Supporting Information

NaxD is a deacetylase required for lipid A modification and *Francisella* pathogenesis.

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Supporting Information Experimental Procedures

***F. tularensis* transformation.** An overnight mMH broth culture of SchuS4 was used to inoculate 50 ml of mMH broth. The resulting culture was then incubated with shaking at 37°C until the culture reached OD_{600} = 0.3 before the culture was transferred to a 50 ml conical tube and the cells were collected by centrifugation at 4°C. The cell pellet was resuspended in 4 ml of 0.5 M sucrose that was pre-chilled to 4°C, equally distributed into two 2 ml screw-capped vials, before the cells were again collected by centrifugation at 4°C. The resulting cells were then washed three additional times with 2 ml of pre-chilled 0.5 M sucrose. Following the final wash, the cell pellet was resuspended to a total volume of 70 μl 0.5 M sucrose and placed on ice. One microgram of purified pΔFTT0453 was added to 70 μl of the electrocompetent SchuS4 cells and the mixture was transferred to a pre-chilled 0.1 cm electroporation cuvette (BTX, Holliston, MA). This mixture was then electroporated at 1.5 kV, 25 uF, and 200 ohms. Next, 1 ml of mMH broth was immediately added to the cuvette and then this cell suspension was transferred to a 50 ml conical tube. An additional 1 ml of mMH broth was added to the electroporated cell suspension and the cells were incubated with shaking at 37°C. After two hours incubation the cells were collected by centrifugation, resuspended in 500 μl of mMH broth and spread onto BHI agar plates containing 10 μg ml^{-1} kanamycin. Following three days incubation at 37°C, several kanamycin resistant colonies were then patched onto fresh BHI agar plates. Among the resulting clones, four colonies were selected and used to inoculate 1 ml of mMH broth without antibiotics. These broth cultures were incubated with shaking at 37°C until early log phase (OD_{600} = 0.4) and then aliquots of the culture were spread onto CHA-5% sucrose plates. When colonies appeared on the sucrose plates (4-5 days later), 24 sucrose resistant colonies were then patched onto fresh CHA sucrose plates and the plates incubated at 37°C until colonies were visible. The resulting colonies were then replica-plated onto BHI agar plates with or without kanamycin. Kanamycin sensitive clones were subsequently assayed for the presence of the *naxD* deletion.

***F. novicida* growth curves.** *F. novicida* overnight cultures were grown at 37°C on a rolling drum in tryptic soy broth (TSB; Difco/BD, Sparks, MD) supplemented with 0.02% L-cysteine (Sigma-Aldrich, St. Louis, MO) and then subcultured in 96 well plates to OD_{600} = 0.03 in either TSB with 0.02% cysteine or Chamberlain's chemically defined minimal medium, prepared as previously described (Chamberlain, 1965). OD_{600} was measured hourly using a BioTek Synergy Mx (Winooski, VT) microplate reader.

Macrophage staining and immunofluorescence. To measure phagosomal escape, primary murine BMM were infected and stained as previously described (Weiss *et al.*, 2007, Jones *et al.*, 2011). Briefly, prechilled macrophages were infected at 4°C, rapidly warmed for 5 min in a 37°C water bath, and then incubated for 10 min at 37°C, 5% CO_2. At 30, 60, or 120 min after infection, cells were fixed with 4% paraformaldehyde and then permeabilized and stained with a rat monoclonal anti-LAMP-1 antibody (1D4B) (Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA) and chicken anti-*F. novicida* antibody (generous gift from Dr. Denise Monack, Stanford University, Stanford, CA) in PBS with 1% saponin and 3% BSA. Next, cells were respectively incubated with Alexa488 (anti-rat) and Alexa594 (anti-chicken) secondary antibodies. The coverslips were then mounted over SlowFade Gold antifade reagent containing DAPI. Images were obtained using a Zeiss Axioscope Z.1 microscope equipped with a Zeiss Imager 2.1 camera, and images were taken at 63x magnification.

