Bacillus anthracis Sortase A (SrtA) Anchors LPXTG Motif-Containing Surface Proteins to the Cell Wall Envelope

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Cell wall-anchored surface proteins of gram-positive pathogens play important roles during the establishment of many infectious diseases, but the contributions of surface proteins to the pathogenesis of anthrax have not yet been revealed. Cell wall anchoring in Staphylococcus aureus occurs by a transpeptidation mechanism requiring surface proteins with C-terminal sorting signals as well as sortase enzymes. The genome sequence of Bacillus anthracis encodes three sortase genes and eleven surface proteins with different types of cell wall sorting signals. Purified B. anthracis sortase A cleaved peptides encompassing LPXTG motif-type sorting signals between the threonine (T) and the glycine (G) residues in vitro. Sortase A activity could be inhibited by thiol-reactive reagents, similar to staphylococcal sortases. B. anthracis parent strain Sterne 34F2, but not variants lacking the srtA gene, anchored the collagen-binding MSCRAMM (microbial surface components recognizing adhesive matrix molecules) BasC (BA5258/BA54884) to the bacterial cell wall. These results suggest that B. anthracis SrtA anchors surface proteins bearing LPXTG motif sorting signals to the cell wall envelope of vegetative bacilli.

Sortase enzymes catalyze transpeptidation reactions on the bacterial surface, utilizing protein precursors with C-terminal sorting signals as substrates (60). Staphylococcus aureus sortase A (SrtA), the prototypic transpeptidase of this class of enzyme (31, 32), cleaves LPXTG motif-type sorting signals between the threonine (T) and the glycine (G) residues to generate an acyl enzyme intermediate (30, 38, 58). Nucleophilic attack of the amino group of cell wall crossbridges resolves the acyl enzyme (62), forming an amide bond between the carboxyl group of the C-terminal threonine of surface proteins and the cell wall crossbridge of lipid II precursor molecules (45, 61). The product of this reaction, surface protein linked to lipid II, is then incorporated into the cell wall envelope via the transpeptidation and transglycosylation reactions of peptidoglycan biosynthesis (39, 51, 57). Twenty different surface proteins with LPXTG motif-type sorting signals have been identified in the staphylococcal genome sequence (34), and deletion of the srtA gene abolishes the cell wall anchoring and surface display of all sortase A substrates (31). As a result, staphylococcal srtA mutants display significant defects in the pathogenesis of murine experimental anthrax (64, 72). For example, corynebacterial sortases cleave precursor proteins in a manner that leads to the assembly of pili, high-molecular-weight polymerization products several microns long on the bacterial surface (65). Two domains of pilus surface proteins, the sorting signal and the pilin motif, are required for this reaction, which occurs in a sortase-specific manner. This results in the assembly of different types of pili by dedicated pairs of sortase enzymes and pilin subunit proteins (59, 65).

The 5.2-Mb genome of B. anthracis strain Ames, a fully virulent isolate, and those of its two virulence plasmids, pXO1 and pXO2, have been sequenced (40, 41, 49). Analysis of the genome sequence identified nine predicted surface protein genes encoding sorting signals with LPXTG motif sequences and three sortase genes (49). One of these genes, BA0688 (srtA), displays striking similarity to the srtA gene of S. aureus, whereas BA4783 (srtB) more closely resembles S. aureus srtB. A third gene, BA5069 (srtC), is homologous to sortase genes in other bacilli (B. cereus, B. halodurans, and B. subtilis) but is not found in staphylococci, listeriae, or corynebacteria (12).

We wondered whether B. anthracis surface proteins are an-
chored to the cell wall envelope by sortases in a manner similar to that observed for staphylococci, streptococci, and listeriae (2–5, 14, 31). If so, analysis of the phenotypic defects of sortase mutants during animal infections may reveal the relative contribution of all of these surface molecules to the pathogenesis of anthrax disease. Herein, we achieved a first step towards this goal by reporting the requirement of the srtA gene for the cell wall anchoring of BasC, an LPXTG motif-type surface protein (71). Furthermore, purified SrtA catalyzed a sortase A-type cleavage reaction with LPETG and LPATG peptides, consistent with the notion that B. anthracis srtA is responsible for the cell wall anchoring of surface proteins with an LPXTG motif.

