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The Mn efflux protein EmfA is required for virulence in *Brucella abortus* 2308

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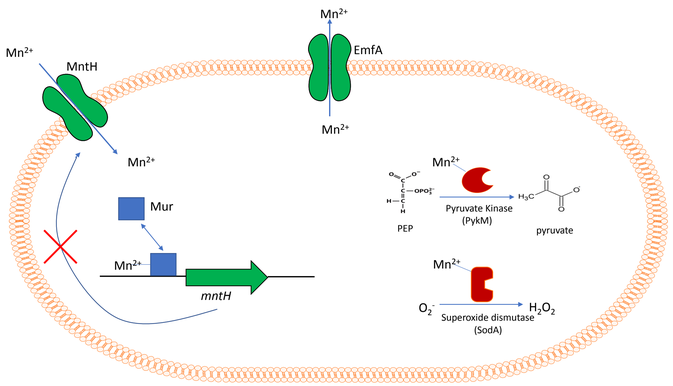
Abstract

Brucella abortus is a Gram-negative bacterium that causes abortion and infertility in food animals and a chronic debilitating febrile disease in humans known as undulant fever. *Brucella* encounter numerous host defenses throughout the course of an infection. One of these is the metal-withdrawal defense, which restricts the availability of these essential micronutrients as a means of preventing microbial growth. To overcome host-mediated metal limitation, bacterial pathogens have evolved high affinity metal acquisition systems to actively compete against the host for available metals. Not surprisingly, the high affinity manganese (Mn) importer MntH plays an essential role in the virulence of *Brucella* strains (1). But we have recently obtained evidence suggesting that a Mn exporter plays an equally important role in the pathogenesis of these bacteria. The cation diffusion facilitator (CDF)-type metal exporter EmfA provides *Rhizobium etli* and *Sinorhizobium meliloti* with an effective means of resisting Mn toxicity (2,3), and *Brucella* strains possess an EmfA homolog. Phenotypic analysis of an *emfA* null mutant suggests that EmfA is a Mn-specific exporter in *Brucella*, and this mutant exhibits significant attenuation in C57BL/6$^{Nramp+/+}$ mice compared to the parent strain. The inability of both Mn import (Δ*mntH*) and export (Δ*emfA*) mutants to sustain chronic spleen infections in experimentally infected mice suggests that the capacity of *Brucella* strains to maintain Mn homeostasis is critical for their virulence. The physiologic basis for the attenuation of the *B. abortus emfA* mutant is unknown. But one possibility based on work that has been done in another pathogen, *Streptococcus pneumoniae* (4), is that the accumulation of excess intracellular Mn interferes with the bacterial cell cycle and disrupts the ability of the brucellae to sustain the intracellular replication they require to sustain their prolonged intracellular residence in host macrophages. Studies are currently underway to determine – a) how EmfA works in conjunction with the Mn-responsive regulator Mur and other metal homeostasis systems to prevent Mn toxicity in these bacteria; and b) how *emfA* expression is regulated in *Brucella.*

Mn is an essential trace nutrient for *Brucella*



This model illustrates Mn acquisition across the cell envelope, factors contributing to the regulation of intracellular Mn levels and the role of Mn as a nutrient for the *Brucella spp*. Mn acquisition is accomplished by the sole high-affinity Mn importer, MntH. The Mn-sensing metalloregulator, Mur, regulates *mntH* expression at the level of transcription, which serves as a negative feedback response for Mn uptake. As with most metals, the primary function of Mn is to serve as a protein cofactor. Several *Brucella* enzymes require Mn to function properly, including its sole pyruvate kinase, PykM, and the superoxide dismutase, SodA, which protects the *Brucella* strains from harmful reactive oxidative species generated by this bacterium's aerobic metabolism (5,6). When intracellular metals are in excess of their optimal concentration, they become toxic to the cell due to loss of protein function via improper metalation. We propose the Mn efflux protein, EmfA, is responsible for resisting Mn-induced toxicity in the *Brucella* spp.

