subjected to affinity chromatography on Strep-tactin Sepharose,

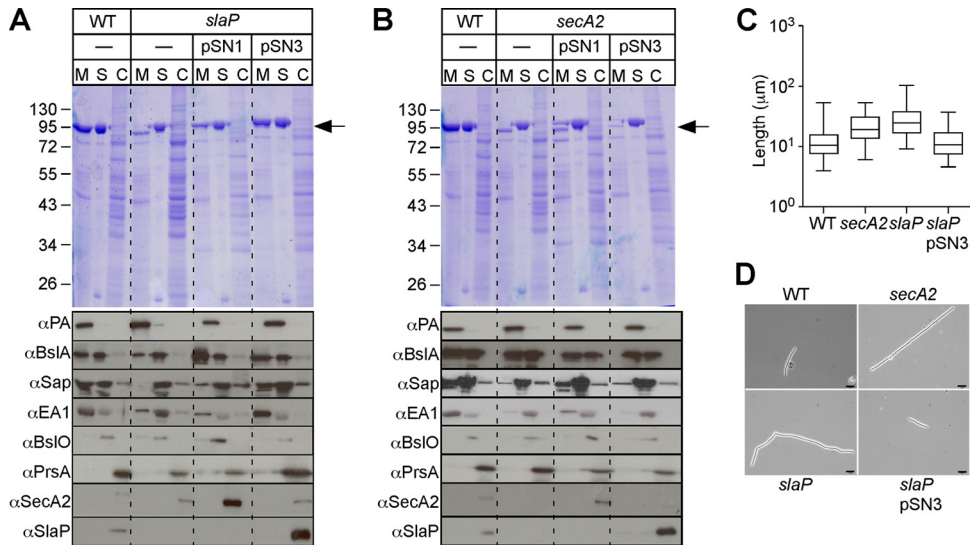


FIG 4 *B. anthracis slaP* mutants are defective for the secretion of S-layer proteins Sap and EA1. (A) Vegetative forms of *B. anthracis* Sterne (WT) and its *slaP* mutant without a plasmid or with pSN1 or pSN3 were grown to the mid-log phase. Cultures were centrifuged to separate the extracellular medium (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). (Top) Proteins were precipitated with TCA, washed in acetone, and analyzed with Coomassie-stained 10% SDS-PAGE gels. The position of Sap/EA1 is indicated by an arrow. (Bottom) Samples from the top panel were subjected to immunoblotting with rabbit antisera raised against purified Sap, EA1, PA, BslA, BslO, PrsA, SecA2, and SlaP. (B) Vegetative forms of *B. anthracis* Sterne (WT) and its *secA2* mutant without a plasmid or with pSN1 or pSN3 were grown to the mid-log phase and analyzed as described above for panel A. (C) Box-and-whisker plot of the chain lengths of *B. anthracis* Sterne (WT), its mutant with mutational lesions in *secA2* or *slaP*, or a *slaP* mutant harboring pSN3 at 3 h postgermination. (D) Chain lengths were measured from DIC micrographs of vegetative bacilli ($n = 100$). Scale bars represent 10 μm. The statistical significances of differences in *B. anthracis* chain lengths were examined with the two-tailed Student *t* test [$P < 0.001$ for the WT versus the *slaP* mutant and $P < 0.001$ for the *slaP* versus the *slaP*(pSN3) mutant].

eluted with desthiobiotin, and analyzed with Coomassie-stained SDS-PAGE gels (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) cells. Mass spectrometry experiments with tryptic peptides derived from the 32-kDa polypeptide confirmed its identity as SlaP_{Strep} (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, and not the eluate of *B. anthracis slaP*(pSN3) cells, harbored SlaP_{Strep} (Fig. 5B). The 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 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 SlaP_{Strep} does not copurify 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* functions.

SlaP associates with the plasma membrane of *B. anthracis*.