### MATERIALS AND METHODS

**Bacterial strains and plasmids.** The bacterial strains and plasmids used in this study are summarized in Table 1. All cultures of bacilli were grown overnight in Luria broth with 0.5% glucose at 30°C and, after dilution, were incubated in fresh medium at 37°C. Antibiotics were added to cultures for plasmid selection as follows: 100 μg/ml ampicillin (E. coli), 5 μg/ml erythromycin (B. anthracis), and 10 μg/ml chloramphenicol (B. anthracis). B. anthracis Sterne 34F5 (56) was used as a parent strain in this study.

The srtA mutant AHG263 was obtained by allelic replacement, introducing an erythromycin resistance gene (ermC) (54) into the srtA locus. Briefly, bacillus template DNA was isolated using the Wizard Genomic DNA purification kit (Promega) after 2 h of vegetative cell treatment with 10 mg/ml lysozyme at 37°C. 5’ and 3’ srtA-flanking sequences were PCR amplified from B. anthracis Sterne template DNA using the primer pairs SrtA-N-BamHI (CGGGATCCAGAT AACGTCGACGGCAGAT AAAGCCTGGCTGTCAG) and SrtA-N-Ala (TCCCCCGGGAAAAGGTTT TTTAATGAAATGACTCAGCTGAGTAG) as well as SrtA-Ala-3 (TCCCCCGGGAAAAGGTTT TTTAATGAAATGACTCAGCTGAGTAG) and SrtA-Ala-N (TCCCCCGGGAAAAGGTTT TTTAATGAAATGACTCAGCTGAGTAG) from SrtA-N-Ala (TCCCCCGGGAAAAGGTTT TTTAATGAAATGACTCAGCTGAGTAG) and SrtA-Ala-N (TCCCCCGGGAAAAGGTTT TTTAATGAAATGACTCAGCTGAGTAG). The srtA 5’-flanking (PstI/XmaI), ermC (released from pAHG316 with XmaI digestion) (35) and srtA 3’-flanking sequences (XmaI/NotI) were cloned in that order, into pLC28 (10) to generate pAHG71. The 3.2-kb 5’-srtA-ermC-3’-srtA cassette was excised with PstI and NotI restriction, and the ends were filled with Klenow polymerase and then cloned into the Smal site of the temperature-sensitive shuttle vector pTS1 (36), thereby generating pAHG107. The plasmid was transformed into B. anthracis Sterne using a previously developed protocol (53) and transformants were selected on LB agar with chloramphenicol. Allochic exchange was induced with a temperature shift to 43°C and erythromycin selection. The mutant allele obtained with this procedure (B. anthracis AHG188) was transduced using the CP-51 phage (20) and verified by Southern blot and Western blot.

For Southern blots, chromosomal DNA from both the wild-type and AHG263 strains was digested with SacI, which cleaves srtA-flanking sequences as well as the ermC coding sequence. The products of the digestion were separated by electrophoresis, transferred to a positively charged nylon membrane (Roche), and probed with either srtA or ermC nucleic acid sequences. Probes were generated by PCR in the presence of digoxigenin-dUTP (DIG System, Roche Molecular Biochemicals) to label the reaction products. The srtA probe was amplified using primers SrtA-5-EcoRI and SrtA-3-PstI (see below), whereas primers Erm5 (TACACCTCCGGATAATAAA) and Erm3 (CACAAGACACTCTTTT TAC) were used to generate the ermC probe. Hybridization products were detected by chemiluminescence.

To analyze bacilli for the presence of sortase A in immunoblot assays, wild-type and mutant strains were grown in 4-ml cultures to an optical density of 600 nm (OD600) 0.8. Cells were harvested by centrifugation at 13,000 × g for 5 minutes and suspended in 1 ml TSM (50 mM Tris-HCl, pH 7.5, 500 mM sucrose, 10 mM MgCl₂) and treated with 10 μg/ml lysozyme at 37°C for 2 h. Proteins were precipitated with trichloroacetic acid (TCA) and subjected to immunoblotting with anti-SrtA rabbit antibody raised from purified SrtA₃⁴ (see below).

