Supporting Information

Crawford et al. 10.1073/pnas.1108495108

SI Materials and Methods

Bacterial Strains and Growth Conditions. Unless otherwise noted, *B. anthracis* Sterne strain 7702 ($pXO1^+$ $pXO2^-$) vegetative bacilli (BEI Resources) were cultured at 37 °C with shaking in brain-heart infusion (BHI) medium. Stationary phase bacilli were prepared from liquid cultures grown overnight (12–16 h incubation); log phase organisms were prepared after subculture and were harvested at an optical density of ≈0.6 at 600 nm. *B. anthracis* spores were prepared in Difco sporulation medium and were purified over a Percoll gradient as described (1). The *E. coli* strain Alpha-select was grown in Luria-Bertani (LB) medium for use in plasmid construction and propagation; *E. coli* strain GM119 was similarly cultured and used to produce unmethylated DNA for *B. anthracis* transformation. The following antibiotics were included in growth medium as appropriate: erythromycin, 1–5 μg/mL; lincomycin, 2 5 μg/mL; tetracycline, 5 μg/mL; kanamycin, 20 μg/mL (*E. coli*) and 100 μg/mL (*B. anthracis*).

Transposon Library Generation, Insertion Site Determination, and Phage-Transduction. For library generation, 1 μg of unmethylated pJZ037 was added to 0.2 mL of electrocompetent *B. anthracis*, prepared as described (2). Suspensions were incubated in a 0.2-cm gap electroporation cuvette on ice for 15 min before being exposed to a single 2.5-kV, 25-μF, 200-Ω pulse from a BioRad Genepulser XCell. Immediately, 1 mL of BGGM medium (BHI supplemented with 10% glycerol, 0.4% glucose, and 10 mM $MgCl_2$) was added to the cells; transformation cultures were incubated at 30 °C with shaking for 1.5 h before being plated onto BHI agar. After incubation overnight at 30 °C, the bacterial lawn was replica plated onto BHI agar plates containing erythromycin and lincomycin by using sterile felt and incubated overnight at 30 °C. The following day, transformants were replica plated a second time as above, but then incubated at 42 °C overnight to eliminate the temperature-sensitive plasmid pJZ037 and select those bacteria that had transposition of the transposon-associated antibiotic resistance cassette into the chromosome. The resulting bacterial lawn was harvested by washing the plate with BHI medium containing erythromycin and lincomycin; glycerol was added to 10%, and the transposon mutant library was stored at −80 °C. The library was initially assessed by isolating DNA from 20 individual colonies and identifying the transposon insertion sites by PCR as discussed below.

Transposon insertion sites were identified by using a described method (3) with modification. Chromosomal DNA from each Tnx mutant was isolated by using the GenElute bacterial genomic DNA kit (Sigma-Aldrich) according to the manufacturer's instructions for Gram-positive bacterial preparation. DNA was digested with HinP1I and ligated with a partially double stranded Y-linker, prepared as described (3). After MinElute purification (Qiagen), an initial PCR was performed to enrich for single-stranded DNA fragments flanking the transposon insertion; 20-μL reactions were prepared by using GoTaq Green Master Mix (Promega) and 0.5 μM primer FIG6 (AACATGACGAATCCCTCCTTCT) and FIG7 (GCCTACGGGGAATTTGTATCG). Reactions were incubated at 95 °C for 3 min; incubated at 94 °C for 1 min, 61 °C for 45 sec, and 72 °C for 1 min (×20 cycles); and then incubated at 72 °C for 7 min. Subsequently, 0.5 μM primer JZ99 (ACTACGCACCGGACGAGACGT) was added for double-stranded amplification, and reaction volumes were increased to 100 μL total with fresh GoTaq Green Master Mix. PCR amplification was then repeated as described above, except that the number of cycles was increased from 20 to 25. PCRs were run out on a 1.5% agarose gel; visible products were excised and purified by using the QIAquick gel extraction kit (Qiagen). Twenty nanograms of DNA per 100 bp of PCR product was submitted to the University of Virginia Biomolecular Research Facility for sequencing with primers FIG6 and FIG7.

