The Characterization of the Potential Iron-acquisition Gene *dUTPase* in *Bacillus anthracis.*

By

Kyle R. Gallegos

Bachelor of Arts, 2019

University of North Texas

Denton, Tx

Submitted to the Graduate Faculty of the College of Science and Engineering

Texas Christian University

In partial fulfillment of the requirements

For the degree of

Master of Science

May 2023

THE CHARACTERIZATION OF THE POTENTIAL IRON-ACQUISITION GENE DUTPASE IN BACILLUS ANTHRACIS

by

Kyle R. Gallegos

Dissertation approved:

Major Professor

For The College of Science and Engineering

Acknowledgements

I would like to thank my phenomenal advisor Dr. Shauna McGillivray for taking me into her lab and giving me the wonderful opportunity to take part in her research. She not only provided me with more knowledge of the life sciences, but the skills it takes to become a better scientist and mentor. For this, I am forever grateful. I would also like to thank my committee members, Dr. Meredith Curtis and Dr. Mikaela Stewart, for their wisdom and assistance in the completion of this project. To the whole TCU Biology staff, I cannot thank you all enough. You all have made my time hear at TCU a grand experience. Carmella, thank you for everything. You were not only my friend, but one of my biggest supporters throughout my time here at TCU. Lastly, I would like to thank all individuals in the McGillivray Lab. You all have been a major part of my life over the course of my masters career and I will forever cherish our times together.

Table of Contents

List of Figures

Introduction

Bacillus anthracis is a gram-positive, rod-shaped, and spore-forming bacterium found in soils throughout the world, especially in areas associated with tending to livestock (Romero et al., 2020). As of now, it is estimated that 1.8 billion people are at risk of exposure to *B. anthracis*. However, it is most common in poverty-stricken parts of Africa, Asia, and Eurasia, with millions of rural individuals experiencing economic decline or life-threatening illness associated with *B. anthracis* (Carlson et al., 2019). *B. anthracis* is recognized as the causative agent of the disease anthrax. Despite its association as a zoonotic disease, humans are also susceptible to the disease via exposure to spores. Spores are the metabolically inactive forms of bacteria that are resistant to external stresses found in nature or in the host. Spores also sense when ideal nutrients are available and can rapidly return to vegetive growth, which results in increased risk of infection (Driks et al., 2009). In humans, there are three variations of anthrax based on the location of the infection in the body and they vary in severity. The first and most common infection is cutaneous, which arises from an exposed external wound to the bacterial spore. The second is gastrointestinal, which results from the consumption of spores found on raw meat from an animal that was not vaccinated against anthrax. Lastly, the rarest and deadliest form is inhalational anthrax, which results from the intake of spores into the lungs (Shafazand et al., 1999). Once in the lungs, the spores bypass the host's immunity, germinate and rapidly multiply, resulting in severe septicemia and possibly death (Shafazand et al., 1999). Even with aggressive treatment the mortality rate of inhalational anthrax is almost 50% (Bowers et al., 2019). Therefore, the National Institute of Allergy and Infectious Diseases classifies *B. anthracis* as a Category A Priority Pathogen. Consequently, due to the high mortality rate and ease of transmission, *B. anthracis* has the

potential as a biological weapon. One case of this was the releasing of spores in the United States mail service in 2001 resulting in 18 confirmed cases and 5 deaths (Jernigan et al., 2002). Following the attacks, there was renewed interest in studying *B. anthracis* virulence.

The most characterized virulence factors are the pX01 and pX02 plasmids. The pX01 plasmid has genes that encode for the lethal toxin and edema toxin, which destroy the host's immune cells by inducing apoptosis or interfering with cellular signaling (Perry 2020). The pX02 plasmid has genes that encode for capsule synthesis, which serves as a protective shield that prevents the host's macrophages from binding to the vegetative bacterium and phagocytizing it (Friebe et al., 2016). However, there has been a shift in interest into the characterization of novel virulence factors encoded in the chromosome. Although sequencing of the *B. anthracis* genome has identified multiple genes that could contribute to virulence by counteracting a range of host defenses (Read et al., 2003), we chose to focus our research on chromosomal genes associated with the acquisition of the essential micronutrient iron.