**Purification of SrtA₃⁴ protein.** Primers SrtA-N-BamHI (CGGGATCCAGAT AACGTCGACGGCAGAT AAAGCCTGGCTGTCAG) and SrtA-C-BamHI (CGGGATCCATTTATGAGATATGATC) were used to PCR amplify the srtA gene from B. anthracis Sterne template DNA. The DNA fragment was digested with BamHI and cloned into pQE30 (Qiagen) to generate pAHG316, which was then transformed into Escherichia coli XL1-Blue. Overnight cultures grown in the presence of 100 μg/ml ampicillin were diluted into 1 liter of fresh culture and then induced for expression with 1 mM isopropylthiogalactopyranoside (IPTG). When the culture reached an OD₆₀₀ of 1.2, cells were harvested by centrifugation at 6,000 × g for 20 min, washed, and suspended in 20 ml of buffer A [50 mM Tris-HCl, 150 mM NaCl (pH 7.5)]. Bacteria were lysed in a French pressure cell at 6,000 psi, washed, and suspended in 20 ml of buffer B [50 mM Tris-HCl, 150 mM NaCl, 300 mM sucrose, 10 mM MgCl₂, 10 μM EDTA, 1% Triton X-100]. Proteins were precipitated with trichloroacetic acid (TCA) and subjected to immunoblotting with anti-SrtA rabbit antibody raised from purified SrtA₃⁴ (see below).

**In vitro analysis of sortase A activity.** Sortase activity was assayed in buffer A at 37°C for 15 h. The 2-aminobenzoyl (Abz)-LPETG-diaminopropionic acid (TCA) and subjected to immunoblotting with anti-SrtA rabbit antibody raised from purified SrtA₃⁴ (see below).
The active-site signature sequence is boxed. The N-terminal residues encompassing the deletion of *B. anthracis* Ames (BA-Ames) BA0688 (SrtA), BA4783 (SrtB), and BA5069 (SrtC). Identical residues are highlighted in red and conserved residues are in blue.

Characterization of sortase A cleavage products. Sortase reactions containing 10 μM of Abz-LPETG-Dnp and 15 μM of recombinant enzyme in a final volume of 1 ml of buffer A were incubated at 37°C for 15 h. The enzyme was removed by centrifugation on Centricon-10 (Millipore) at 7,500 x g for 15 min. Proteins in the supernatant (cleared lysate) were purified by Ni-NTA affinity chromatography as described above. All purified proteins were separated by SDS-PAGE, transferred to a polyvinylidene difluoride membrane, and BasCFLAG/MH6 was detected using a FLAG-specific monoclonal antibody (Stratagene) and chemiluminescence.

Infection of A/J mice with *B. anthracis* spores. *B. anthracis* Sterne as well as AHG263 mutant spores were obtained by transferring 2 ml of an overnight culture grown in LB-0.5% glucose to 30 ml of 2X SP medium (28). Cells were incubated for 4 days at 37°C with vigorous shaking, and the development of spore preparations. Inbred A/J mice (Harlan) were used to investigate the virulence of the *sortA* mutant strain AHG263. Mice were infected subcutaneously with 1.56 x 10^2, 1.27 x 10^3, 2.03 x 10^4 and 1.98 x 10^5 wild-type spores or 1.36 x 10^2, 1.67 x 10^3, 1.93 x 10^4 and 1.66 x 10^5 mutant spores. Ten mice per dose and strain were infected, and the 50% lethal dose (LD₅₀) for each strain was calculated following the Reed and Muench method (50).

