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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 34F$_2$, but not variants lacking the *srtA* gene, anchored the collagen-binding MSCRAMM (microbial surface components recognizing adhesive matrix molecules) BasC (BA5258/BAS4884) 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 organ abscesses, infectious arthritis, or endocarditis (25, 26, 31, 66).

The genomes of most gram-positive bacteria encode two or more sortase enzymes, which fulfill different functions (12, 42). For example, *S. aureus* sortase B is involved in anchoring IsdC (iron-regulated surface determinant C), a polypeptide with an NPQTN motif sorting signal, to the cell wall envelope (29, 33, 35). *Streptococcus pyogenes* sortases A and B both anchor surface proteins with LPXTG motif sequences to the envelope. These two sortases appear to recognize unique surface protein substrates using the LPXTG motif as well as other features of cell wall sorting signals (2). Streptococcal SrtC2 recognizes surface protein substrates with QVPTGV motif sorting signals, consistent with the view that different sortases recognize unique sets of substrates (1).

Perhaps the most astonishing sortase-catalyzed reaction is the assembly of pili on the surface of corynebacteria, actinomycetales, enterococci, group B streptococci, and pneumococci (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-

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TABLE 1. Bacterial strains, plasmids, and phages used in this study

Strain plasmid, or phage	Property	Reference or source
Strains		
E. coli XL-1 Blue	Expression of $srtA_{\Delta N}$	8
E. coli DH5α	Plasmid transformation	21
E. coli K1077	*dam dcm* mutant, plasmid purification for *B. anthracis* transformation	P. Model/M. Russel collection
B. anthracis Sterne $34F_2$	Vaccine strain, $pXO1^+$ and $pXO2^-$	56
B. anthracis AHG188	*ermC* replacement of *srtA* (BA0688/BAS0654) in strain Sterne	This work
B. anthracis AHG263	Δ(*srtA*)::*ermC* CP-51 transduced into strain Sterne	This work
Plasmids		
pOS1	Multicopy shuttle vector	52
pAHG279	Full-length *srtA* cloned in pOS1	This work
pAHG277	$basC_{FLAG/MH6}$ cloned in pOS1	This work
pAHG322	$basC_{FLAG/MH6}$ and *srtA* cloned in pOS1	This work
pTS1	Temperature-sensitive shuttle vector	36
pAHG107	5′-*srtA*-*ermC*-3′-*srtA* cassette in pTS1	This work
pQE30	Vector for expression cloning of His_6 fusions	Qiagen
pAHG316	$srtA_{\Delta N}$ cloned in pQE30	This work
Phage		
CP-51	*B. anthracis* transducing phage	20

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 $34F_2$ (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-51-PstI (AACTGCAGGCCAGAT AAAGCTTCGTCTAG) and SrtA-31-XmaI (TCCCCCCGGGTTCAGTCTGT TTCTTTTCAGC) as well as SrtA-52-XmaI (TCCCCCCGGGAAAGGTGTTT TATTTAGTGATATAGCTTC) and SrtA-32-NotI (ATTTGCGGCCGCAACA TTATCAAATCGCTTCC). The *srtA* 5′-flanking (PstI/XmaI), *ermC* (released from pErmC 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 SmaI 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. Allelic 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 ClaI, 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 TTC) 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 at 600 nm (OD_{600}) 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 $MgCl_2$) and treated with 10 mg/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 $SrtA_{\Delta N}$ (see below).

Purification of $SrtA_{\Delta N}$ protein. Primers SrtA-N-BamHI (CGGGATCCAAG CCATTTTATGATGGATATCA) and SrtA-C-BamHI (CGGGATCCTTATT TCTTCGCCTTCGTTCCT) 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 OD_{600} 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 14,000 lb/in^2 and extracts were centrifuged twice at 32,600 x *g* for 15 min. The supernatant was applied to 1 ml of Ni-nitrilotriacetic acid (NTA) Sepharose (QIAGEN), pre-equilibrated with buffer A. After two 10-ml washes with buffer A and one 10-ml wash with buffer B (buffer A supplemented with 10 mM imidazole), $SrtA_{\Delta N}$ was eluted in 4 ml of buffer A containing 0.5 M imidazole. The purified polypeptides were detected on Coomassie-stained sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE).

