Discovery of a novel iron-acquisition gene in *Bacillus anthracis* Sterne

R. Samuel Baugh, Jacob A. Malmquist, Shauna M. McGillivray

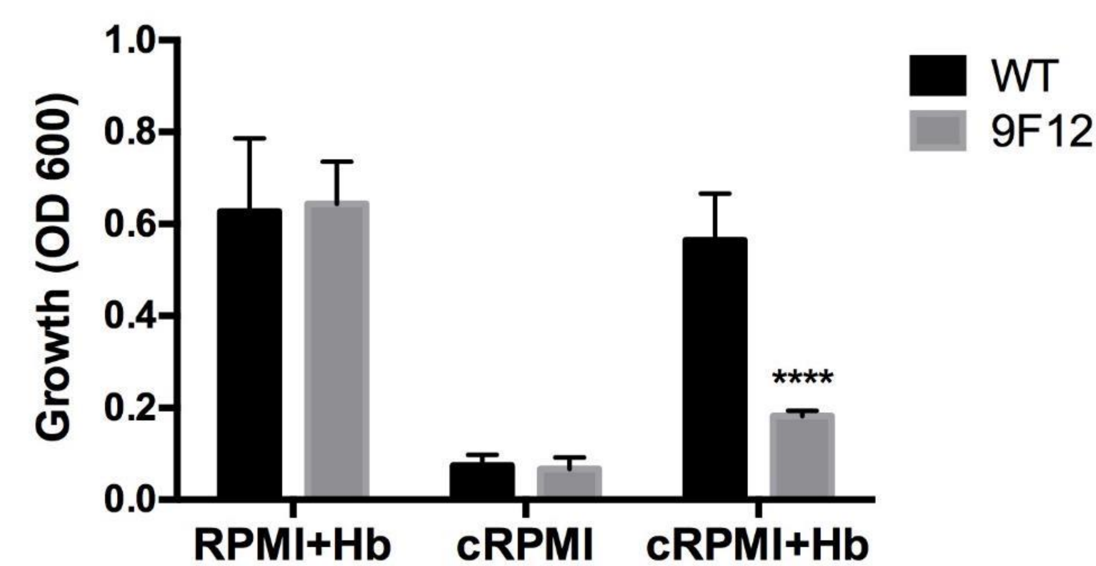
Department of Biology, Texas Christian University, Fort Worth, TX

Introduction

As the threat of antimicrobial-resistant infections continues to rise, the need for novel antibiotics grows. Targeting virulence factors in bacterial pathogens is one potential strategy for antibiotic development because inhibiting virulence would decrease the ability of the pathogen to evade the host immune response. This strategy may decrease the development of resistance since the treatment is not directly bactericidal and there is less selective pressure put on the bacteria population. Our goal is to discover new virulence genes in *Bacillus anthracis* Sterne that could potentially be a therapeutic target. Specifically, we are interested in finding genes that allow *B. anthracis* Sterne to acquire iron from the host. For bacterial pathogens, iron is critical for growth and often a limiting nutrient in the host. It has been linked with proper functioning of electron transfer proteins and superoxide dismutase enzymes. In a *B. anthracis* infection, iron is acquired from host hemoglobin through a hemolytic pathway, but the complete mechanism of this is unknown. Approximately 1000 transposon mutants of *B. anthracis* Sterne were screened for the inability to acquire iron from hemoglobin, and five were deficient in acquiring iron from hemoglobin in *in vitro* assays. Of those five mutant strains, only one (9F12) also exhibited an in vivo phenotype using the wax worm model of infection. The gene disrupted in the 9F12 transposon mutant is the *dUTPase/aminopeptidase* gene. Our aim in this study is to confirm that the disruption of the *dUTPase* gene alone, rather than aminopeptidase, leads to the inability to acquire iron from hemoglobin in *B. anthracis* Sterne.