Phage transduction was performed by using the bacteriophage CP-51ts45 according to the method of Giorno et al. (4). Tnx18 was used as the donor strain and Sterne as the recipient strain. Infected recipient cells were plated onto BHI plates supplemented with erythromycin to select those cells containing the transduced transposon-associated antibiotic resistance cassette.

Antimicrobial Assays and Microscopic Visualization. *B. anthracis* bacilli ($\approx 3 \times 10^4$ total per sample well) or spores ($\approx 1 \times 10^5$ total per sample well) were treated with individual chemokines reconstituted in sterile water stabilized with 0.3% human serum albumin (ZLB Bioplasma AG) or an equal volume of 0.3 % human serum albumin (vehicle, untreated control). Also, where indicated, *B. anthracis* vegetative cells were treated with protamine (Sigma) reconstituted in sterile water (5). Alamar blue reduction and cfu determination were performed 6 h after treatment as described (6). Light microscopy was used for bacterial cell visualization; camera control and image capture were performed by using QCapture Pro-5.1 software as described (6).

Markerless Gene Deletion and Complementation. Deletion of *ftsX* was performed as described in detail (7). Briefly, *ftsX* gene replacement constructs were cloned into plasmid pBKJ236 for integration into the chromosome by homologous recombination; plasmid integrants were isolated by shifting bacterial growth to replication-nonpermissive temperature. Plasmid pBKJ233 was then introduced by electroporation and maintained in the presence of tetracycline to affect allelic exchange. Individual colonies were scored for loss of erythromycin resistance and PCR was used to confirm gene deletion, *ftsX* PCR conditions are included below. Plasmid pBKJ233 was then lost spontaneously through passage in medium lacking tetracycline.

For *ftsX* complementation, the native *B. anthracis* Sterne strain *ftsX* gene (BAS5033), carrying its native ribosomal binding site, was amplified by using Phusion high fidelity polymerase (New England BioLabs) according to the manufacturer's instructions and using primer MCX1 containing a SphI restriction site (GCCTAGGCATGCAGCGTGTGATGCCTTTACCT) and primer MCX3 containing a PspXI restriction site (CCGGATACTCGAGTCGGAAAAATTGTTCGAGATGA); PCR cycle conditions: 98 °C for 30 sec; 98 °C for 10 sec, 66 °C for 20 sec, and 72 °C for 30 sec (×30 cycles); and then 72 °C for 7 min. The *ftsX* amplification products were double-digested with SphI-HF and PspXI, purified, and ligated into pUTE973 (8) that had been digested with SphI-HF and SalI-HF (leaves a cohesive end compatible with PspXI). The prepared vector was transformed into Alpha-select *E. coli* for propagation; the cloning site was sequenced for appropriate *ftsX* insertion and, subsequently, transformed into GM119 *E. coli*. This *ftsX* complementation vector (pUVA113) and the empty vector control (pUTE973) were isolated from GM119 transformants grown in the presence of kanamycin by using the QIAprep spin miniprep kit (Qiagen) and individually transformed into *B. anthracis* Δ*ftsX* bacilli as described above. Diagnostic PCR and sequencing of the plasmid cloning sites were used to validate *B. anthracis* transformants.

Examination of Bacterial Growth Rates. Log phase *B. anthracis* Sterne and Δ*ftsX* bacilli were grown as described above to an optical density of ≈0.6 at 600 nm. For growth determination in DMEM + 10% FBS, equal amounts of Sterne and Δ*ftsX* bacilli, normalized by cfu determination (≈3×10^4 bacilli total per sample well), were plated in triplicate into the wells of a 96-well plate. Alamar blue was added 1:10 by volume, and samples were incubated at 37 °C, 5% CO_2. Alamar blue reduction, as judged by increasing fluorescence, was determined every 30 min until both strains had reached maximal reduction. For growth determination in BHI medium, log phase Sterne and Δ*ftsX* bacilli were each diluted to an optical density at 600 nm of ≈0.005 in fresh BHI medium. Every 30 min for 8 h, and then at 10, 12, and 24 h, the culture optical densities at 600 nm were recorded; also at these times, aliquots of each culture were diluted and plated onto BHI agar to determine viable cell density by cfu determination.