Bacteria use iron for cellular functions such as growth, DNA replication, metabolism, and energy generation; however, iron is only found in the external environment. To survive, *B. anthracis* must obtain iron from its host. Despite the abundance of iron in a healthy individual, iron is made unavailable through tight sequestering to hemoglobin or is bound to transfer proteins, such as transferrin and ferritin, when released from dead red blood cells. Nevertheless, *B. anthracis* has found efficient strategies to acquire iron. When iron is bound to transfer proteins, one of the strategies used are the siderophores bacillibactin and petrobactin (Hotta et al., 2010 and Nusca et al., 2012). Siderophores are low molecular weight iron chelators released that shuttle hazardous free-radical iron in the blood into the

cell (Lee et al., 2011) as well as strip iron from transfer proteins and shuttle it into the bacteria (Zawadzka et al., 2010). Strategies to acquire iron from hemoglobin include the use of hemolysins to release hemoglobin from red blood cells (Callegan et al., 1999), hemoglobin receptors (Pishchany et al., 2012), proteases and heme oxygenase to degrade hemoglobin and expose iron (Honsa et al., 2011). However, these are most effective if there is an abundance of hemoglobin in the environment which the bacteria can easily access. Therefore, one scavenging technique used by *B. anthracis* are Isd proteins. Isd proteins are a family of NEAT proteins anchored to the cell wall that capture hemoglobin as it passes by, remove heme and transfer it into the cell to be degraded by heme oxygenase (Ellis-Guardiola et al., 2021). IsdX1 and IsdX2 are two Isd proteins that have gained interest because of their correlation with increased survival in iron-poor environments as well as their high affinity for iron bound to hemoglobin (Maresso et al., 2008). However, the deletion of the IsdX1 and IsdX2 proteins has shown to have no attenuated virulence phenotype *in vivo* (Balderas et al., 2012). This has sparked interest to discover other chromosomal genes associated with iron acquisition from hemoglobin that may also contribute virulence.

The discovery of genes associated with iron acquisition from hemoglobin started with the creation of our mutant library through transposon mutagenesis Dr. Shauna McGillivray (McGillivray et al., 2009). From this library we are able to select random mutants and test for virulence-related phenotypes, such as iron acquisition from hemoglobin. To test for iron acquisition, we chelated the media to remove divalent-cations, including iron, and then supplemented with hemoglobin as the sole source of iron. Previous students grew mutants in the chelated media supplemented with hemoglobin and measured growth after 24 hrs. They screened for lack of growth as this is indicative of the inability to acquire iron from

hemoglobin. After screening approximately 2000 mutants, 5 mutants were found that were unable to acquire iron from hemoglobin (Manceras & Green unpublished data). However, these mutants at the time were only tested *in vitro*. To assess virulence, an *in vivo* model is required. Therefore, to further prioritize these 5 mutants, they were tested in an invertebrate animal model of infection with *Galleria mellonella* to discover which of these mutants truly had attenuated virulence. Only 1 of the 5 transposon mutants was significantly different from the parent strain (*B. anthracis*-Sterne) in its ability to kill the injected host (Malmquist et al., 2018). This mutant is known as 9F12 Tn. The 9F12 Tn mutant has a transposon inserted into the first gene of a putative two-gene operon containing *dUTPase* and *aminopeptidase* (*ysdC*) genes. The dUTPase enzyme functions by hydrolyzing dUTP into dUMP, which prevents its utilization by DNA polymerases during replication and repair (Ladner 2001) and decreases the chances of misincorporation of uracil into DNA (Kerepesi et al., 2016). Aminopeptidases are a branch of enzymes that break terminal bonds in a peptide chain. This is essential for protein maturation, protein turnover, and modulation of gene expression (Cheng et al., 2015). To our knowledge neither have been characterized in relation to iron-acquisition in *B. anthracis.* Therefore, the goal of this study is to use the 9F12 Tn mutant and an independently created insertional mutant to confirm whether the *dUTPase* gene is necessary for *B. anthracis'* ability to acquire iron from hemoglobin and whether it is important for virulence in *B. anthracis*.