The *Brucella spp*. encode a putative Mn efflux protein

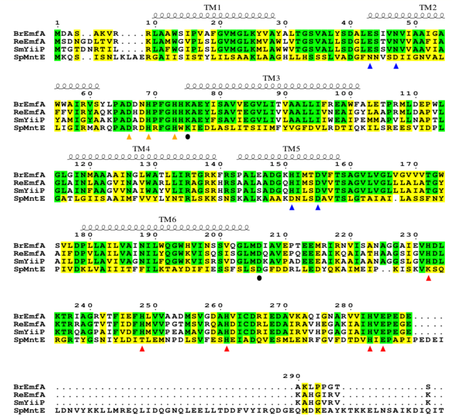


Figure 1: Multiple sequence alignment of the proposed *B. abortus* Mn efflux protein (designated EmfA), *R. etli* EmfA, and *S. meliloti* smYiiP infers a high degree of homology with 76% amino acid similarity. MntE is another CDF-type exporter that confers Mn resistance in other bacterial species. While essential amino acid residues are conserved among the EmfA homologs and MntE, lower amino acid similarity and the absence of an elongated C-terminus in the EmfA homologs indicates the two efflux protein types are not identical.

B. abortus EmfA confers resistance to excess Mn

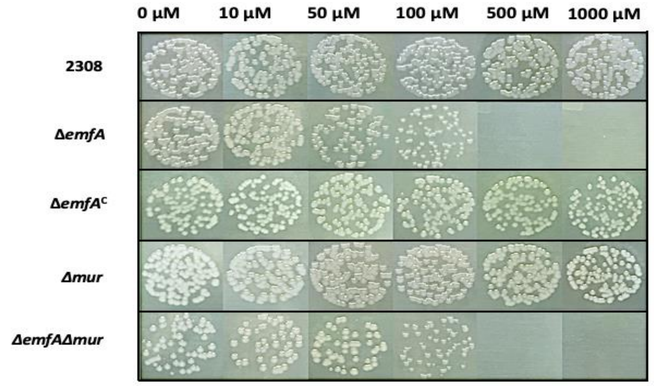


Figure 2: An Mn toxicity assay was performed on Schaedler agar supplemented with increasing concentrations of $MnCl_2$. Deletion of the Mn-responsive transcriptional regulator, Mur, does not increase *B. abortus* sensitivity to excess environmental Mn. However, the *B. abortus emfA* mutant exhibits increased sensitivity to excess environmental Mn. These results suggest that EmfA is the primary determinant of Mn resistance in *B. abortus* 2308.

The *B. abortus emfA* mutant accumulates increased cellular Mn following exposure to excess Mn

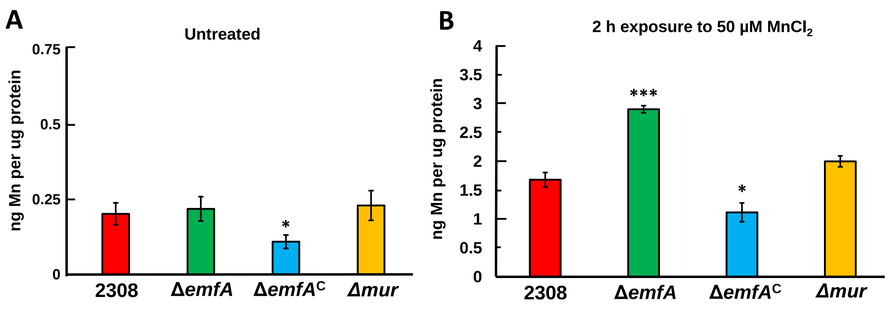


Figure 3: Total cellular Mn content of *B. abortus* 2308 and derivative strains before (A) and after (B) exposure to a 50 μM $MnCl_2$ 'metal shock'. Each tested strain was grown to mid-log phase prior to the addition of excess Mn. Inductively coupled plasma mass spectrometry was employed to determine the total cellular Mn for each sample and a Bradford Assay was performed to determine the amount of total protein present in each sample. *= p<0.05, ***= p<0.005 for comparison of 2308 versus the other strains using the Student T-test.

