SUPPORTING INFORMATION

Roles and Organization of BxpB (ExsFA) and ExsFB in the Exosporium Outer Basal Layer of *Bacillus anthracis*

Jorge Durand-Heredia, Hsin-Yeh Hsieh, Krista A. Spreng, and George C. Stewart*

Department of Veterinary Pathobiology and Bond Life Sciences Center
University of Missouri
Columbia, Missouri, 65211

*To whom correspondence should be addressed. Email: stewartgc@missouri.edu

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Table S1. *Bacterial strains and plasmids*

Strain	Relevant characteristic(s)	Source or reference
Bacillus anthracis		
ΔSterne	plasmid-free derivative of Sterne	Lab stock
MUS7999	Sterne *ΔexsFB::spc*	This study
MUS8134	Sterne *ΔexsFB*	This study
MUS8135	Sterne *bxpB::kan ΔexsFB*	This study
MUS8227	Sterne *bxpB::kan ΔcotE ΔexsFB*	This study
MUS8228	Sterne *ΔexsY*	This study
MUS8229	Sterne *ΔcotY*	This study
MUS8230	Sterne *ΔcotY ΔexsY*	This study
MUS8232	Sterne *ΔcotE::kan ΔcotY ΔexsY*	This study
RG56	Sterne *ΔcotE::kan*	(1)
RG124	Sterne *ΔbxpB::kan*	(2)
Sterne	pXO1^{+} pXO2^{-}	Lab stock
E. coli		
BTH101	*cya-99, rpsL1 (Str r), hsdR2, mcrA1, mcrB1*	(3)
DH5α	*φ80dlacZΔM15 recA1 endA1 gyrA96 thi-1 hsdR17* (r_K^- m_K^-) *supE44 relA1 deoR Δ(lacZYA-argF)U169*	Lab stock
GM48	*F^{-} thr leu thi lacY galK galT ara fhuA tsx dam dcm glnV44*	Lab stock
M15	*str lacZΔM15*	Qiagen
MUS4186	M15 pREP4 pKS4186	This study
MUS4691	M15 pREP4 pJD4886	This study

Plasmids

Plasmid	Description	Source or reference
pDG4099	pMK4 *egfp* fusion vector	(4)
pDG4100	pMK4 *mcherry* fusion vector	(5)
pGS4260	pMK4 *bclA* promoter - *bclA NTD-mcherry*	(5)
pGS4294	Allele replacement ts shuttle vector Amp^r, Ery^r	Lab stock
pGS4709	pMK4-*bclA promoter - rbs - mcherry*	Lab stock
pGS4900	pMK4 - P_{bclA} - *bclA NTD-his*$_{12}$	This study
pGS4618	pGS4294 - *ΔexsFB::spc*	This study
pGS6328	pGS4295 - *amyS::spc*; Amp^R, Ery^R, Spc^R ts shuttle vector	(6)
pHPS2	Shuttle vector; Spc^r, Ery^r	(7)
pJD4693	pMK4 - P_{bxpB} - *exsFB*	This study
pJD4695	pMK4 - P_{exsFB} - *bxpB*	This study
pJD4696	pMK4 - P_{bxpB} - *bxpB* (NT) - *exsFB* (CT)	This study
pJD4697	pMK4 - P_{exsFB} - *exsFB* (NT) - *bxpB* (CT)	This study
pJD4698	pMK4 - P_{bxpB} - *exsFB* (NT) - *bxpB* (CT)	This study
pJD4699	pMK4 - P_{exsFB} - *bxpB* (NT) - *exsFB* (CT)	This study
pJD4730	pMK4 - P_{bxpB} - *bxpB*	This study
pJD4732	pMK4 - P_{exsFB} - *exsFB*	This study
pJD4813	pDG4099 - P_{bxpB} - *bxpB* - *egfp*	This study
pJD4814	pDG4099 - P_{exsFB} - *exsFB* - *egfp*	This study
pJD4886	pQE30 - *exsFB*	This study
pJD6139	pHPS2 - P_{bxpB} – *bxpB* - *mcherry*	This study
pJD6163	pHPS2 - P_{exsFB} – *exsFB* - *mcherry*	This study
pJD6268	pKT25 - *T25* - *bxpB*	This study
pJD6269	pKT25 - *T25* - *exsFB*	This study

pJD6443	pUT18 - *bxpB - T18*	This study
pJD6444	pKNT25 - *exsFB - T25*	This study
pJD6445	pUT18 - *exsFB - T18*	This study
pJD6446	pUT18C - *T18 - bxpB*	This study
pJD6447	pUT18C - *T18 - exsFB*	This study
pJD6448	pKT25 - *T25 - aw20_5669*	This study
pJD6450	pUT18C - *T18 - exsY*	This study
pJD6471	pKNT25 - *bxpB - T25*	This study
pKNT25	Kan^r, in-frame fusions at the C-terminal end of the T25 adenylate cyclase polypeptide	(3)
pKS4186	pQE30- *bxpB*	(8)
pKT25	Kan^r, in-frame fusions at the N-terminal end of the T25 adenylate cyclase polypeptide	(3)
pKT25-Zip	pKT25 with Zip domain	(3)
pMK4	Shuttle plasmid Amp^r, Cm^r	(9)
pQE30	His-tag cloning vector; Amp^r	Qiagen
pREP4	p15a origin, LacI expression plasmid; kan^r	Qiagen
pUT18	Amp^r; in-frame fusions at the N-terminal end of the T18 adenylate cyclase polypeptide	(3)
pUT18C	Amp^r; in-frame fusions at the C-terminal end of the T18 adenylate cyclase polypeptide	(3)
pUT18C-Zip	pUT18C with Zip domain	(3)