Materials & Methods

Bacterial strains

The parental *B. anthracis* strain used in this study is capsule-deficient *B. anthracis* Sterne ($pX01^+$, $pX02^-$). *B. anthracis* 9F12 Tn was isolated from a previously described transposon mutant library (McGillivray 2009). *B. anthracis* dUTPase IM was constructed by a previous student (Sam Baugh unpublished data). Overnight *B. anthracis* cultures were grown in brain heart infusion (BHI; Hardy Diagnostics) and supplemented with kanamycin 50 µg/mL or erythromycin 5µg/mL (all Sigma) when growing the transposon or insertional mutant (respectively).

Media & solutions

200 µM hemoglobin (Sigma) was created by adding 129 mg bovine hemoglobin to 10mL sterile water. It was not sterile filtered unless specifically indicated otherwise. All hemoglobin assays were carried out in Roswell Park Memorial Institute medium supplemented with 5 % Luria-Bertani (RPMI-LB) or in RPMI-LB that had undergone chelation. To achieve chelation, 3 g of Chelex salt (Sigma) was added to 100 mL RPMI-LB shaking at room temperature for 1 hr and then sterile filtered. This process was repeated to make chelated-RPMI-LB (cRPMI-LB). To make iron-free cation supplemented media, the following stock cation solutions were made: 100 mM $ZnCl_2$ stock was created by adding 136 mg $ZnCl_2$ to 10 mL pure water, 100 mM $MnCl_2$ stock was created by adding 126 mg $MnCl_2$ to 10 mL pure water, 1 M $MgCl_2$ stock was created by adding 950 mg $MgCl_2$ to 10mL pure water, 100 mM $CaCl_2$ was created by adding 110 mg $CaCl_2$ to 10 mL pure water (all Sigma). These were diluted into c-RPMI-LB to a final concentration of 25 µM $ZnCl_2$, 25 µM $MnCl_2$, 1 mM $MgCl_2$, and 100 µM $CaCl_2$ to make cation-supplemented c-RPMI-LB, hereby denoted

as (c-RPMI-LB^{2+}). 100 mM ferric iron stock was created by adding 127 g $FeCl_3$ to 10 mL pure ethyl alcohol then diluted into c-RPMI-LB^{2+} to make a final concentration of 100μM to make ferric iron supplemented c-RPMI-LB^{2+} hereby denoted as (c-RPMI-LB^{2+}+ $FeCl_3$). To create a stock solution of 1 mM 2,2'-bipyridyl, 781 mg of 2,2'-bipyridyl was added to 10 mL pure ethyl alcohol. 50 uL of 1 mM 2,2'-bipyridyl was added to 25 mL cRPMI-LB^{2+} to achieve a final concentration of 500 μM.

Growth curves

Log phase cultures were first generated by adding 100 μL of overnight cultures in 3 mL BHI and growing to an optical density of 0.4. Log cultures were washed and resuspended in 1 mL of PBS. 100 μL of washed log-phase culture was added to 3 mL of BHI or RPMI-LB. Strains were incubated at 37^{o}C shaking under aerobic conditions. Optical density (OD) at wavelength 600 nm was recorded every hour.

Hemoglobin assay

The parental *B. anthracis* strain (WT *Ba*), 9F12 Tn, and dUTPase IM were grown overnight in BHI. 10 μL of unwashed overnight cultures were placed in in 5 mL plastic culture tubes containing 965 μL c-RPMI-LB and 350 μL of 200 μM unfiltered hemoglobin for a final concentration of 53 μM (c-RPMI-LB + Hb) and incubated at 37^{o}C shaking under aerobic conditions for 48-hrs. A positive control containing RPMI-LB and negative controls containing c-RPMI-LB were also included. 200 μL of the 48-hr culture was transferred to a 96-well plate and the OD600 read on the plate reader. During cation supplementation, the assay was conducted identically with the inclusion of the c-RPMI-LB^{2+} with or without 500 μM 2,2'-dipyridl.

Galleria mellonella survival assay.