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 (Dpn), Abz-LPATG-Dpn, Abz-LGATG-Dpn, Abz-NPKTG-Dpn, and Abz-LPNTA-Dpn peptides were dissolved in dimethyl sulfoxide and added to the reaction at a final concentration of 10 μM. Purified $SrtA_{\Delta N}$ was added to the reaction at a final concentration of 15 μM. Reactions were quenched by boiling the samples for 5 min and peptide cleavage was monitored by fluorescence at 420 nm after excitation at 320 nm. The mean and standard deviation of three independent measurements are reported. Inhibition of $SrtA_{\Delta N}$ activity was achieved by addition of MTSET [2-(trimethylammonium)ethyl]methanethiosul-

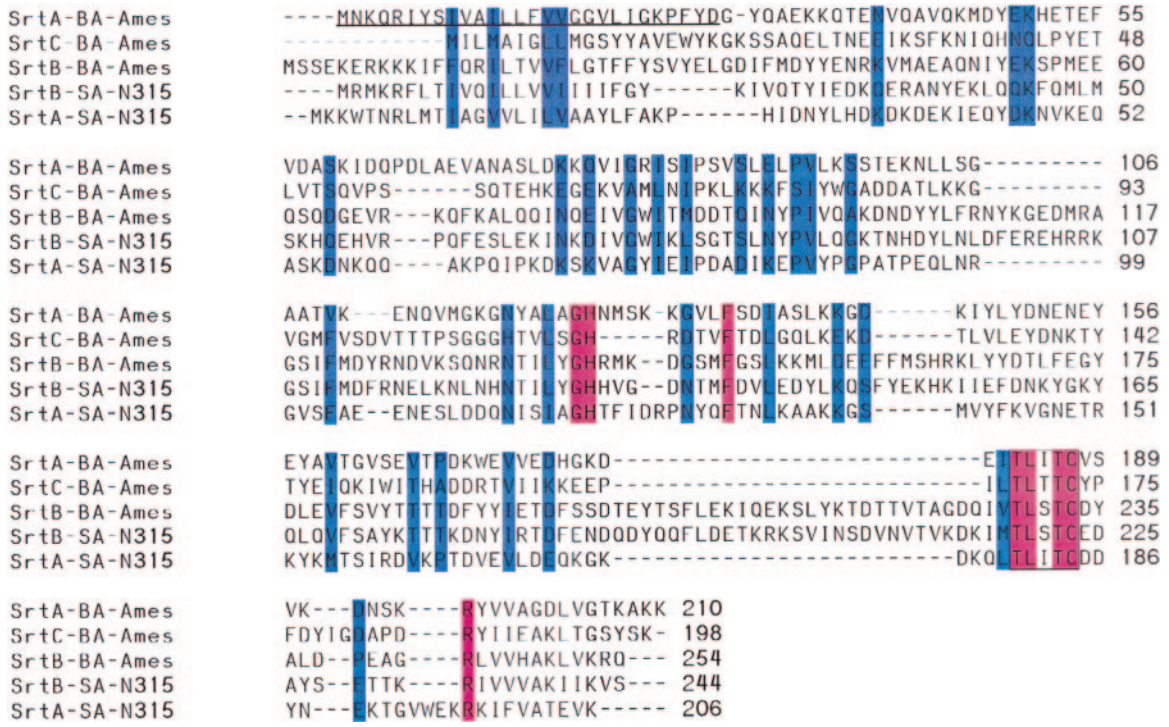

FIG. 1. Multiple sequence alignment of *Staphylococcus aureus* N315 (SA-N315) sortase A (SrtA) and sortase B (SrtB) and *Bacillus anthracis* Ames (BA-Ames) BA0688 (SrtA), BA4783 (SrtB), and BA5069 (SrtC). Identical residues are highlighted in red and conserved residues are in blue. The active-site signature sequence is boxed. The N-terminal residues encompassing the deletion of *B. anthracis* $SrtA_{\Delta N}$ generated for affinity chromatography purification of soluble sortase enzyme are underlined.

fonate at a concentration of 5 mM. This inhibition was relieved by supplementing the reaction with 10 mM dithiothreitol.