Statistical Analysis. Statistically significant differences among treatment groups were determined by using one-way analysis of variance with a Bonferroni multiple comparison test; logarithmic ($\log_{10}$) transformation of cfu values was performed before statistical evaluation. Experimental groups demonstrating statistically significant differences were subsequently analyzed by using an unpaired, two-tailed *t* test. Curve comparisons were performed by nonlinear regression by using an F test comparison method. Reported EC_{50} concentrations were determined by using the sigmoidal dose–response equation of nonlinear regression and are presented as EC_{50} ± 95% confidence interval.

1. Crawford MA, et al. (2010) Interferon-inducible CXC chemokines directly contribute to host defense against inhalational anthrax in a murine model of infection. *PLoS Pathog* 6:e1001199.
2. Koehler TM, Dai Z, Kaufman-Yarbray M (1994) Regulation of the Bacillus anthracis protective antigen gene: CO_2 and a trans-acting element activate transcription from one of two promoters. *J Bacteriol* 176:586–595.
3. Zemansky J, et al. (2009) Development of a mariner-based transposon and identification of Listeria monocytogenes determinants, including the peptidyl-prolyl isomerase PrsA2, that contribute to its hemolytic phenotype. *J Bacteriol* 191: 3950–3964.
4. Giorno R, et al. (2009) Localization and assembly of proteins comprising the outer structures of the Bacillus anthracis spore. *Microbiology* 155:1133–1145.
5. Johansen C, Verheul A, Gram L, Gill T, Abee T (1997) Protamine-induced permeabilization of cell envelopes of gram-positive and gram-negative bacteria. *Appl Environ Microbiol* 63:1155–1159.
6. Crawford MA, et al. (2009) Antimicrobial effects of interferon-inducible CXC chemokines against Bacillus anthracis spores and bacilli. *Infect Immun* 77:1664–1678.
7. Janes BK, Stibitz S (2006) Routine markerless gene replacement in Bacillus anthracis. *Infect Immun* 74:1949–1953.
8. Pflughoeft KJ, Sumby P, Koehler TM (2011) Bacillus anthracis sin locus and regulation of secreted proteases. *J Bacteriol* 193:631–639.

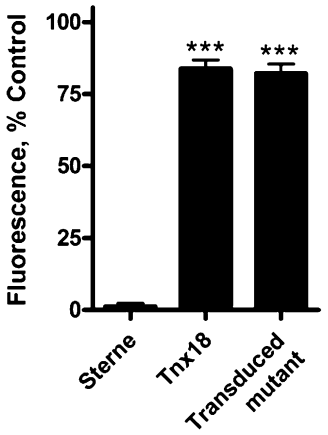

Fig. S1. Phage transduction of CXCL10 resistance from Tnx18 to *B. anthracis* Sterne strain. Indicated bacilli were treated with 12 μg/mL CXCL10 for 6 h. Data are expressed as a percentage of the strain-specific untreated control and represent mean values ± SEM; $n = 3$ independent experiments, ***$P < 0.001$ compared with CXCL10-treated Sterne bacilli.