G. mellonella survival assay was conducted according to the method previously developed (Malmquist 2018) with small modifications. *G. mellonella* were obtained from Rainbow Mealworms. Larvae weighing 170-230 mg were placed into groups of ten for injection. Larvae were kept at room temperature prior to injection. *B. anthracis* strains were grown overnight, diluted 1:20, and grown to log phase (OD 0.4) in BHI. Once OD 0.4 was reached, bacteria were washed and suspended in PBS at a 1:2 dilution and the cfu/ml of the diluted culture was enumerated using serial dilution. Larvae were injected with 10 µL of 1:2 diluted bacteria Following injection, larvae were observed at room temperature to ensure they recovered from injection and were transferred to an incubator at 37^{o}C. Surviving larvae were counted at 24, 48, and 72-hrs post injection.

Statistical analysis

Statistical analysis was performed using GraphPad Prism. Hemoglobin assays were evaluated with a one-way ANOVA followed by a Tukey's *post hoc* analysis using GraphPad Prism software. The Kaplan-Meir survival assay was assessed using a log-rank test to generate a p-value using GraphPad Prism software.

Results

Establishing hemoglobin assay conditions with wild-type *B. anthracis*

Prior to determining that the *dUTPase* gene is necessary for iron acquisition from hemoglobin in *B. anthracis*, we needed to solidify our assay conditions as a previous undergraduate in the lab had struggled to get consistent results once the assay was expanded from growing bacteria in 96-wells to larger-volume culture tubes. RPMI was originally selected as the media because, as a mammalian cell culture media, it more closely resembles the host's physiological environment including being naturally low in iron. However, there

are different types of RPMI available, including the presence and absence of phenol red and supplemented glutamine. We saw no differences between RPMI and RPMI with phenol red, but due to inconsistencies with growth in chelated media with phenol red we decided to use plain RPMI. Surprisingly, we saw that adding glutamine as a bacterial-protein source was detrimental to growth (figure 1A). Because RPMI is low in nutrients and challenging for bacteria to grow in, we supplemented with 5% the bacterial media Luria-Bertani (LB) broth to make RPMI-LB as the base media for our assays.

Our next objective was to optimize the hemoglobin used in our assays. First, we wanted to determine if sterile filtering the hemoglobin stock solution was necessary after it was dissolved in water. We measured growth (OD 600) after growing wild-type in chelated RPMI-LB (c-RPMI-LB) with increasing concentrations (μM) of sterile-filtered or unfiltered hemoglobin for 48 hrs. We found that wild-type grew better in c-RPMI-LB with supplemented unfiltered hemoglobin (figure 1B). Therefore, this indicates that sterile filtering hemoglobin is unnecessary for completing our assays. Another factor of potential contamination that needed to be addressed was nutrient contamination from growing cultures overnight in nutrient rich BHI. To reduce contamination in c-RPMI-LB we tested whether washing the overnight culture was necessary. To wash bacteria, we centrifuged the overnight culture, removed the supernatant and resuspended the culture in sterile saline buffer (PBS) and then repeated the process a second time. We then added either 10 μL of bacteria from BHI overnight cultures or 10 μL from washed cultures to c-RPMI-LB with increasing amounts of supplemented hemoglobin and measured growth after 48 hrs. We observed no difference in growth between washed and unwashed wild-type bacteria (figure 1C). Therefore, we chose to use unwashed overnight culture as this simplified assay set up.

Throughout the preliminary assays we noticed a dose-dependency with the amounts of hemoglobin supplemented. Therefore, we wanted to determine the ideal amount of hemoglobin by using higher amounts of hemoglobin than previously used. We grew wild-type in c-RPMI-LB with 41 µM, 53 µM, or 63 µM of supplemented hemoglobin and measured growth after 48 hrs. We found no difference in growth between the three amounts of hemoglobin, so we decided to select the middle dose of 53 µM (figure 1D). Therefore, all following assays used unwashed bacteria in c-RPMI-LB supplemented with 53 µM of unfiltered hemoglobin.