RESULTS

Sortase genes in the genomes of *B. anthracis* strains Ames and Sterne. A bioinformatic approach was used to identify homologs of *S. aureus* sortA in the genome of *B. anthracis* strain Ames. BLAST searches identified BA0688, BA4783, and BA5069 as homologs of staphyloccocal sortA (Fig. 1). BLAST searches were also used for pairwise comparison between the three *B. anthracis* sortases and *S. aureus* SortA and SortB (Fig. 1).
BA0688, here putatively assigned as sortase A (SrtA), displayed 27% amino acid identity with *S. aureus* SrtA and 25% identity with *S. aureus* SrtB, whereas BA4783 [sortase B (SrtB)] encompasses 21% identity with staphylococcal SrtA and 42% identity with *S. aureus* sortase B. The third homolog, BA5069, displayed 27% identity with *S. aureus* SrtA and 29% identity with SrtB, but significantly higher degrees of identity were observed with homologs from *B. subtilis*, *B. halodurans*, or *B. cereus* (12). BA5069 was assigned as sortase C (SrtC).

The genome sequence of the vaccine strain *B. anthracis* strain Sterne 34F2 has been completed (NCBI). As the present work focused on characterizing sortase genes in the attenuated vaccine strain, we examined the Sterne genome for sortase genes using BLAST searches. Again, three sortases were identified, BAS0654 (SrtA), BAS4438 (SrtB), and BAS4708 (SrtC), and their amino acid sequences were identical to those of *B. anthracis* strain Ames (Fig. 1).

**Purified *B. anthracis* sortase A cleaves LPXTG peptides between the threonine and the glycine residues.** To characterize the gene product of BA0688, the coding sequence of *B. anthracis* *srtA* was PCR amplified with oligonucleotide primers in a manner that deleted the first 27 amino acid residues, encoding the signal peptide/membrane anchor of sortase A (Fig. 1). The DNA fragment was cloned into the plasmid vector pQE30 (QIAGEN), and expression was induced with IPTG via the *lac* promoter. The resulting hexahistidyl gene fusion product, *B. anthracis* SrtA<sub>ΔN</sub>, was purified from cleared lysates via affinity chromatography on nickel-NTA-Sepharose and eluted with imidazole. After dialysis, protein concentration was determined and purity was assessed by Coomassie-stained SDS-PAGE (Fig. 2A). The identity of the purified protein was verified by matrix-assisted laser desorption ionization (MALDI) mass spectrometry (data not shown).

Fluorescence of the Abz fluorophore (a) within the peptide *a*-LPETG-*d* is quenched by the close proximity to Dpn (d), a fluorescence quencher (58). When the peptide is cleaved by *S. aureus* sortase A and the Abz fluorophore is separated from Dpn, an increase in fluorescence is observed (22). Incubation of purified *B. anthracis* SrtA<sub>ΔN</sub> with *a*-LPETG-*d* substrate resulted in an increase in fluorescence similar to that observed for purified *S. aureus* sortase A (58) (Fig. 2B). To test whether cleavage required the only thiol moiety (Cys187) of *B. anthracis* SrtA, the enzyme was incubated with [2-(trimethylammonium)-ethyl] methane-thiosulfonate (MTSET), a reagent that reacts rapidly with the active-site thiol of *S. aureus* SrtA (63, 74). Indeed, MTSET treatment abolished all *B. anthracis* SrtA cleavage of *a*-LPETG-*d* substrate. Methanethiosulfonate forms disulfide with sulfhydryl groups, and this product of inhibition can be resolved by reducing agents such as dithiothreitol. MTSET-inactivated *B. anthracis* SrtA<sub>ΔN</sub> was incubated in the presence of 10 mM dithiothreitol, which restored most of the enzymatic activity (Fig. 2B). Together with the observation that *B. anthracis* SrtA<sub>ΔN</sub> failed to cleave scrambled peptide sequence (GLETTP) or *S. aureus* sortase B substrate (NPQTN) (data not shown), these observations suggest that *B. anthracis* sortase A cleaves the LPXTG motif of surface protein sorting signals in vitro. The specific activity of *B. anthracis* SrtA<sub>ΔN</sub> is similar to that of *S. aureus* sortase A, which should render this enzyme useful for future biochemical studies (30, data not shown).