To examine the membrane association of SlaP, a 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 ultracentrifugation to separate soluble cytoplasmic components in the supernatant from integral membrane proteins and membrane-associated proteins in the sediment (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 from integral membrane proteins in the sediment (pellet). Proteins in all fractions were precipitated with trichloroacetic acid (TCA) and analyzed with Coomassie-stained SDS-PAGE gels and by immunoblotting (Fig. 6). Following fractionation, most of SlaP sedimented with the membranes of *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).

Membrane association of SlaP does not require S-layer proteins or SecA2. We wondered whether the membrane association of SlaP is caused by an 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_{Strep} from the bacterial membrane (Fig. 7). This was tested; however, mutants lacking *secY2*, *secA2*, *sap*, *eag*, or both S-layer protein genes (*sap eag*) displayed similar patterns of SlaP_{Strep} associations with the membranes of bacilli; 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 (*secA2* and *secY2*) nor the genes for the S-layer protein substrates of SlaP and SecA2-mediated secretion are required for the association of SlaP_{Strep} with the bacterial membrane.

BslO is mislocalized in *B. anthracis secA2* and *slaP* mutants.

Spores of *B. anthracis* strain Sterne and its *secA2* and *slaP* mutants were germinated for 3 h. Cells were fixed in 4% paraformaldehyde and stained for Sap and BslO by using specific rabbit antisera and a secondary antibody conjugated to a fluorophore. BODIPY-van

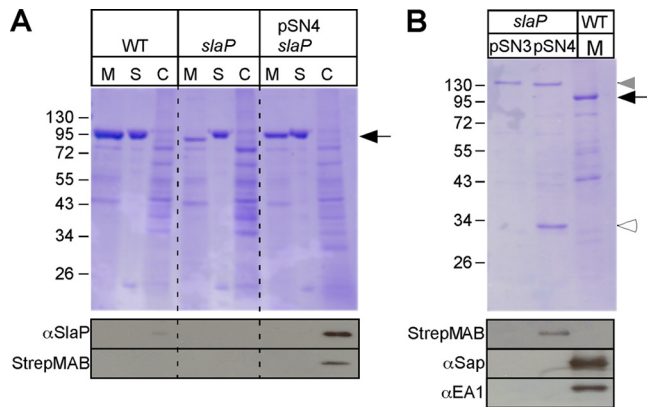


FIG 5 *B. anthracis* *slaP* mutants can be complemented by affinity-tagged *slaP*, which encodes a cytoplasmic protein. (A) Vegetative forms of *B. anthracis* Sterne (WT) and its *slaP* mutant without a plasmid or with pSN4, harboring *slaP* with a 3' extension of its open reading frame specifying a Strep-tagged peptide, were grown to the 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). (Top) Proteins in all fractions were precipitated with TCA, washed in acetone, and analyzed with Coomassie-stained 10% SDS-PAGE gels. The position of Sap/EA1 is indicated by a black arrow. (Bottom) Samples were subjected to immunoblotting with a Strep-tag-specific monoclonal antibody, which identified SlaP_{Strep} 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 were subjected to affinity chromatography on Strep-tactin Sepharose, eluted with desthiobiotin, and analyzed with Coomassie-stained SDS-PAGE gels. A medium (M) sample from *B. anthracis* Sterne cultures was added as a control. The black arrow identifies the position of Sap/EA1, and the white arrowhead identifies that of SlaP_{Strep}. The gray arrowhead identifies the position of a *B. anthracis* protein in *slaP*(pSN3) and *slaP*(pSN4) cells that copurified during chromatography on Strep-tactin Sepharose but that did not react with the Strep-tag-specific monoclonal antibody (see below). Shown at the bottom are samples that were subjected to immunoblotting with a Strep-tag-specific monoclonal antibody or with rabbit antisera raised against purified Sap or EA1.

comycin was used to stain peptidoglycan lipid II, which accumulates in the septum between adjacent vegetative cells (1). Bacilli were imaged with a Leica SP5 Tandem Scanner Spectral 2-Photon confocal microscope. As noted in the accompanying article by Kern et al. (29a), 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 signals for BslO and BODIPY-vancomycin (Fig. 8B) (1). The distribution of Sap in the envelope of *secA2* and *slaP* mutant cells appeared uneven, with patches of strong fluorescence intensity (Fig. 8A). Unlike its physiological septal localization in wild-type bacilli, BslO was found to be deposited in patches throughout the envelope of *B. anthracis* *secA2* and *slaP* mutant vegetative forms. The localization of BslO in the *secA2* and *slaP* mutants was restored to the septal position when the wild-type *secA2* or *slaP* gene products were expressed from complementing plasmids (Fig. 8B). These data suggest that the secretion defect of the *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).