Western blot. For *F. novicida*, protein concentrations were normalized to colony forming units of initial cultures and amounts equaling 1 x 10^8 bacteria from each fraction were run on protein gels (Bio-Rad, Hercules, CA) along with a broad range protein standard (Bio-Rad) for 35 minutes at 200 volts using a Bio-Rad PowerPac HC. Next, the proteins were transferred onto nitrocellulose membranes (GE Osmonics, Trevose, PA) for 30 minutes at 100 volts. Western blots were then performed. Briefly, at room temperature, membranes were blocked 2 hours using 5% skim milk tris-buffered saline with 0.005% Tween-20 (TBST). Next, membranes were

incubated for 2 hours with primary antibodies diluted in 5% skim milk TBST against His-tag (Abgent, San Diego, CA), FopA (generous gift from Dr. Michael Norgard, University of Texas Southwestern Medical Center, Dallas, TX)(Huntley *et al.*, 2010), or IglA (BEI Resources, Manassas, VA)(de Bruin *et al.*, 2007). The membranes were then incubated with secondary antibodies against IgG from mouse (Cell Signaling, Danvers, MA), rat (Cell Signaling), or rabbit (Cell Signaling). Finally, membranes were developed using SuperSignal (Bio-Rad) and imaged using a UVP BioSpectrum 600 (Upland, CA). UVP Vision Work CS Image Acquisition and Analysis Software 6.7.2 was used to merge white light and fluorescent images.

Supporting Information References

Chamberlain, R.E. (1965) Evaluation of Live Tularemia Vaccine Prepared in a Chemically Defined Medium. *Appl Microbiol* **13**: 232-235.

de Bruin, O.M., Ludu, J.S., and Nano, F.E. (2007) The *Francisella* pathogenicity island protein IglA localizes to the bacterial cytoplasm and is needed for intracellular growth. *BMC Microbiol* **7**: 1.

Huntley, J.F., Robertson, G.T., and Norgard, M.V. (2010) Method for the isolation of *Francisella tularensis* outer membranes. *J Vis Exp*.

Jones, C.L., and Weiss, D.S. (2011) TLR2 signaling contributes to rapid inflammasome activation during *F. novicida* infection. *PLoS One* **6**: e20609.

Weiss, D.S., Brotcke, A., Henry, T., Margolis, J.J., Chan, K., and Monack, D.M. (2007) *In vivo* negative selection screen identifies genes required for *Francisella* virulence. *Proc Natl Acad Sci U S A* **104**: 6037-6042.

Supporting Information Figure Legends

Figure S1. The *naxD* deletion mutant exhibits wild-type growth kinetics in both rich and minimal media. Wild-type (WT, black) and *naxD* deletion mutant strains (ΔnaxD, red) were grown in either (A) rich media (tryptic soy broth) or (B) minimal media (Chamberlain's broth) and the OD_{600} was measured hourly for 16 hours.

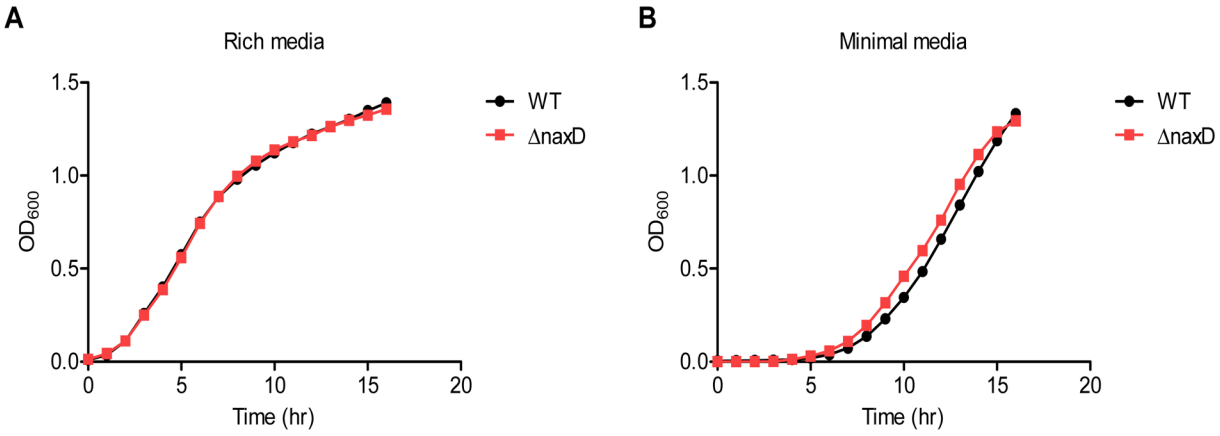
Figure S2. NaxD is not required for phagosomal escape. Flourescence microscopy imaging was used to quantify the escape of wild-type *F. novicida* (WT) and *naxD* deletion mutant (ΔnaxD) bacteria. Primary murine BMM were infected at 100:1 MOI and fixed with 4% paraformaldehyde at 30, 60, and 120 minutes post-infection. After sample staining and preparation, bacteria within phagosomes were identified by colocalization of LAMP-1 and *F. novicida*. The percentage of bacteria that escaped from the phagosome was determined by counting a minimum of 200 bacteria per strain from three different experiments. Bars represent the average and error bars represent the standard deviation.