[a] Amp^r, Cm^r, Ery^r, Kan^r and Spc^r denote resistance to 100 μg ampicillin ml^{-1}, 10 μg chloramphenicol ml^{-1}, 5 μg erythromycin ml^{-1}, 50 μg kanamycin ml^{-1}, and 100 μg spectinomycin ml^{-1}, respectively.

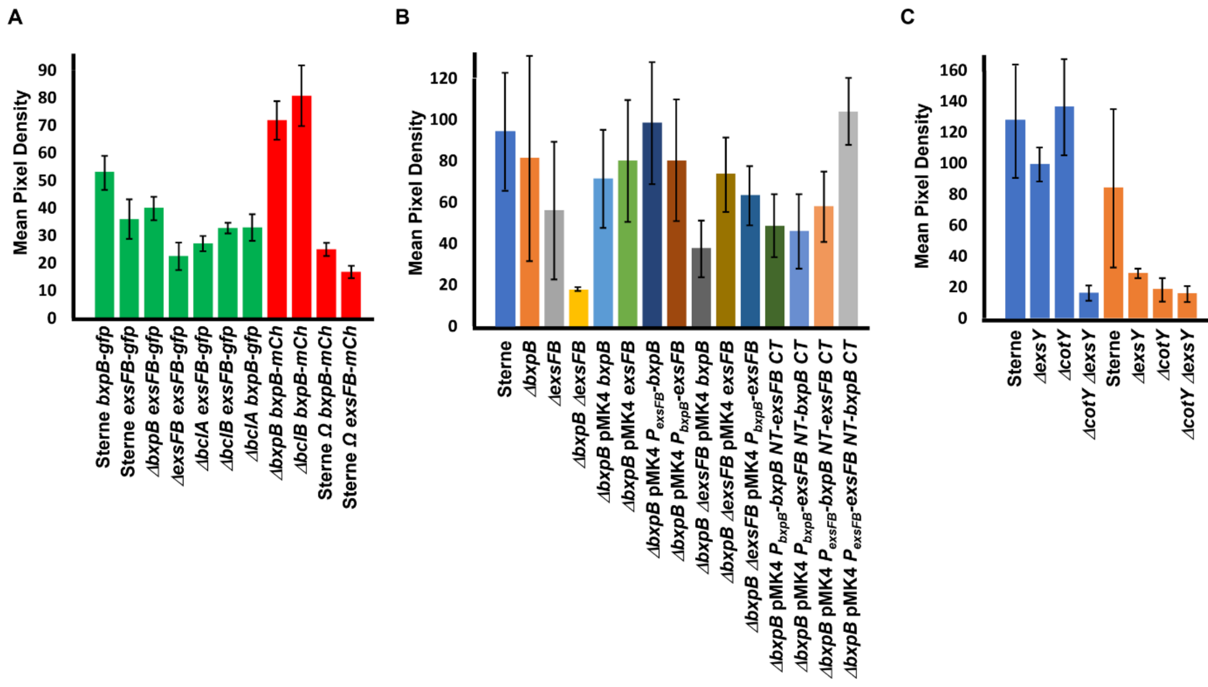

Figure S1. Fluorescence intensity of the spore micrographs used in this study were measured as mean pixel density with the NIH ImageJ program. Approximately 25 fluorescent spores from each experimental condition were included in the assessment. The bars represent the mean pixel density of this population and the error bars are the standard deviation. Panel A are the results from the spores containing eGFP fusion proteins (green bars) and mCherry fusion proteins (red bars) corresponding to the Figure 1-based experiments. Panel B shows the results of the anti-BclA fluorescence experiments from Figures 2- and 3-based experiments. Panel C shows the results of the anti-BclA fluorescence (blue bars) and anti-BxpB fluorescence from Figure 6-based experiments.