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* and the filtrate was subjected to reverse-phase high-performance liquid chromotography purification on a C-18 column (2 by 250-mm, C18 Hypersil, Keystone Scientific). Cleaved products were eluted following the application of a linear gradient of acetonitrile from 1 to 100%. Eluted peptides were monitored at 215 nm.

Cell wall anchoring of $BasC_{FLAG/MH6}$. Plasmids used throughout this study were generated by ligating PCR-amplified DNA fragments at unique restriction sites into the shuttle vector pOS1 (52). To generate pAHG279, the *srtA* gene was amplified using primers SrtA-5-EcoRI (AAGAATTCTGTATCGACTGTTCTTTATAAAG) and SrtA-3-PstI (AACTGCAGTATTAATGGGAGTATGGTTAGC). For the construction of pAHG227, *basC* sequences specifying promoter nd Flag-tagged N-terminal portions of the polypeptide were amplified with the primers BasC-51-E (AAGAATTCTTGTATTAAGATGGGTTCGTTT) and BasC-31-K-Flag (CGGGATCCTTTGTCATCGTCGTCCTTGTAGTCATTATGAGTACTAGGTTGTTGCA). Amplification of C-terminal *basC* coding sequence with a six-His-tag and cell wall sorting signal required the primers BasC-52-K-MH6 (GGGGTACCATGCACCATCACCATCACCATGAAGTGCGTTTACCAGCTACT) and BasC-32-B (CGGGATCCCTACATTTCTTTTCTATTTTTCAT). pAHG322 was generated after insertion of the *srtA* sequence pAHG227. All ligation products were transformed into *E. coli* DH5α, and plasmid DNA of isolated transformants was analyzed by restriction analysis and DNA sequencing. Plasmids were then transformed into the *dam dcm E. coli* K1077 strain, DNA was purified without methylation products and finally transformed into *B. anthracis* cells (53).

Isolation of $BasC_{FLAG/MH6}$ was achieved by growing 400 ml of cells harboring either pAHG227 or pAHG322 to an OD_{600} of 1.0. Bacteria were harvested by centrifugation, suspended in TSM-lysozyme buffer (50 mM Tris-HCl, pH 7.5, 0.5 M sucrose, 10 mM $MgCl_2$, 10 mg/ml lysozyme), incubated for 5 h at 37°C, and sedimented by centrifugation at 32,600 x *g* for 15 min. To purify lysozyme-solubilized cell wall-anchored $BasC_{Flag/MH6}$, cleared lysates were applied to 0.5 ml of Ni-NTA-Sepharose pre-equilibrated with buffer A and bound proteins were washed once with 10 ml of buffer A followed by a second wash with 10 ml buffer B. Proteins were eluted in 4 ml of buffer C. For the preparation of whole-cell sample extracts, sedimented cells were treated with TSM-lysozyme and lysed in a French pressure cell at 14,000 lb/in^2. The lysate was centrifuged at 32,600 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 $BasC_{FLAG/MH6}$ 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 2× SG medium (28). Cells were incubated for 4 days at 37°C with vigorous shaking, and the development of spores was examined by microscopic inspection of culture aliquots. Residual vegetative cells were heat killed by incubation at 65°C for one hour. Spores were sedimented by centrifugation at 6,000 x *g*, washed three times with 50 ml of sterile distilled water, and suspended in 1 ml of sterile distilled water. CFU were enumerated after serial dilution, plating on agar medium, and colony formation of spore preparations. Inbred A/J mice (Harlan) were used to investigate the virulence of the *srtA* mutant strain AHG263. Mice were infected subcutaneously with 1.56×10^2, 1.27×10^3, 2.03×10^4 and 1.98×10^5 wild-type spores or 1.36×10^2, 1.67×10^3, 1.93×10^4 and 1.66×10^5 mutant spores. Ten mice per dose and strain were infected, and the 50% lethal dose (LD_{50}) 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 srtA* in the genome of *B. anthracis* strain Ames. BLAST searches identified BA0688, BA4783, and BA5069 as homologs of staphylococcal *srtA* (Fig. 1). BLAST searches were also used for pairwise comparison between the three *B. anthracis* sortases and *S. aureus* SrtA and SrtB (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 $34F_2$ 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_{uv5} promoter. The resulting hexahistidyl gene fusion product, *B. anthracis* $SrtA_{\Delta N}$, 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* $SrtA_{\Delta N}$ 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* $SrtA_{\Delta N}$ 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* $SrtA_{\Delta N}$ failed to cleave scrambled peptide sequence (GLETP) 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* $SrtA_{\Delta N}$ is similar to that of *S. aureus* sortase A, which should render this enzyme useful for future biochemical studies (30, data not shown).