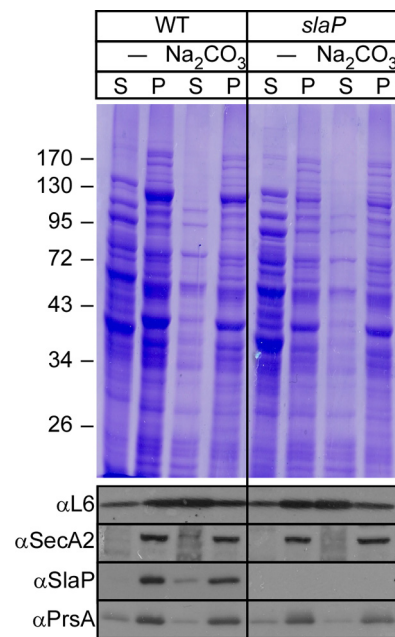


FIG 6 SlaP occurs as soluble and membrane-associated species in *B. anthracis*. Vegetative forms of *B. anthracis* Sterne and the *slaP* mutant were grown to the mid-log phase. Cultures were centrifuged to sediment vegetative forms, which were subsequently lysed in a bead beater. Beads and unbroken cells were removed by slow-speed centrifugation, and crude extracts were subjected to ultracentrifugation at 100,000 × g, separating soluble cytoplasmic proteins in the supernatant (S) from membrane proteins in the pellet (P). Membranes were extracted on ice with 0.1 M Na₂CO₃ and again subjected to ultracentrifugation at 100,000 × g, separating peripheral membrane proteins in the supernatant from integral membrane proteins in the pellet. All samples were analyzed with Coomassie-stained 10% SDS-PAGE gels and by immunoblotting with SlaP-specific rabbit antiserum.

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 for *E. coli*, is operational in *B. anthracis*, signal peptide-bearing precursors would likely use the ATPase SecA and the membrane translocon SecY/SecE/SecG complex as well as the SecD/SecF/YajC complex for their travels across the plasma membrane (54). The 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 an 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 cotranslational 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 the *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*

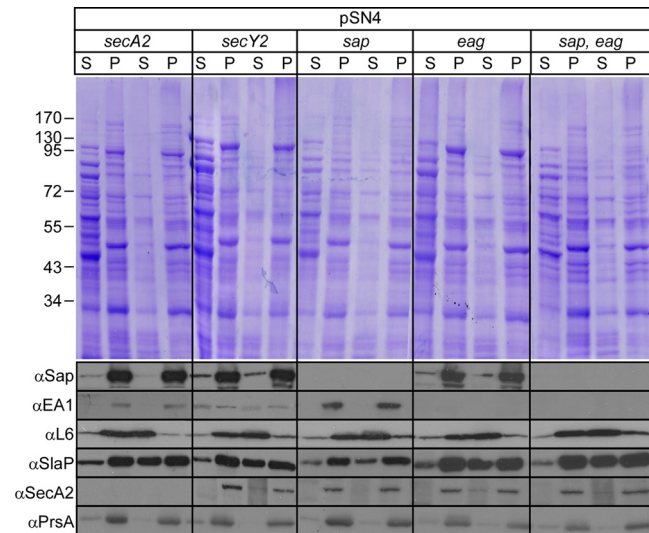


FIG 7 SlaP_{Strep} association with the membranes of *B. anthracis* does not require S-layer proteins (Sap/EA1) or SecA2. *B. anthracis* mutants with mutational lesions in *secY2*, *secA2*, *sap*, *eag*, or *sap* and *eag* were transformed with pSN4. Lysates of vegetative forms that had been broken in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

gene but harbors 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). Due to posttranslational glycosylation, GspB cannot be secreted via the canonical SecA pathway (4, 58). A 90-residue N-terminal signal peptide as well as the first 20 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 to Asp5 (5, 56). Several Gram-positive microbes also use the 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 broader 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 a specific substrate by SecA2 or its presumed interaction with other components of the secretory pathway, are still unknown (52).