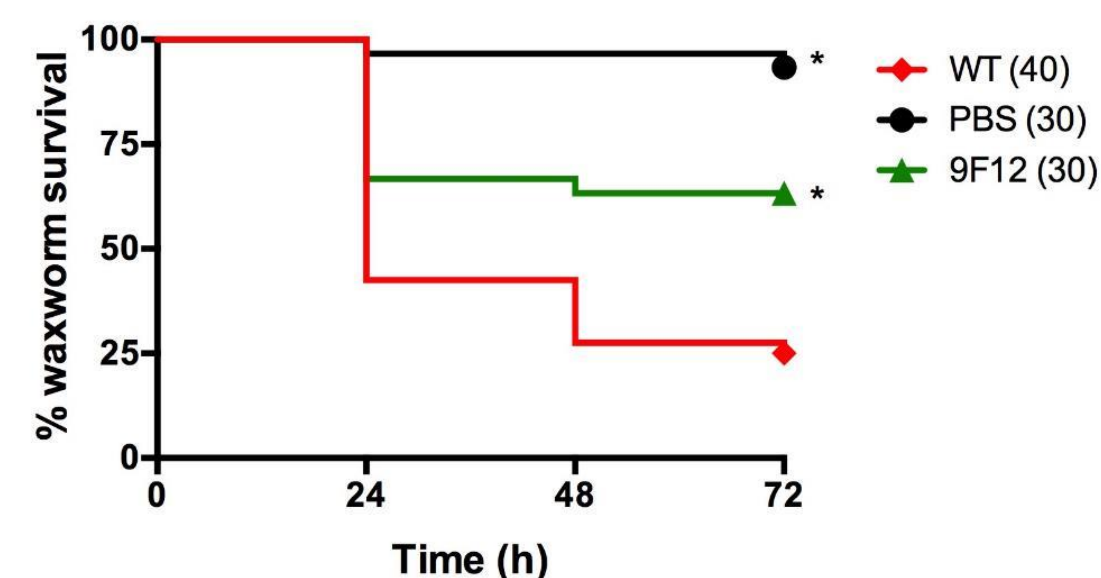
Background

Growth of 9F12 Tn mutant in iron-deficient media



RPMI and chelated RPMI (cRPMI) supplemented with 50 µl of 200 µM stock lyophilized bovine hemoglobin (Hb) yielding a final concentration of 10 µM. 10 µl of overnight culture were used to inoculate 990 µl of RPMI+Hb, cRPMI, and cRPMI+Hb. Cultures were incubated at 37°C under shaken, aerobic conditions. Optical density (OD) was read after 48 hours. ****, p<0.0001 by one-way ANOVA followed by Tukey's multiple comparisons test.

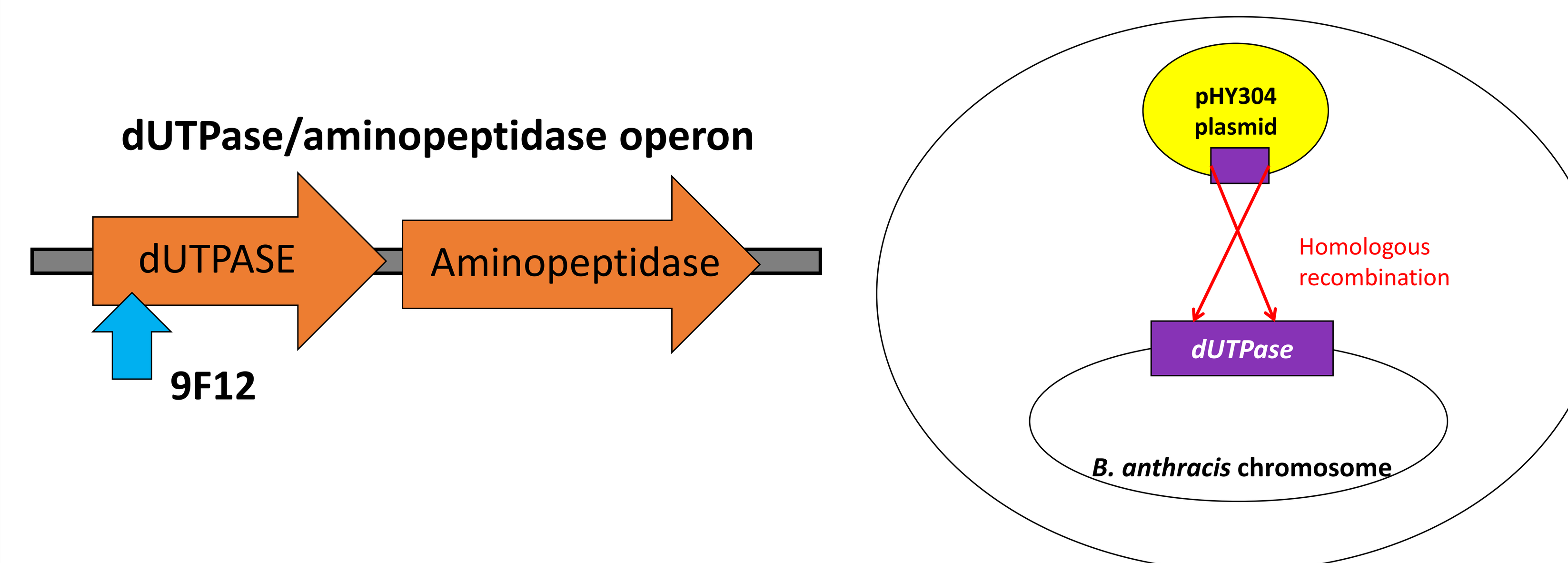
Increased survival of G. mellonella infected with 9F12