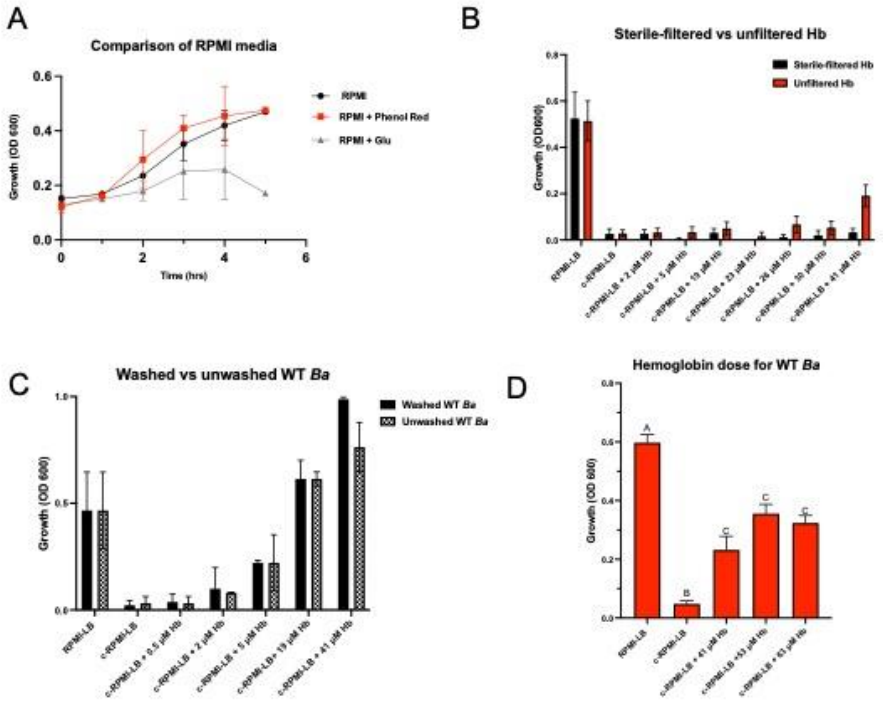

Figure 1: Establishing hemoglobin assay conditions for *B. anthracis*.
A, Wild-type *B. anthracis* Sterne (WT *Ba)* grown in RPMI, RPMI + phenol red, and RPMI supplemented with glutamine. Data represents 2 independent experiments. **B,** WT *Ba* grown in regular or chelated RPMI (c-RPMI) supplemented with LB (c-RPMI-LB) using sterile-filtered or unfiltered hemoglobin (Hb) at increasing concentrations. Data represents 3 independent experiments. **C,** Overnight cultures were washed in PBS or left unwashed before grown in the indicated conditions. Data represents 2 independent experiments. **D,** Unwashed overnight cultures WT *Ba* was grown in c-RPMI-LB + unfiltered Hb at increasing concentrations. Data represents 5 independent experiments. All data is presented as mean $^{+}$/- SEM. Same letters indicate $p > 0.01$, different letters indicate $p < 0.01$ by one-way ANOVA followed by Tukey's post-hoc test was used.

Verification of the disrupted *dUTPase* gene in 9F12 Tn and dUTPase IM

The *dUTPase* is one of two genes found in a putative operon, the other gene being *ysdC*, an aminopeptidase. To ensure that phenotypes observed in the 9F12 Tn mutant were due to the disruption of the *dUTPase* gene rather than due to an unknown mutation in another gene, a previous student used a technique known as insertional mutagenesis to create a second independent mutant known as dUTPase IM. Insertional mutagenesis involved the insertion of the 5,000 bp pHY304 plasmid into the middle of the *dUTPase* gene via homologous recombination (figure 2A). To validate the specific disruption of the *dUTPase* gene, we performed PCR on genomic DNA from wild-type, 9F12 Tn, and dUTPase IM using specific primers for the *dUTPase* and *ysdC* genes. The primers were designed to amplify the middle of either the *dUTPase*, indicated by the black arrows, or *ysdC* genes, indicated by the pink arrows. The results of amplification for the *dUTPase* primers should be approximately 485 bp, whereas the results of amplification for *ysdC* should be approximately 430 bp. As expected, we only observed amplification of the *dUTPase* gene in wild-type indicated by the band at ~ 400bp due to no disruption of the *dUTPase* gene. Our 9F12 Tn and dUTPase IM mutants had no amplification of the *dUTPase* gene despite using the same primers due to the insertion of a 10,000 bp transposon or 5,000 bp plasmid in the middle of the gene. Therefore, this confirms the disruption of the *dUTPase* gene in our dUTPase mutants. We observed amplification of the *ysdC* gene across all strains confirming that the only gene disrupted in our dUTPase mutants is *dUTPase* and not *ysdC* (figure 2B)