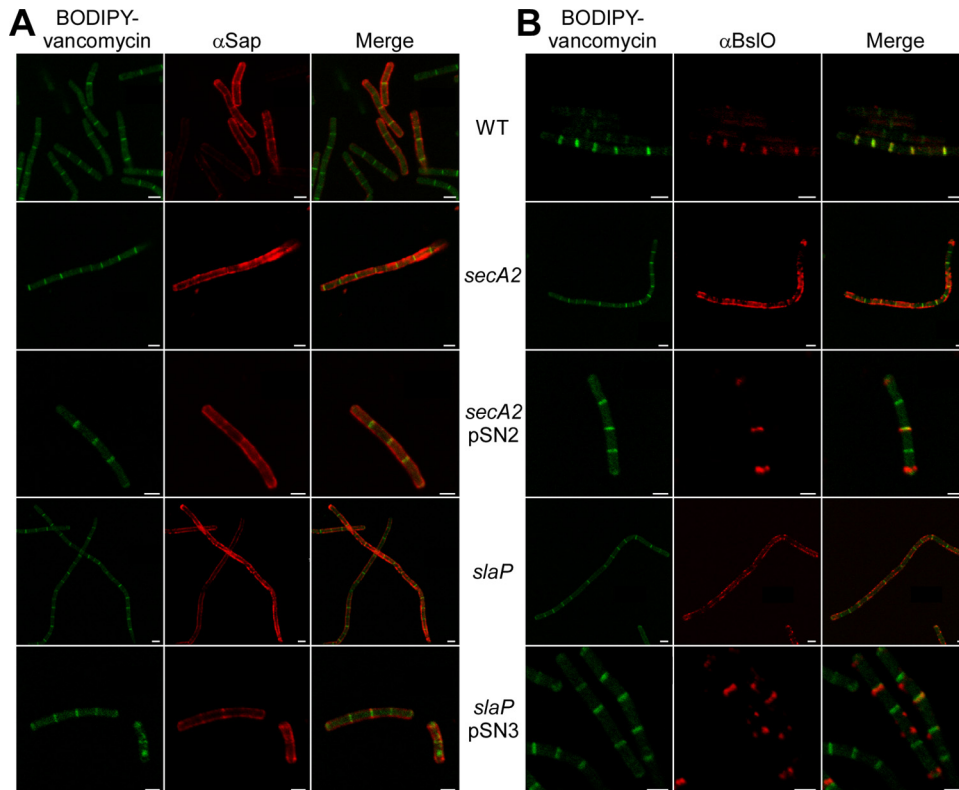


FIG 8 Localization of Sap and BsI O in the envelope of *B. anthracis* *secA2* and *slaP* mutants. *B. anthracis* strain Sterne and its mutants with mutational lesions in *secA2* and *slaP* were fixed in 4% buffered formalin at 3 h postgermination. The localizations of Sap (A) and BsI O (B) were observed 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 \times objective). Scale bars represent 2 μ m.

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, contains two *secA* genes, of which the noncanonical 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), which includes *B. cereus*, *B. anthracis*, and *B. thuringiensis*, harbor the *slaP* gene and require SLH domains within S-layer proteins for their envelope deposition via a noncovalent 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). Furthermore, 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 a dedicated S-layer secretion/assembly pathway could further involve folding catalysts enabling the transfer of proteins into the envelope and the 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 above-mentioned models for S-layer protein secretion and assembly.

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