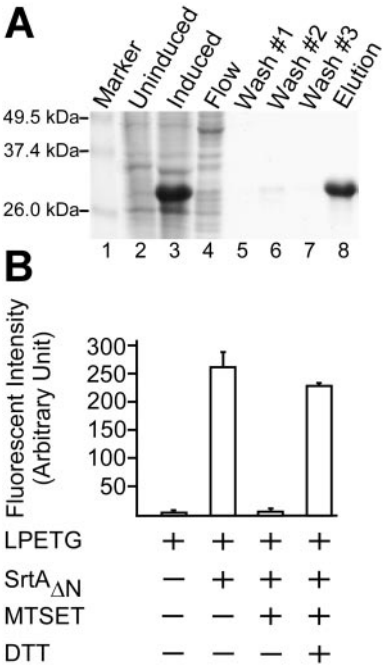

FIG. 2. Purification and characterization of *B. anthracis* sortase A (SrtA). (A) *E. coli* XL-1 Blue(pAHG316) expressing *B. anthracis* $SrtA_{\Delta N}$, in which the N-terminal membrane anchor of sortase had been replaced with a six-histidyl tag, was lysed by French press. $SrtA_{\Delta N}$ was purified by affinity chromatography on Ni-NTA and analyzed on Coomassie-stained SDS-PAGE. Lanes: 1, molecular weight markers; 2, French press extract of uninduced culture; 3, French press extract of 1 mM isopropyl β-D-thiogalactoside-induced culture; 4, cleared lysate of French press extract of 1 mM isopropyl-β-D-thiogalactoside-induced culture was loaded on the Ni-NTA column and flowthrough was collected; 5 to 7, wash buffer was used to remove contaminating protein; and 8, 1-ml fraction was eluted with 0.5 M imidazole. (B) Purified $SrtA_{\Delta N}$ was incubated with Abz-LPETG-Dpn substrate (LPETG), and cleavage was monitored as an increase in fluorescence. The reaction was inhibited by the addition of 1 mM MTSET. MTSET-treated $SrtA_{\Delta N}$ could be rescued by incubation with 10 mM dithiothreitol (DTT).