The *B. abortus emfA* mutant does not exhibit increased sensitivity to other biologically-relevant metals

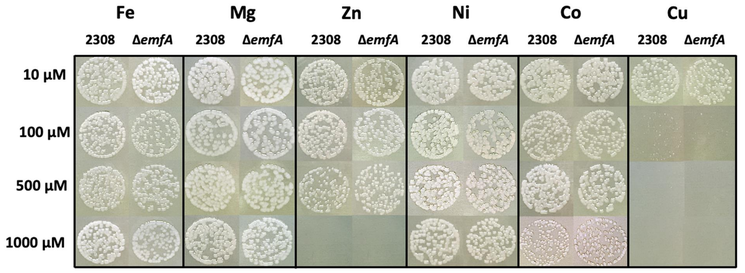


Figure 4: A metal toxicity assay was performed on Schaedler agar supplemented with increasing concentrations of biologically-relevant metals other than Mn. The *emfA* mutant does not display increased sensitivity to excess concentrations of other biologically-relevant metals compared to the parental 2308 strain. These data suggest that *B. abortus* EmfA possesses a specific role in preventing Mn toxicity.

The *B. abortus emfA* mutant displays attenuation in a murine infection model

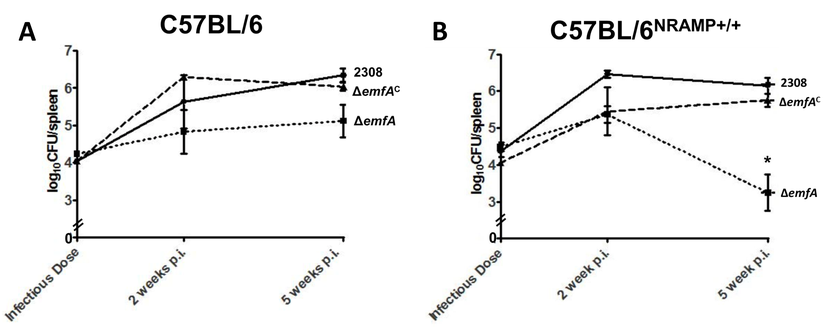


Figure 5: Spleen colonization profiles of *Brucella abortus* 2308, an *emfA* mutant, and the *emfA* mutant complemented with a plasmid-borne copy of *emfA* in C57BL/6 (A) and C57BL/6$^{NRAMP+/+}$ (B) mice. Mice were infected with $5x10^4$ brucellae via the intraperitoneal route and evaluated at two weeks and five weeks post infection. While the *emfA* mutant strain displays attenuation at five weeks post-infection in both strains of mice, the attenuation is greater in C57BL/ 6$^{NRAMP+/+}$ mice that encode a functional NRAMP1 protein. *= p<0.05 for comparison of 2308 versus the other strains using the Student T-test.

Summary/Conclusion

- The *B. abortus* Mn-responsive transcriptional regulator, Mur, is not required for resisting excess environmental Mn.
- The *Brucella spp*. employ a CDF-type metal efflux protein, EmfA, to confer resistance to excess environmental Mn.
- The *emfA* mutant accumulates increased cellular Mn following exposure to excess Mn which is consistent with the proposed role of EmfA as a Mn efflux protein.
- EmfA is required for wild-type virulence of *B. abortus* 2308 in mice.

References

1. Anderson et al. 2009. Infect. Immun. **77**:3466-3474.
2. Cubillas et al. 2014. Metallomics **6**:1808-1815.
3. Raimunda and Elso-Berberián. 2014. Biochim. Biophys. Acta **1838**:3203-3211.
4. Martin et al. 2017. Mol. Microbiol. **104**:334-348.
5. Pitzer et al. 2018. J. Bacteriol. doi: 10.1128/JB.00471-18
6. Martin et el. 2012. Microbiology. **158**:1767-1774

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