The *B. anthracis* SrtA cleavage product of the *a*-LPETG-*d* substrate was analyzed in an effort to ascertain whether sortase cleaved between the threonine and the glycine residues, similar to *S. aureus* SrtA. Reaction products were separated by reverse-phase high-performance liquid chromatography, and absorbance at 215 nm was recorded (62). Eluted peaks were retrieved from collected fractions, and the molecular identity of compounds was verified by mass spectrometry. Mock-treated *a*-LPETG-*d* eluted at 58 min, but incubation of substrate with *S. aureus* SrtA<sub>ΔN</sub> generated two product peaks, which eluted at 42 min (G-d) and 47 min (*a*-LPET) from reverse-phase high-performance liquid chromatography (Fig. 3). Incubation of *a*-LPETG-*d* substrate with *B. anthracis* SrtA<sub>ΔN</sub> generated product species identical to those observed for *S. aureus* SrtA<sub>ΔN</sub> by reverse-phase high-performance liquid chromatography and mass spectrometry (Fig. 3). Together these data reveal that *B. anthracis* SrtA cleaves LPETG peptides between the threonine and glycine residues.

**B. anthracis** vegetative cells express sortase A. Purified *B. anthracis* SrtA<sub>ΔN</sub> was injected subcutaneously into rabbits to raise specific antibodies and the immune sera were used in immunoblotting experiments of bacterial extracts obtained...
from vegetative cells of \textit{B. anthracis} strain Sterne. An immune-reactive species with an estimated mobility of 23 kDa was detected on 15\% SDS-PAGE (Fig. 4). Further, detection of the 23-kDa species occurred by immune but not by preimmune sera, consistent with the notion that this species may represent sortase A (BA0688).

To confirm the identity of the 23-kDa immune-reactive species, the sortase A gene was deleted by allelic replacement with the \textit{ermC} marker, and the mutation was then transduced into \textit{B. anthracis} strain Sterne with bacteriophage CP-51 (20), generating strain AHG263. DNA extracted from parent strain Sterne was subjected to nucleotide hybridization (Southern blot) analysis with the \textit{srtA} probe, spanning gene coding and flanking sequences (Fig. 4A). A 4,361-bp ClaI fragment was detected with the \textit{srtA} probe, whereas Southern blotting of ClaI-restricted DNA from the mutant strain AHG263 detected 2,331- and 3,124-bp fragments. Probing ClaI-digested chromosomal DNA with labeled \textit{ermC} DNA also revealed the 2,331- and 3,124-bp \textit{Δ(srtA):ermC} fragments. As expected, the \textit{ermC} probe did not detect hybridizing species in the chromosomal DNA of \textit{B. anthracis} strain Sterne (Fig. 4B). These data demonstrate that the \textit{srtA} coding sequence in strain AHG263 had been replaced with \textit{ermC}.

Immunoblotting with anti-SrtA failed to detect the 23-kDa immune-reactive species in strain AHG263, but transformation of the mutant strain with plasmid encoded sortase A (pAHG279) not only restored the appearance of the 23-kDa immune-reactive species but also led to the overexpression of sortase A (48) (Fig. 4C). As a control, transformation of strain AHG263 with pOS1 vector DNA did not affect the expression of the 23-kDa immune-reactive species (Fig. 4C). Together these results indicate that anti-SrtA detects sortase A expression in \textit{B. anthracis} strain Sterne vegetative cells.

\textit{B. anthracis} strain Sterne encodes eleven putative surface protein genes. Previous work used amino acid sequences of mature, anchored surface proteins or their cell wall sorting signals as queries in BLAST searches to identify protein genes in newly sequenced genomes of Gram-positive bacteria (12, 49). Höök and colleagues used a similar approach to identify two \textit{B. anthracis} homologs (BA0871 and BA5258) of \textit{S. aureus} CNA, a collagen-binding MSCRAMM (microbial surface components recognizing adhesive matrix molecules) expressed by \textit{S. aureus} strains that have been isolated from osteomyelitis and connective tissue infections (43, 44). Recently published work demonstrated that purified recombinant surface proteins...
signals with LPXTG motifs. Equal efficiency, whereas the LGATG and NPKTG peptides were not cleaved. Further, only a small amount of cleavage of LPATG peptides was observed, suggesting that the preferred substrates of sortase A are surface protein sorting signals with LPXTG motifs.