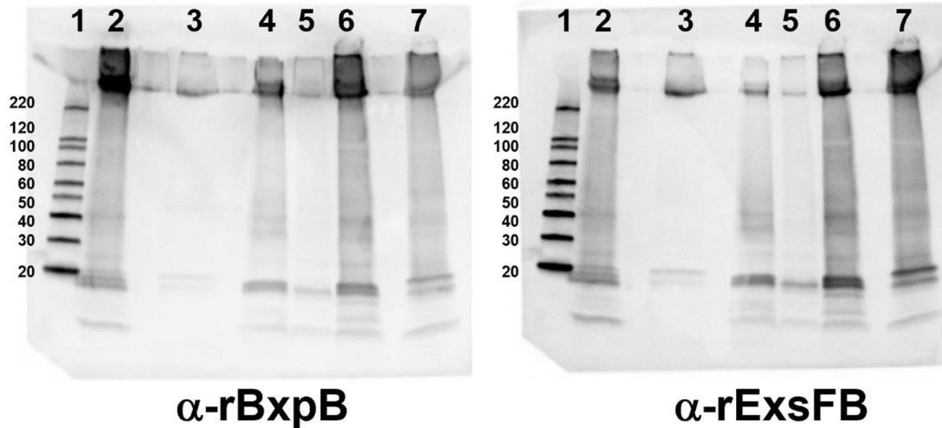

Figure S2. Western blots of spore extracts probed with rabbit polyclonal antiserum against rBxpB (left panel), or rExsFB (right panel). Ten mg of spores in a volume of 100 µl were extracted by boiling in the presence of SDS and urea and 15 µl of the supernatant loaded onto an SDS-PAGE gradient gel and the proteins resolved by electrophoresis. The proteins were electro-transferred to an Immobilon membrane. The membrane was probed with the anti-ExsFB antiserum, then stripped and re-probed with the anti-BxpB antiserum. Lane 1 is the protein size standard with the molecular weights in kilodaltons listed to the left. Lanes 2-7 are the spore extracts: 2, Sterne; 3, Sterne *ΔbxpB*; 4, Sterne *ΔexsFB*; 5, Sterne *ΔbxpB ΔexsFB*; 6, Sterne *ΔbxpB ΔexsFB* pMK4 *bxpB*; and 7, Sterne *ΔbxpB ΔexsFB* pMK4 *exsFB*.

The molecular masses of BxpB and ExsFB are 17.33 kDa and 17.48 kDa, respectively. However, the two proteins resolved into distinct bands, migrating below the 20 kDa marker, in this electrophoresis system. Unfortunately, rabbit serum recognizes a band of approximately the same size in the *B. anthracis* spore extracts, resulting in triplet bands in the Sterne extract and one remaining species in the *ΔbxpB ΔexsFB* extracts. The BxpB and ExsFB proteins are found in the extracts in both monomer and oligomeric forms that are stable to the denaturation conditions employed. The high molecular weight species are characteristic of exosporium structural proteins. ImageJ was used to quantify the amount of BxpB and ExsFB in the extracts from the parent Sterne strain and Sterne bearing the pMK4-based complementation plasmids. The plasmid-expressed determinants resulted in higher levels of expression of the proteins. BxpB was found to elevated 3.7-fold relative to the single copy determinant in Sterne and the elevation of ExsFB as 2.7-fold. It was not surprising that the elevated gene dosage from the plasmid-based would result in higher expression level and consequently elevated protein levels in the spores. The BxpB and ExsFB proteins were elevated to similar extents overall in the spores from the strains bearing the complementation plasmids.

REFERENCES

1. Giorno R, Bozue J, Cote C, Wenzel T, Moody KS, Mallozzi M, Ryan M, Wang R, Zielke R, Maddock JR, Friedlander A, Welkos S, Driks A. 2007. Morphogenesis of the *Bacillus anthracis* spore. J Bacteriol. 189:691-705.

2. Giorno R, Mallozzi M, Bozue J, Moody KS, Slack A, Qiu D, Wang R, Friedlander A, Welkos S, Driks A. 2009. Localization and assembly of proteins comprising the outer structures of the *Bacillus anthracis* spore. Microbiol. 155:1133-1145.

3. Karimova G, Gauliard E, Davi M, Ouellette SP, Ladant D. 2017. Protein-protein interaction: Bacterial two-hybrid. Meth Molec Biol. 1615:59-176.

4. Boone TJ, Mallozzi M, Nelson A, Thompson B, Khemmani M, Lehmann D, Dunkle A, Hoeprich P, Rasley A, Stewart G, Driks A. 2018. Coordinated assembly of the *Bacillus anthracis* coat and exosporium during bacterial spore outer layer formation. mBio 9(6):e01166-18. doi: 10.1128/mBio.01166-18.

5. Hermanas TM, Subramanian S, Dann CE 3rd, Stewart GC. 2021. Spore-associated proteins involved in c-di-GMP synthesis and degradation of *Bacillus anthracis*. J Bacteriol. 203(17):e0013521.

6. Durand-Heredia J, Stewart GC. 2022. Localization of the CotY and ExsY proteins to the exosporium basal layer of *Bacillus anthracis*. Mol Microbiol (submitted for publication).

7. Thompson BM, Hsieh HY, Spreng KA, Stewart GC. 2011. The co-dependence of BxpB/ExsFA and BclA for proper incorporation into the exosporium of *Bacillus anthracis*. Mol Microbiol. 79:799-813.

8. Spreng, KA. 2012. Identification and Characterization of *Bacillus anthracis* spore-associated proteins. PhD thesis. University of Missouri, Columbia, MO.

9. Sullivan MA, Yasbin RE, Young FE. 1984. New shuttle vectors for *Bacillus subtilis* and *Escherichia coli* which allow rapid detection of inserted fragments. Gene 29:21-26.