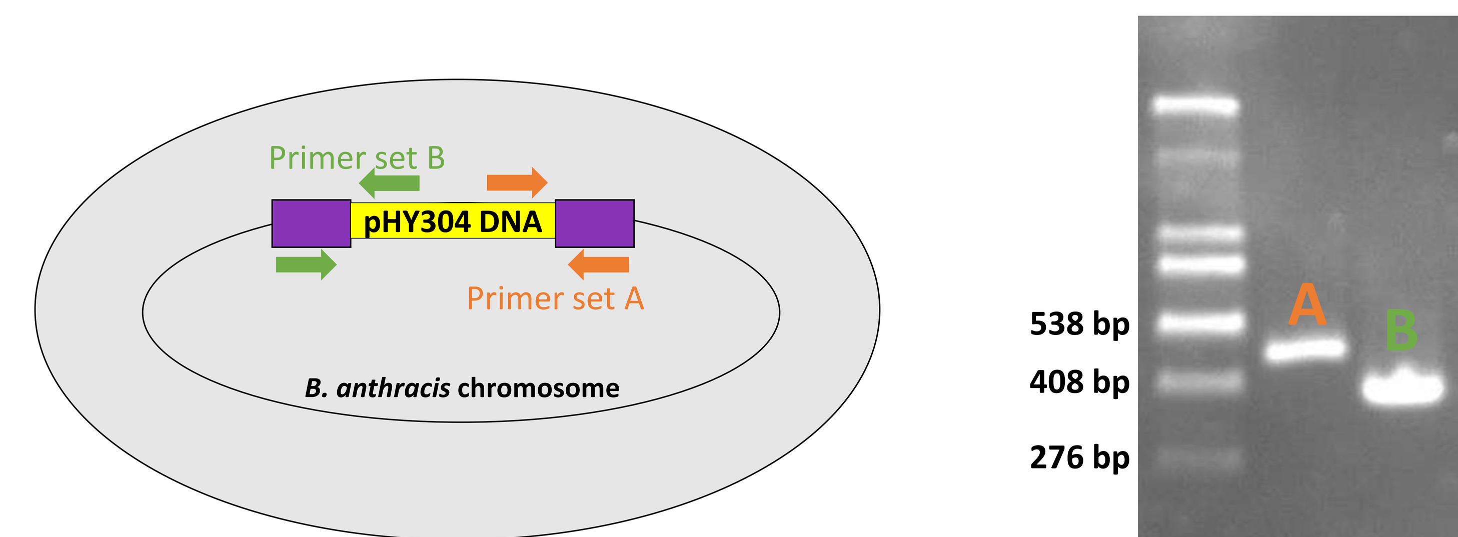
Bacterial strains were grown to OD 0.4 and diluted 1:2 in PBS. 10 ul of bacteria were injected in each worm. Worms incubated at 37°C for 72 hours. Survival assessed every 24 hours for 3 days. *, p<0.01 by Mantel-Cox test in comparison to the WT survival curve.