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* $SrtA_{\Delta N}$ 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* $SrtA_{\Delta N}$ generated product species identical to those observed for *S. aureus* $SrtA_{\Delta N}$ 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* $SrtA_{\Delta N}$ was injected subscapularly into rabbits to raise specific antibodies and the immune sera were used in immunoblotting experiments of bacterial extracts obtained

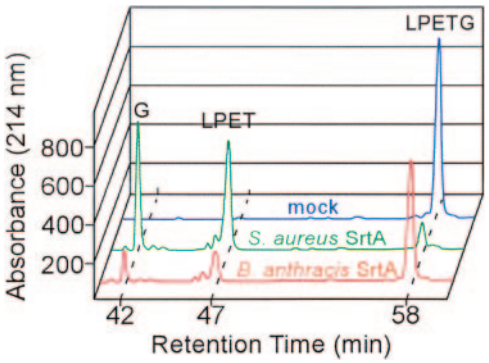

FIG. 3. *B. anthracis* sortase A (SrtA) cleaves the LPETG peptide between the threonine and glycine residues. Purified *B. anthracis* $SrtA_{\Delta N}$ was incubated with LPETG peptide (Abz-LPETG-Dpn), and cleavage products were separated by reversed-phase high-pressure liquid chromatography. Sortase substrates and products were detected by absorbance at 215 nm and characterized by mass spectrometry. As a control, mock-treated and *S. aureus* $SrtA_{\Delta N}$-treated peptides were separated by reversed-phase high-pressure liquid chromatography. Data generated from three independent experiments were averaged and standard deviations were calculated.

from vegetative cells of *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 on the 23-kDa immune-reactive species, the sortase A gene was deleted by allelic replacement with the *ermC* marker, and the mutation was then transduced into *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 *srtA* probe, spanning gene coding and flanking sequences (Fig. 4A). A 4,361-bp ClaI fragment was detected with the *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 *ermC* DNA also revealed the 2,331- and 3,124-bp Δ(*srtA*):*ermC* fragments. As expected, the *ermC* probe did not detect hybridizing species in the chromosomal DNA of *B. anthracis* strain Sterne (Fig. 4B). These data demonstrate that the *srtA* coding sequence in strain AHG263 had been replaced with *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 *B. anthracis* strain Sterne vegetative cells.

***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 *B. anthracis* homologs (BA0871 and BA5258) of *S. aureus* CNA, a collagen-binding MSCRAMM (microbial surface components recognizing adhesive matrix molecules) expressed by *S. aureus* strains that have been isolated from osteomyelitis and connective tissue infections (43, 44). Recently published work demonstrated that purified recombinant surface proteins