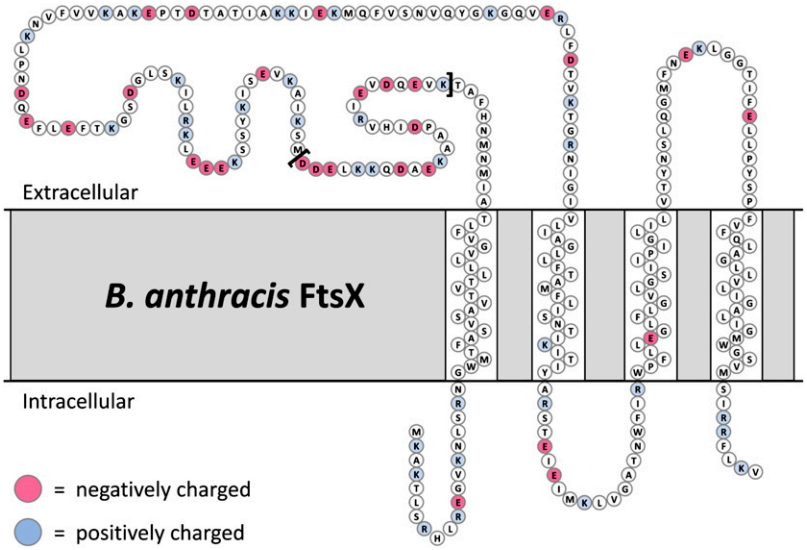

Fig. S2. Predicted topology of *B. anthracis* FtsX. Membrane topology was determined by using the Center for Biological Sequence Analysis TMHMM server v. 2.0; charged amino acid residues are indicated in color. The extracellular region of *B. anthracis* FtsX that demonstrates homology to the N-terminal chemokine binding domain of human CXCR3 is enclosed with brackets **[ ]**; 45% similarity was determined by BLASTp analysis.

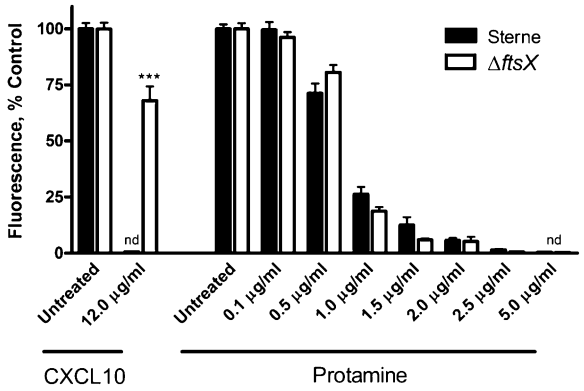

Fig. S3. Sterne and Δ*ftsX* bacilli susceptibility to the cationic antimicrobial peptide protamine. Indicated bacilli were treated with 12 μg/mL CXCL10 or increasing concentrations of protamine. Data are expressed as a percentage of the strain-specific untreated control and represent mean values ± SEM; $n = 3$–4 independent experiments; nd, none detected; ***$P < 0.001$. No statistically significant differences were observed between Sterne and Δ*ftsX* bacilli susceptibility to protamine at any concentration tested.

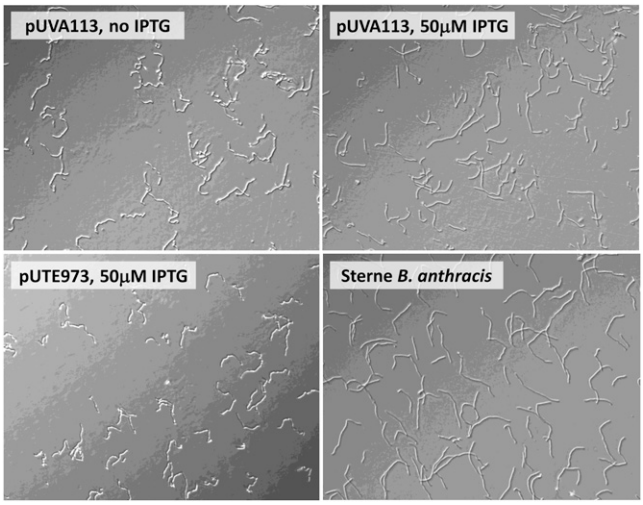

Fig. S4. Complementation of *ftsX* corrects the irregular cellular morphology of Δ*ftsX* bacilli. Transformation of the *ftsX* complementation vector pUVA113 into *ftsX*-null *B. anthracis* bacilli did not restore normal cellular morphology (*Upper Left*); IPTG-induced expression of this vector, however, restored typical bacilli morphology (*Upper Right*). For reference, images of *ftsX*-null bacilli expressing the pUTE973 empty vector control (*Lower Left*) and *B. anthracis* Sterne strain bacilli (*Lower Right*) are included. Representative fields from three independent experiments are shown at 200× magnification.