Figure S3. NaxD is required for *F. tularensis* proliferation within murine BMM. Primary murine bone marrow-derived macrophages (BMM) were infected with a 20:1 MOI of wild-type *F. tularensis* (WT) or the *naxD* deletion strain (ΔnaxD). CFU from lysates 30 minutes post-infection were compared to those from 24 hours post-infection to determine fold intracellular replication (n=3 biological replicates). Bars represent the average and error bars represent the standard deviation. Asterisks indicate significance as compared to wild-type. (*) = $P < 0.05$.

Figure S4. NaxD localizes to the membrane fraction. Western blot analysis of whole cell lysates, soluble fractions, and membrane fractions of wild-type *F. novicida* (WT) and a wild-type strain in which NaxD was tagged with an 8x His tag (HIS). αHis, αIglA (a cytosolic protein), or αFopA (a membrane protein) antibodies were used with the appropriate secondary antibodies.

Figure S5. Exogenously expressed NaxD localizes to the membrane fraction in *E. coli*. Coomassie stain was used to analyze whole cell lysate (WCL), membrane (Mem) or soluble (Sol) protein fractions from *E. coli* transformed with either the vector control pET-21a or pET-21a encoding *naxD*, with or without IPTG induction.

Figure S1


A
Rich media
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1.0
0.5
0.0
OD600
0
5
10
15
20
Time (hr)
WT
ΔnaxD
B
Minimal media
1.5
1.0
0.5
0.0
OD600
0
5
10
15
20
Time (hr)
WT
ΔnaxD

Figure S2

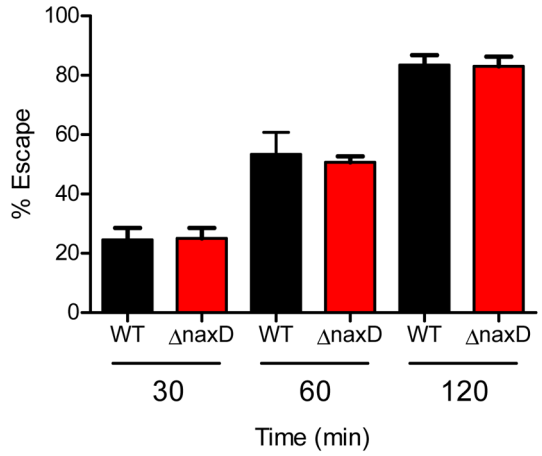
100
80
60
40
20
0
% Escape
WT
ΔnaxD
WT
ΔnaxD
WT
ΔnaxD
30
60
120
Time (min)

Figure S3

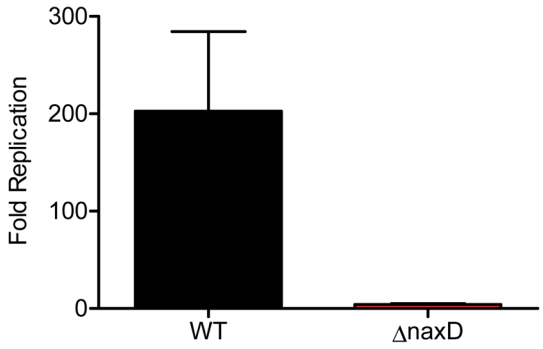

Figure S4

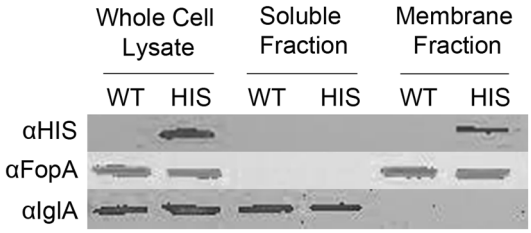
Whole Cell Lysate
Soluble Fraction
Membrane Fraction
WT
HIS
WT
HIS
WT
HIS
αHIS
αFopA
αIglA