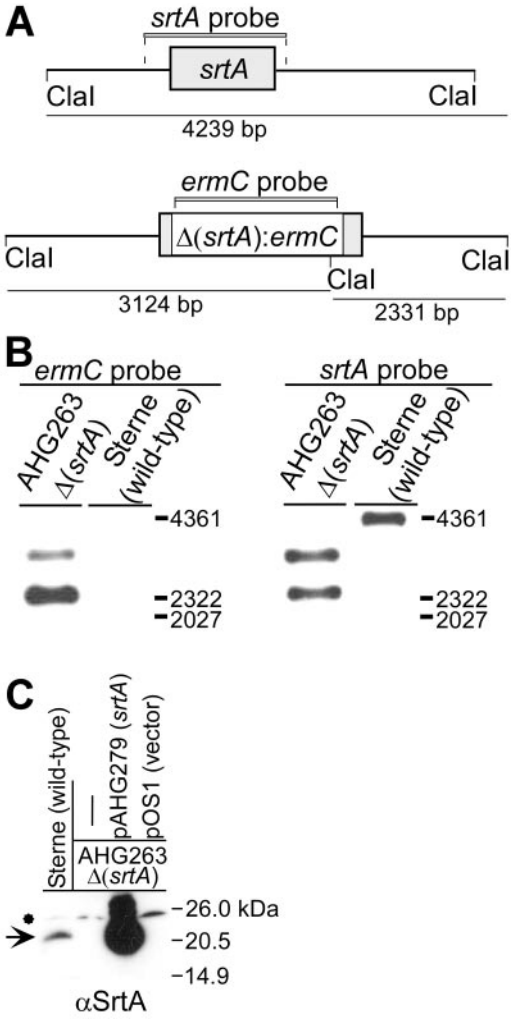

FIG. 4. *B. anthracis* vegetative cells express sortase A (*srtA*). (A) Drawing depicts the *srtA* gene (211-codon open reading frame with a predicted molecular mass of 23.1 kDa for the SrtA polypeptide) on the chromosome of *B. anthracis* strain Sterne, flanked by ClaI restriction sites that release a 4,239-bp *srtA* probe-hybridizing fragment. A *srtA* mutant was generated by allelic replacement with the *ermC* gene, thereby generating two ClaI restriction fragments (3,124 and 2,331 bp in size), each of which hybridize with the *ermC* probe and with the *srtA* probe. (B) Southern blot hybridization of ClaI-restricted chromosomal DNA fragments and their hybridization signals with labeled *srtA* and *ermC* probes. (C) The cell wall envelope of vegetative cells of *B. anthracis* strain Sterne $34F_2$ and its isogenic Δ(*srtA*)::*ermC* mutant, strain AHG263, were cleaved with lysozyme, and proteins were solubilized in sample buffer prior to SDS-PAGE and immunoblot analysis. The arrow denotes the SrtA immune-reactive species, whereas the asterisk indicates a cross-reactive species. Strain AHG263 was transformed with multicopy vector pOS1 or pAHG279, i.e., recombinant pOS1 harboring the *srtA* gene.

TABLE 2. Bioinformatic identification of *B. anthracis* surface proteins

Protein[a]	Ames[b]	Sterne[b]	Function	No. of amino acids[c]	Motif	Predicted sortase[d]	Reference(s)
BasA	BA4346	BAS4031	2′,3′ cyclic nucleotide 2′ phosphodiesterase	774	LPKTG	SrtA	49
BasB	BA0871	BAS0828	Collagen adhesin	969	LPATG	SrtA	49
BasC	BA5258	BAS4884	Collagen adhesin	627	LPATG	SrtA	49
BasD	BA3367	BAS3121	Unknown	595	LPKTG	SrtA	49
BasE	BA3254	BAS3021	Unknown	372	LPNAG	SrtA	49
BasF		BAS5207	Collagen adhesin	882	LPNTG	SrtA	This work
BasG		BAS4798	Unknown	2,025	LPKTG	SrtA	This work
BasH	BA0397	BAS0383	Unknown	257	LPNTA	SrtC	12, 49
BasI	BA5070	BAS4709	Unknown	148	LPNTA	SrtC	12, 49
BasJ	BA0552	BAS0520	Internalin/LRR[e]	1,070	LGATG	?	49
BasK	BA4789	BAS4444	Heme transport	237	NPKTG	SrtB	49

[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.
[e] LRR, leucine-rich repeats.

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 motif sequences, 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 gene (BAS5207) and BAS4798, a gene with unknown function. Of note, BA3254/BAS3021 (LPNAG motif) was assigned as the sortase A substrate, because amino acid substitution of threonine with alanine in the LPXTG motif does not interfere with *S. aureus* sortase A substrate recognition (22, 61).

The *B. anthracis isd* locus encodes IsdC, 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 BA5070) and were assigned as putative sortase C substrates, as one of the two genes is positioned immediately adjacent to *srtC* (BA5069) (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 $SrtA_{\Delta N}$ 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 fluroescence 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.