BA0871 and BA5258 indeed bind collagen in a manner that resembles collagen binding of S. aureus CNA (70).

A bioinformatics approach was used here for the preliminary identification of surface protein genes in B. anthracis strains Ames and Sterne. In addition to BA0871 and BA5258, BLAST searches with the sorting signals from 20 known staphylococcal surface protein genes identified nine other genes in the genome of strain Sterne. Table 2 summarizes the identified surface protein genes of B. anthracis, their sorting signal motifs, predicted functions, and NCBI identification numbers. In addition to the nine known surface protein genes from the Ames strain, B. anthracis strain Sterne encodes a collagen adhesin (BA5207) (12). The surface protein gene BA5070 encodes the heme-binding protein substrate of S. aureus sortase A substrate recognition (22, 61).

The B. anthracis isd locus encodes IsdB, the heme-binding protein substrate of S. aureus sortase B (33, 35, 55). However, in contrast to S. aureus IsdC substrate with an NPQTN motif, B. anthracis SrtB is presumed to cleave its substrate at an NPKTG motif (73). Two predicted surface proteins encompass C-terminal sorting signals with LPNTA motifs (BA0397 and BA0383) and were assigned as putative sortase C substrates, as one of the two genes is positioned immediately adjacent to srtC (BA0699) (12). The surface protein gene BA0552, with an LGATG motif sorting signal, displays homology to the leucine-rich domain of Listeria monocytogenes internalins (19). A prediction for sortase recognition of this presumed gene product has thus far not been achieved (12, 17) (Table 2).

To examine substrate recognition by B. anthracis sortase A in vitro, purified SrtAAN was incubated with a-LPETG-d, a-LPATG-d, a-LGATG-d, a-NPKTG-d, and a-LPNTA-d peptides and substrate cleavage was measured by fluorescence resonance energy transfer analysis (Fig. 5). B. anthracis sortase A cleaved the LPETG and LPATG peptides with equal efficiency, whereas the LGATG and NPKTG peptides were not cleaved. Further, only a small amount of cleavage of LPNTA peptide was observed, suggesting that the preferred substrates of B. anthracis sortase A are surface protein sorting signals with LPXTG motifs.

### Table 2. Bioinformatic identification of B. anthracis surface proteins

<table>
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<tr>
<th>Protein</th>
<th>Signal</th>
<th>Function</th>
<th>No. of amino acids</th>
<th>Motif</th>
<th>Predicted sortase</th>
<th>Reference(s)</th>
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<tr>
<td>BasA</td>
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<td>2',3' cyclic nucleotide 2' phosphodiesterase</td>
<td>774</td>
<td>LPKTG</td>
<td>SrtA</td>
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<td>LPATG</td>
<td>SrtA</td>
<td>49</td>
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<tr>
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<td>Collagen adhesin</td>
<td>627</td>
<td>LPATG</td>
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<td>595</td>
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<td>372</td>
<td>LPNAG</td>
<td>SrtA</td>
<td>49</td>
</tr>
<tr>
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<td>882</td>
<td>LPNTG</td>
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<td>LPNIA</td>
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<td>148</td>
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<td>BasJ</td>
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<td>Internalin/LRR</td>
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<td>LGATG</td>
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<td>Heme transport</td>
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<td>NPKTG</td>
<td>SrtB</td>
<td>49</td>
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</table>

**a** BAS, Bacillus anthracis surface protein.
**b** NCBI identification number.
**c** Number of codons in the entire open reading frame.
**d** See text for details on the assignment of sortase substrates.