Figure S5

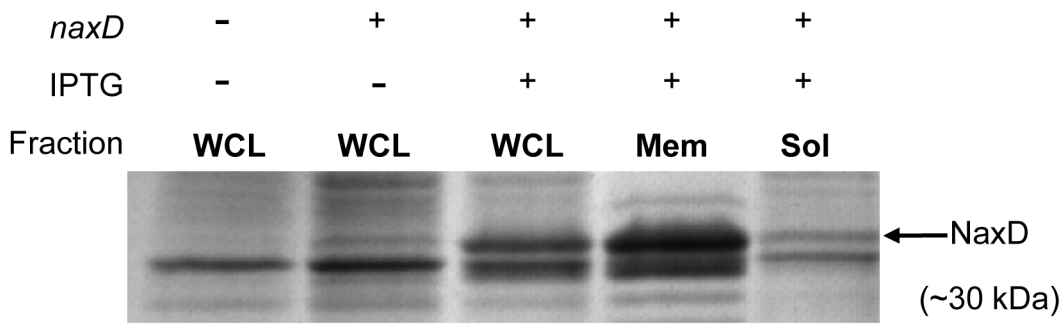

Table S1. Primers used in this study.

Primer/Plasmid name	Sequence/Specifications
F. novicida FTN_0544	
FTN_0544 deletion check F1	atctgcaacctcaaatggta
FTN_0544 Arm 1 FWD	gtcctctgataaaacgacta
FTN_0544 Arm 1 REV	ttatcgataccgtcgacctcaacttttatttgctaattgaatta
FTN_0544 frt_sKAN_frt FWD	taattcaattagcaaataaaagttgaggtcgacggtatcgataa
FTN_0544 frt_sKAN_frt REV	ttataagaaagtaaaatagcagttatgcatagctgcaggatcga
FTN_0544 Arm 2 FWD	tcgatcctgcagctatgcataactgctattttactttcttataa
FTN_0544 Arm 2 REV	aacaaagtacgttggtatgg
FTN_0544 deletion check R1	gggatgtatcgcaatattgt
FTN_0544 comp Arm 1 REV	tgagtgacaacccaaagagatttatagtacaatattatgatctt
FTN_0544 comp sKAN FWD	aagatcataatattgtactataaatctctttgggttgtcactca
FTN_0544 comp sKAN REV	taagaaagtaaaatagcagttatacaaccaattaaccaattctg
FTN_0544 comp Arm 2 FWD	cagaattggttaattggttgtataactgctattttactttctta
FTN_0544 comp check R2	tggtcatcaacatgttcatc
F. tularensis FTT_0453	
pXB186	Kanamycin resistance cassette (*aph)*, sucrose sensitivity cassette (*sacB)*
FTT0453c checkF1	aaaggtactaatgttggagc
FTT0453c checkF2	tatccaagttgcagaattgg
FTT0453c Arm 1 FWD	atatatatggatccttattattgggtggtgtagc
FTT0453c Arm 1 REV	aggtgataattcaattagcaaaataactgctattttactttct
FTT0453c Arm 2 FWD	agaaagtaaaatagcagttattttgctaattgaattatcacct
FTT0453c Arm 2 REV	atatatatggatccaaatgttgttgtcagtgttg
FTT0453c checkR1	tattattatcttcacgccag
FTT0453c checkR2	acagatatcaatctaagcgg
B. bronchiseptica BB4267	
pSS4245	Allelic exchange plasmid for *Bordetella* species. Tetracycline, streptomycin, ampicillin, bleomycin and kanamycin resistance cassettes
pXQ026	pSS4245 containing *bb4267* deletion fragment (used to generate strain RBXQ24)
pXQ042	pUC18-Mini-TN7 containing *bb4267* complement fragment [used to generate strain RBXQ27 (RBXQ24/pXQ042)]
4267F1	aagatgcctggccgacttc
4267R1	ctggcagccgtttatgagt
LK-4267F2	taaacggcttgccagtcgttcactggtcatttcag
4267R2	cctgcatacctatccgttcg
pUC18-Mini-TN7	ampicillin and kanamycin resistance cassettes
pTNS3	Helper plasmid for pUC18-Mini-TN7 vectors, ampicillin resistance cassette.
E. coli	
pET-21a	Empty expression vector plasmid
pET-21a-*FTN_0544*	pET-21a encoding *FTN_0544*