***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 ($BasC_{FLAG/MH6}$) (35) (Fig. 6A). Plasmid pAHG227 encoding $BasC_{FLAG/MH6}$ was transformed into *B. anthracis* strain Sterne or its isogenic variant AHG239 (Δ*srtA*). 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 $BasC_{FLAG/MH6}$ species. *B. anthracis* parent strain Sterne immune-reactive species migrated more slowly on SDS-PAGE than $BasC_{FLAG/MH6}$ 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

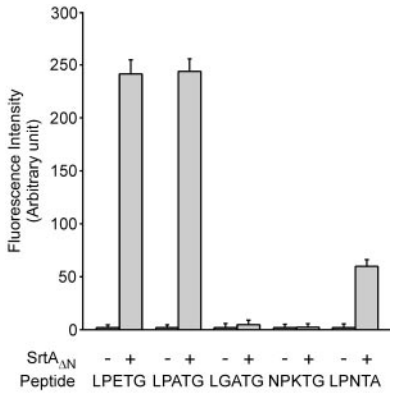

FIG. 5. Substrate specificity of *B. anthracis* sortase A (SrtA). *B. anthracis* $SrtA_{\Delta N}$ was purified as described in the legend to Fig. 2 and incubated with presumed substrate peptides Abz-LPETG-Dpn (LPETG), Abz-LPATG-Dpn (LPATG), Abz-LGATG-Dpn (LGATG), Abz-NPKTG-Dpn (NPKTG), and Abz-LPNTA-Dpn (LPNTA). Substrate cleavage was monitored as an increase in fluorescence. Data generated from three independent experiments were averaged and standard deviations were calculated.