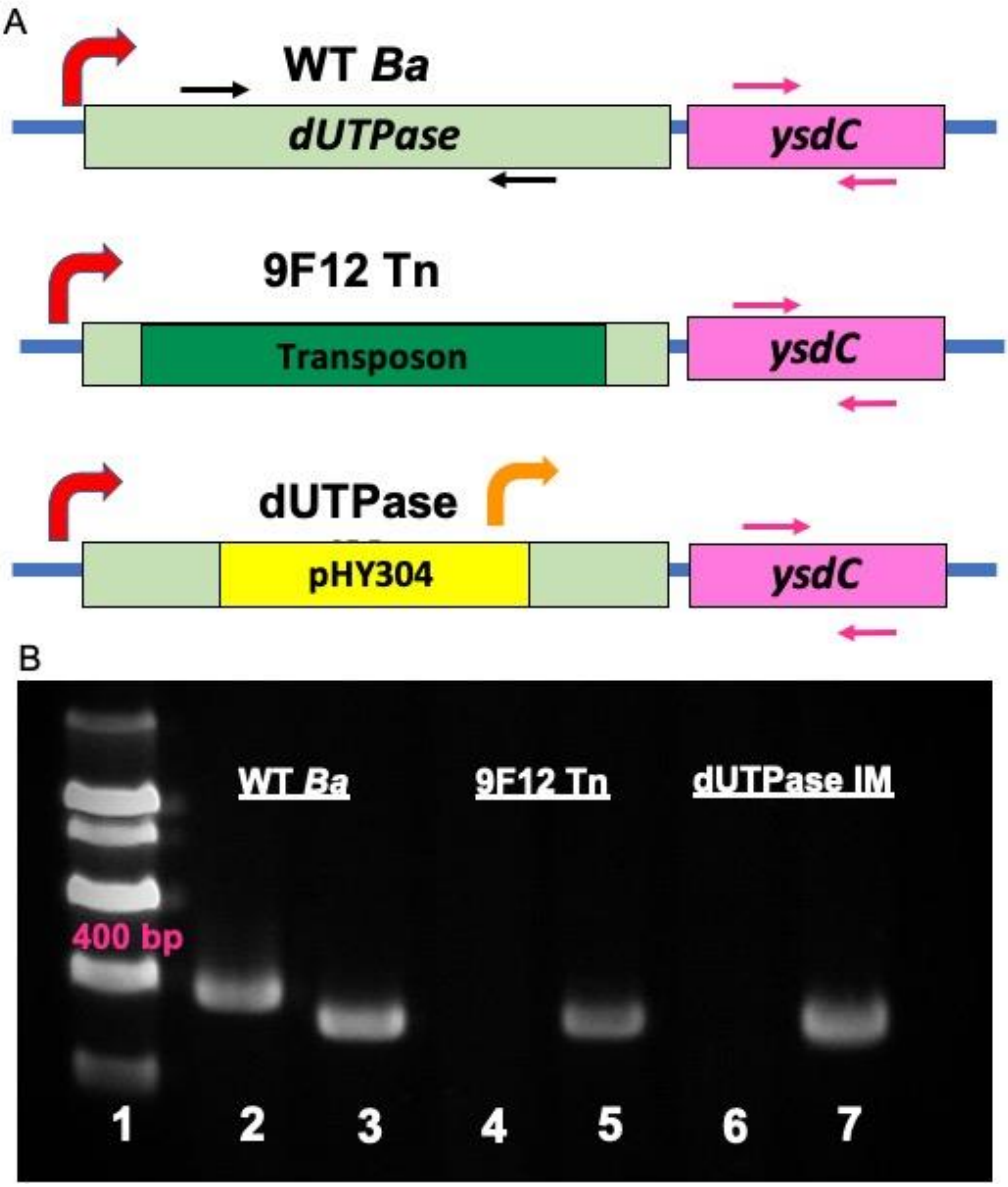

Figure 2: Genetic disruption of the *dUTPase* gene
A, Schematic of wild-type *B. anthracis* (WT *Ba*), 9F12 Tn and dUTPase IM. Blue line represents *B. anthracis* chromosome. Light green and pink rectangles represent the *dUTPase* or *ysdC* gene respectively. Dark green and yellow rectangles represent the inserted transposon or pHY304 plasmid in the *dUTPase* gene respectively. Black arrows represent the locations of dUTPase primers. Pink arrows represent the location of *ysdC* primers. Red arrow represents the operon promoter. Orange arrow represents the pHY304 promoter. **B**, Gel electrophoresis of the disruption of the dUTPase gene in the WT *Ba*, 9F12 Tn and dUTPase IM genome. Lane 1 is ladder. Lane 2, 4, 6 are amplified *dUTPase* gene. Lane 3, 5, 7 are amplified *ysdC* gene.

The loss of the *dUTPase* gene leads to the inability to acquire nutrients from hemoglobin