### B. anthracis srtA is required for the cell wall anchoring of BasC (BA5258).

To test whether B. anthracis sortase A is required for the cell wall anchoring of surface proteins in vivo, we focused on BasC (BA5258). Our experimental design employed a recombinant basC gene, modified by in-frame insertion of a short nucleotide sequence encoding the FLAG epitope and a six-histidyl tag (BasCFLAG/MH6) (35) (Fig. 6A). Plasmid pAHG227 encoding BasCFLAG/MH6 was transformed into B. anthracis strain Sterne or its isogenic variant AHG239 (ΔsrtA). Whole-cell extracts of the transformants were subjected to affinity chromatography on Ni-NTA and eluted fractions were analyzed by immunoblotting with anti-FLAG monoclonal antibody, revealing immune-reactive BasCFLAG/MH6 species. B. anthracis parent strain Sterne immune-reactive species migrated more slowly on SDS-PAGE than BasCFLAG/MH6 expressed by AHG239, an observation that is consistent with the posttranslational anchoring of surface protein to the cell wall envelope (39) (Fig. 6). As a control, transformation of...
strain AHG239 with the plasmid vector pOS1 did not generate anti-FLAG immune-reactive species.

To further examine the cell wall anchoring of BasCFLAG/MH6, the cell wall envelope was digested with muramidase under conditions that stabilized the resulting proteolipids. Following sedimentation of bacilli via centrifugation, the cell wall lysate was removed with the supernatant and subjected to affinity chromatography on Ni-NTA. Immunoblotting of eluted fractions revealed the presence of BasCFLAG/MH6 in the cell wall fraction of B. anthracis strain Sterne but not in the cell wall envelope of the sortase mutant strain AHG239 (Fig. 6B). However, transformation of strain AHG239 with pAHG322 (wild-type srtA) restored the cell wall anchoring of BasCFLAG/MH6 as immuno-reactive species could be purified from cell wall lysates of pAHG316 transformants, but not from lysates of strains harboring the vector control plasmid pOS1 (Fig. 6B).

As a control for proper fractionation, ribosomal protein L6 was detected by immuno-blotting in whole-cell lysates but not in the cell wall compartment of bacilli (Fig. 6D).

srtA gene of B. anthracis strain Sterne is not required for the development of acute anthrax disease in A/J mice. B. anthracis secretes lethal toxin and edema toxin to cause anthrax disease (11). Three pXO1 virulence plasmid genes encode subunits for both toxins, pag (protective antigen), lef (lethal factor), and cya (edema factor), as protective antigen performs binding and host cell transport functions for both lethal factor and edema factor (11). The toxin genes are known to be essential for disease progression in multiple animal models of anthrax cutaneous infection, including guinea pigs and A/J mice (6, 7, 46). Further, antibodies against protective antigen appear to be a critical factor in protective immunity (23, 67).

B. anthracis strain Sterne lacks the pXO2 virulence plasmid, encoding the capABCD genes responsible for synthesis of the poly-D-glutamic acid capsule (18, 40, 56). The glutamate capsule of B. anthracis is essential for the pathogenesis of cutaneous anthrax infections in mice and presumably in many other animal infections (16), but strain Sterne retains significant virulence in the A/J mouse model, as these animals display significant defects in phagocytic killing of bacterial pathogens (68, 69).

B. anthracis strain Ames LD50 doses of 50 spores are required for the development of lethal anthrax disease in mice (46), whereas a subcutaneous LD50 dose of 106 Sterne spores is required to generate a similar disease (46). Welkos, Friedlander and colleagues showed that subcutaneous infection of A/J mice with B. anthracis strain Sterne spores leads to an acute lethal disease at a dose of 102 to 103 spores (68). To test whether sortase A and therefore sortase A-anchored surface proteins play a role in the pathogenesis of anthrax disease, A/J mice were infected subcutaneously with 1.56 × 102, 1.67 × 103, 1.36 × 104, 1.93 × 105 and 1.66 × 105 spores of its isogenic variant AHG239. Figure 7 displays the data of a time-to-death analysis for both strains. Death occurred between days 2 and 4 following inoculation with a calculated LD50 dose of 632 spores for B. anthracis Sterne and 1,110 spores for strain AHG239. The observed difference in LD50 doses between the two strains was not statistically significant. We also analyzed the replication of anthrax bacilli in infected tissues (liver, spleen, brain, and blood) and observed no significant difference in replication between strain Sterne and AHG239 (data not shown).