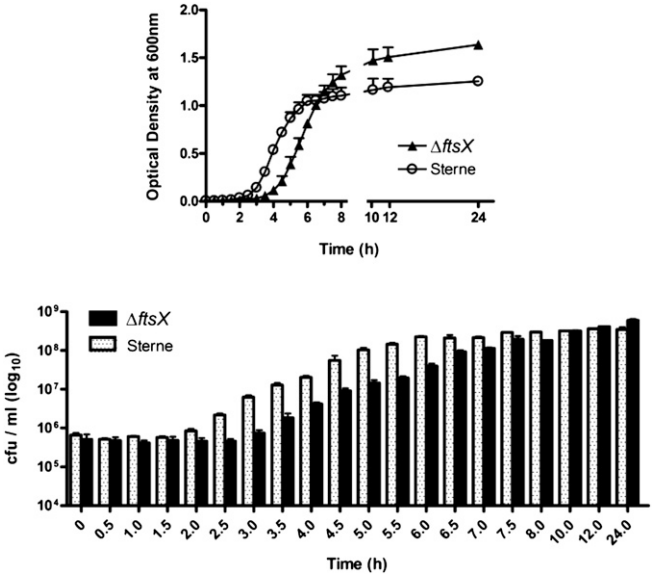

Fig. S5. Bacterial growth rates among Sterne and Δ*ftsX B. anthracis* bacilli in BHI medium. The optical density at 600 nm of each bacterial culture was determined over time (*Upper*). Δ*ftsX* bacilli initially grew more slowly than Sterne organisms but reached higher optical densities at later timepoints. The disparity in stationary phase optical density between the two strains is likely due to the tendency of Δ*ftsX* bacilli to become entangled and grow in clusters; cfu determination (*Lower*) supports this conclusion as similar viable cell densities were observed for each strain during the stationary phase of growth. Optical densities and cfu data represent mean values ± SEM; $n = 3$ independent experiments.

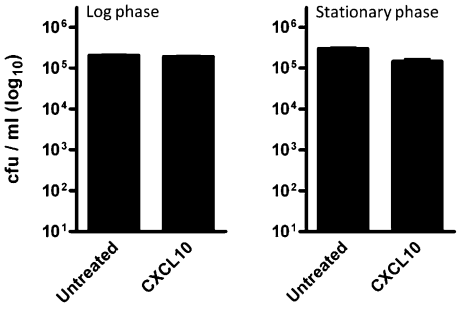

Fig. S6. Growth phase does not affect CXCL10 resistance among Δ*ftsX* bacilli. Log phase (*Left*) and stationary phase (*Right*) Δ*ftsX* bacilli were left untreated or treated with 8 μg/mL CXCL10 for 30 min before bacterial viability was assessed by cfu determination. Viability is expressed as cfu/ml (log_{10} scale) and represents mean values ± SEM; n = 3 independent experiments.

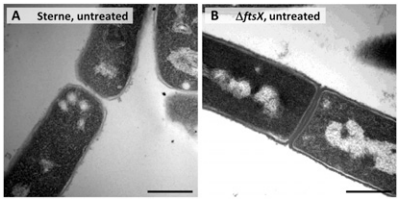

Fig. S7. Electron micrographs of untreated *B. anthracis* Sterne and Δ*ftsX* bacilli. Untreated bacilli demonstrated negligible binding of labeling reagents used for silver-enhanced immunogold labeling of CXCL10. Representative fields from two independent experiments are shown at 30,000× magnification. (Scale bars: 0.5 μm.)

Table S1. Unique transposon insertion sites identified among primary CXCL10 resistance screen isolates

Gene	Name*	Annotation*	Tnx isolate(s)	CXCL10 resistance†
BAS5033	*ftsX*	ABC transporter permease	Tnx1, 4, 6, 8, 14, 15, 16, 18	+++
BAS5033	*ftsX*	ABC transporter permease	Tnx11, 17	+++
BAS0651		Conserved, hypothetical	Tnx2, 3, 7, 13	++
BAS5043	*lytE*	Endopeptidase	Tnx5	+
BAS0716		Fructokinase	Tnx9	—
Intergenic‡			Tnx10	—
BAS5306		Conserved, hypothetical	Tnx12	—

*As identified by NCBI annotation of the *B. anthracis* Sterne strain genome; blank spaces indicate that no specific name has been assigned.

†CXCL10 resistance was determined by comparison with *B. anthracis* Sterne strain bacilli using Alamar blue analysis (also see Fig. 1*B*).

‡Transposon insertion mapped to a noncoding region located between BAS0232 and BAS0233.