Creating *dUTPase* Insertional Mutant

Goal: Insertional mutagenesis of dUTPase gene

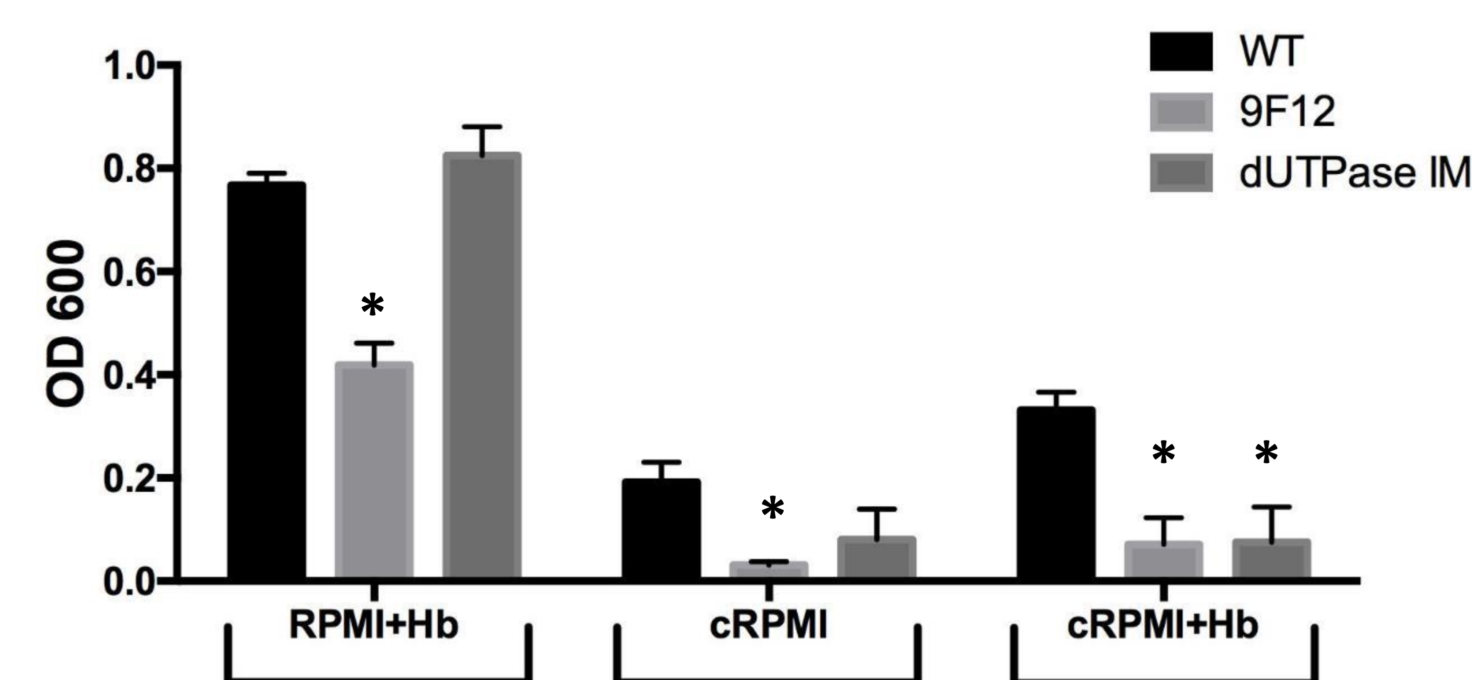


B. anthracis Sterne dUTPase IM



Integration of pHY304 into chromosomal dUTPase gene. Confirmed by amplification of plasmid and genomic DNA via PCR using primers specific for plasmid and genomic DNA and isolated by gel electrophoresis. (A) pHY304 forward primers and dUTPase EV reverse primers. (B) dUTPase EV forward primers and pHY304 reverse primers.