DISCUSSION

Secretion of lethal toxin and edema toxin by vegetative bacilli is critical in the pathogenesis of anthrax disease and crucial
for toxin-mediated killing of infected hosts (11). Early events in anthrax pathogenesis are much less understood (37). For example, the genetic requirement for entry of spores across respiratory or intestinal epithelia has not been fully explored (16). Other fundamental questions, whether spores specifically bind to host cell receptors to mediate entry and germination or use surface proteins on vegetative cells for binding to specific tissues or invasion of host cells, have not been addressed (37). Bioinformatic analysis of the *B. anthracis* genome sequence identified surface proteins such as the collagen adhesin BasC that may function as MSCRAMMs in binding to connective tissues, and an interalin-like molecule that could mediate host cell invasion of anthrax bacilli (49, 71). Although bioinformatic analysis provides a guide for physiological function, the relative contribution of cell wall-anchored surface proteins to disease pathogenesis can therefore be gleaned by comparing the virulence of sortase mutant strains with the wild-type parent. In *S. aureus*, sortase mutants display a three log reduction in the ability to form abscesses, a dramatic defect that remains the largest reduction in virulence for staphylococcal knockout mutants (26, 31, 66). Sortase A mutations in *L. monocytogenes* on the other hand displayed only a modest defect in the pathogenesis of acute listeriosis in mice, although the cell wall anchoring of about 20 different internal-lins is thought to be abrogated (3, 4). It is presumed that a second anchoring mechanisms of listerial surface proteins, binding of internalin B to lipoteichoic acids, plays a critical role in bacterial invasion of mouse cells and is responsible for the manifestation of disease (13, 15, 24). Presumably, sortase A-anchored proteins may play a much larger role during the pathogenesis of listerial infections in humans (27).

In this report we have characterized sortase A of *B. anthracis* and examined *srtA* mutations for their ability to cause disease in the A/J mouse model. Similar to *srtA* mutations in *L. monocytogenes*, we observed no significant defect in the ability to cause acute lethal disease. Does this indicate that sortase A-anchored surface proteins are dispensable for anthrax disease in animals or humans? We think not. Mice are not a physiological host for *B. anthracis*, and mice in fact appear hypersensitive to anthrax disease following the injection of spores (46, 47). For example, mutants lacking the gene for protective antigen, an essential component for the delivery of lethal and edema toxins, do not display a phenotype in a murine infection model with virulent *B. anthracis* strains (9, 46). In contrast, a dramatic defect for protective antigen mutants can be observed in a guinea pig infection model (47). Thus, although our data provide evidence that *srtA* is not required for *B. anthracis* strain Sterne pathogenesis in the A/J mouse model of disease, additional work is needed to reveal the contribution of LPXTG-type surface proteins in other models of anthrax disease.

While the studies here can presumptively assign five or seven surface protein substrates to sortase A in strains Ames and Sterne, the substrates of *B. anthracis* sortases B and C remain unknown. In fact, the identification of four different types of sorting signal motifs and their relationship with three sortases remains an enigma that can only be resolved experimentally. Future work must therefore focus on the identification of surface protein substrates for sortases and their contribution to anthrax disease in several different animal models of infection.

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**REFERENCES**


**FIG. 7.** Sortase A gene of *B. anthracis* strain Sterne is not required for anthrax disease in the A/J mouse model. A/J mice, 10 per group, were infected by subcutaneous injection of 0.1 ml of spore suspension in phosphate-buffered saline and observed for a lethal infection over a period of 14 days (data points did not change after day 6). Both death and time-to-death were recorded and analyzed for spore infection doses of $1.56 \times 10^2$, $1.27 \times 10^3$, $2.03 \times 10^3$ and $1.98 \times 10^3$ for *B. anthracis* Sterne and $1.36 \times 10^2$, $1.67 \times 10^3$, $1.93 \times 10^3$ and $1.66 \times 10^3$ for strain AHG263 (determined by enumerating colonies on Luria agar after incubation at 37°C). The legend identifies data points for parent strain Sterne $34F_2$ (open symbols) and the isogenic *ΔsrtA* variant (solid symbols). Using these data and the Reed and Muench method (50), LD$_{50}$ doses for strain Sterne $34F_2$ (632 spores) and the *ΔsrtA* strain AHG239 variant (1,110 spores) were calculated.

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