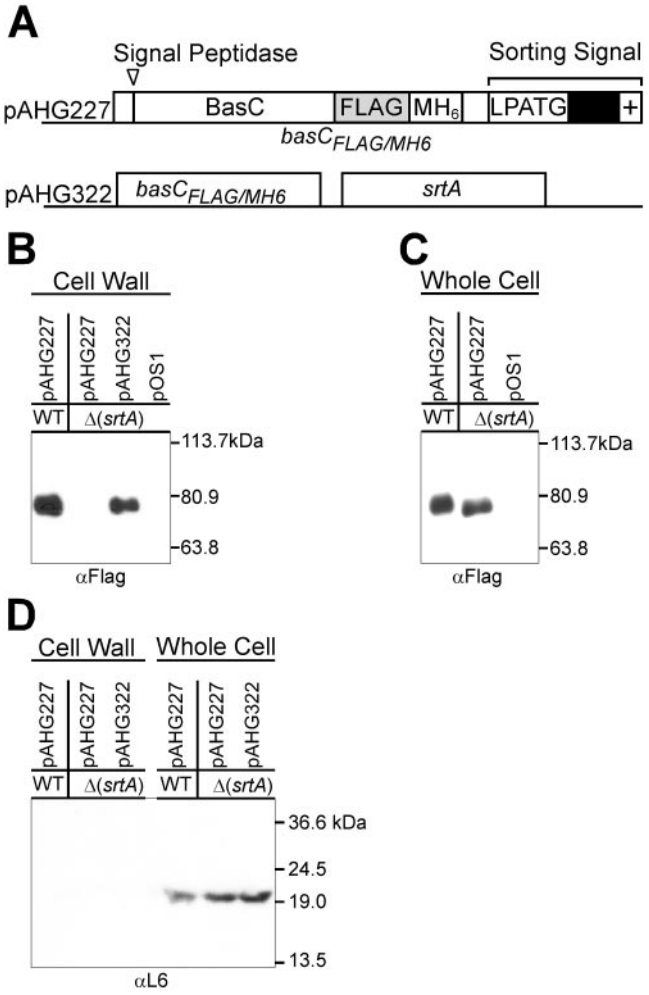

FIG. 6. *B. anthracis srtA* gene is required for cell wall anchoring of BasC. (A) Plasmid pAHG227 expresses the $basC_{FLAG/MH6}$ gene. A FLAG epitope tag and methionyl-six-histidyl (MH_6) affinity chromatography tag were inserted upstream of the cell wall sorting signal (pAHG227). (B) After transformation of vegetative cells with pAHG227 or empty vector plasmid (pOS1), mid-log-phase bacilli were harvested by centrifugation and washed, and the cell wall envelope was digested with lysozyme. Cleared crude lysate of *B. anthracis* vegetative cell extracts was subjected to affinity chromatography, and eluates were analyzed for the presence of $BasC_{FLAG/MH6}$ using FLAG monoclonal antibodies and immunoblotting. As a control, bacilli were transformed with pOS1 vector or with pAHG322, which carries an insertion of the *B. anthracis srtA* gene into pAHG227 and was used for complementation studies. (C) The cell wall envelope of mid-log-phase bacilli was digested with lysozyme. After sedimentation of the resulting protoplasts, the cell wall lysate was applied to affinity chromatography on Ni-NTA and eluted with imidazole. Proteins in the eluate were then subjected to SDS-PAGE and immunoblotting with anti-FLAG monoclonal antibodies. (D) Ribosomal protein L6 was detected by immunoblotting in whole-cell lysates but not in the cell wall compartment of bacilli.

strain AHG239 with the plasmid vector pOS1 did not generate anti-FLAG immune-reactive species.

To further examine the cell wall anchoring of $BasC_{FLAG/MH6}$, the cell wall envelope was digested with muramidase under conditions that stabilized the resulting protoplasts. 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 $BasC_{FLAG/MH6}$ 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 $BasC_{FLAG/MH6}$, 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 LD_{50} doses of 50 spores are required for the development of lethal anthrax disease in mice (46), whereas a subcutaneous LD_{50} dose of 10^6 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 10^2 to 10^3 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 1.56×10^2, 1.27×10^3, 2.03×10^4 and 1.98×10^5 spores of *B. anthracis* strain Sterne or 1.36×10^2, 1.67×10^3, 1.93×10^4 and 1.66×10^5 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 LD_{50} dose of 632 spores for *B. anthracis* Sterne and 1,110 spores for strain AHG239. The observed difference in LD_{50} 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

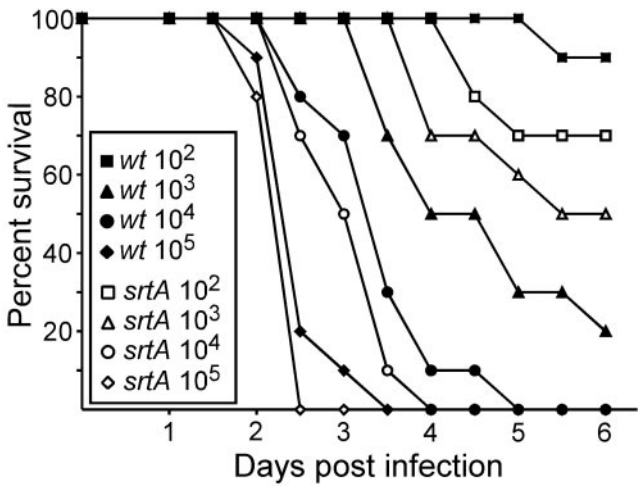

FIG. 7. Sortase A gene of *B. anthracis* strain Sterne is not required for anthrax disease in the A/J mouse model. A/J mice, 10 per group, were infected by subcutaneous injection of 0.1 ml of spore suspension in phosphate-buffered saline and observed for a lethal infection over a period of 14 days (data points did not change after day 6). Both death and time-to-death were recorded and analyzed for spore infection doses of 1.56×10^2, 1.27×10^3, 2.03×10^4 and 1.98×10^5 for *B. anthracis* Sterne and 1.36×10^2, 1.67×10^3, 1.93×10^4 and 1.66×10^5 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 Δ*srtA* 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 Δ*srtA* strain AHG239 variant (1,110 spores) were calculated.

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 internalin-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 various surface proteins to disease pathogenesis cannot be gleaned from the sum of all putative binding activities.

Recent work in *S. aureus* suggests that sortase A and sortase B mutations abolish the cell wall anchoring and surface display of all proteins bearing sorting signals with LPXTG or NPQTN motifs (34). The relative contribution of cell wall-anchored surface proteins to disease pathogenesis can therefore be examined 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 internalins 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* mutants 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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