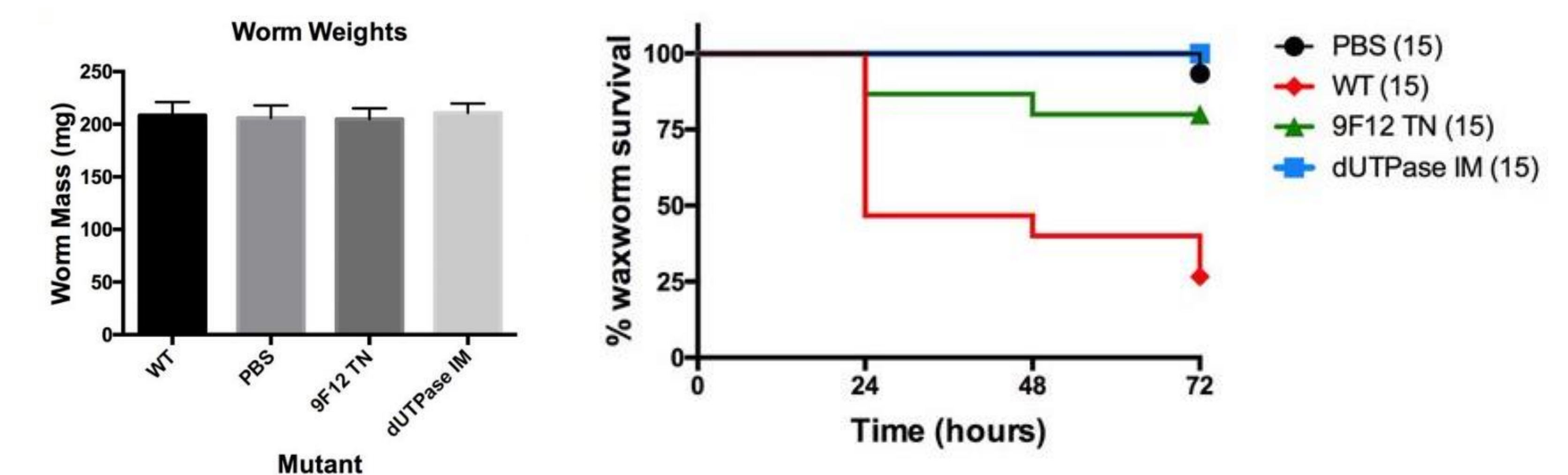
Iron Acquisition Assay



RPMI and chelated RPMI (cRPMI) supplemented with 50 µl of 200 µM stock lyophilized bovine hemoglobin (Hb) yielding a final concentration of 10 µM. 10 µl of overnight culture were used to inoculate 990 µl of RPMI+Hb, cRPMI, and cRPMI+Hb. Cultures were incubated at 37°C under shaken, aerobic conditions. Optical density (OD) was read after 48 hours. n = 5. *, p<0.05 by one-way ANOVA followed by Dunnett's post-hoc test in comparison to WT growth in each growth condition.

G. mellonella Survival Assay

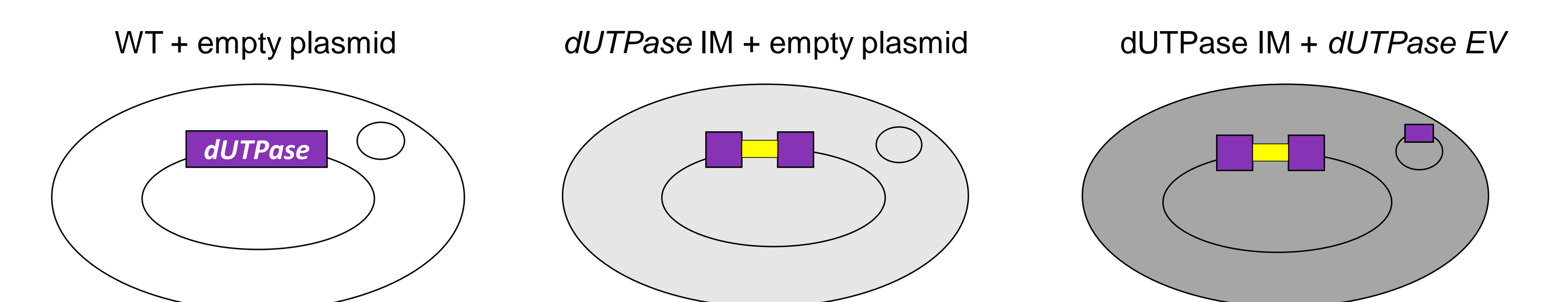
dUTPase gene necessary for *B. anthracis* Sterne pathogenesis



Bacterial strains were grown to OD 0.4 and diluted 1:2 in PBS. 10 ul of bacteria were injected in each worm. Worms incubated at 37°C for 72 hours. Survival assessed every 24 hours for 3 days.

Future Directions

- **Complementing Δ*dUTPase* in *B. anthracis* Sterne**



- **Optimize hemoglobin assay**
- **Test in mammalian model of infection**

Conclusions

Disruption of the dUTPase gene in *B. anthracis* Sterne results in a decreased ability to grow in an iron-deficient environment. Results from the hemoglobin assay were inconsistent, unlike those conducted by the original graduate student. Measures will be taken to yield a more consistent result in the future. The *G. mellonella* assay showed strong results supporting the importance of the dUTPase gene in *B. anthracis* pathogenesis. This gene codes for a protein that shares homology with the UTPase family of proteins found in other strains of bacteria, and has yet to be fully characterized. Directly downstream from this dUTPase gene is an aminopeptidase gene. Due to the proximity of these two genes to each other, we hypothesize that they share an operon. We believe that the 9F12 TN insertion may disrupt the downstream aminopeptidase gene as well. Once this method yields consistent results seen in the first tests on the transposon mutant strains, the next step will be to make a complement strain with a dUTPase expression vector. If future Hb assays and *G. mellonella* survival assays continue to yield promising results, the next step will be a mammalian model of infection test.

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