To ensure that the loss of the *dUTPase* gene does not affect the ability for *B. anthracis* to grow in standard media, we grew wild-type, 9F12 Tn, dUTPase IM in both BHI (figure 3A) and RPMI-LB (figure 3B). We measured the amount of bacterial growth in both medias every hr for at least 8 hrs and found no differences between wild-type, 9F12 Tn, dUTPase IM in BHI or RPMI-LB. This indicates that there are no inherent growth defects in our mutants.

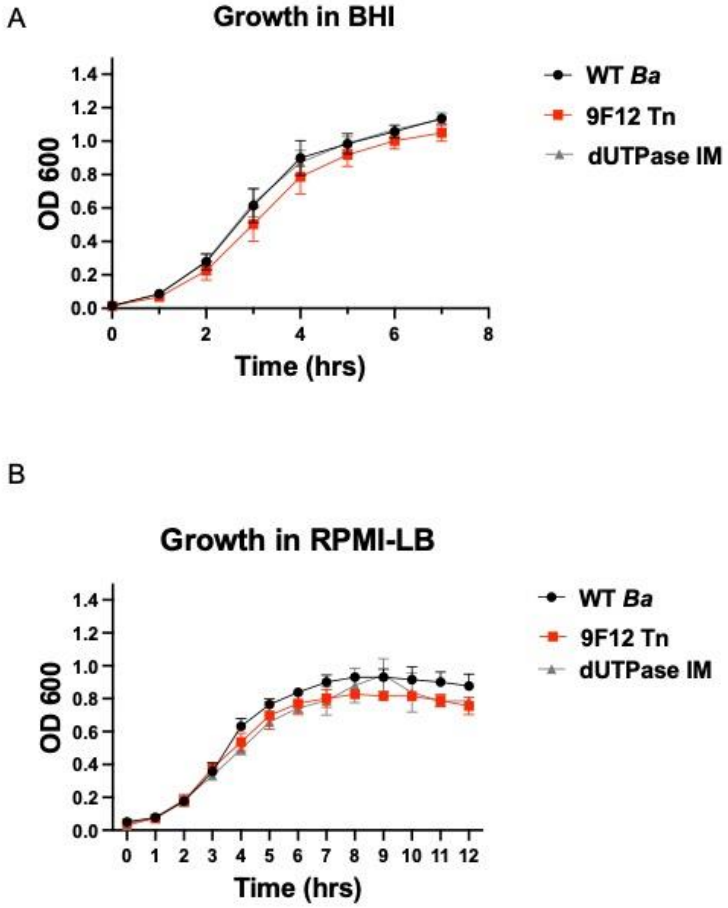

Figure 3*:* Loss of *dUTPase* gene does not affect growth in unchelated growth media.
Growth assay of wild-type *B. anthracis* (WT *Ba*), 9F12 Tn, and dUTPase IM mutants in **A**) BHI and **B**) RPMI-LB. Data is presented as mean +/- SEM from at least 3 independent experiments.

We next wanted to determine whether the *dUTPase* gene is necessary for the acquisition of divalent-cations from hemoglobin using both of our mutants. We grew wild-type, 9F12 Tn, and dUTPase IM in RPMI-LB, c-RPMI-LB, and c-RPMI-LB with supplemented hemoglobin (c-RPMI-LB + Hb) and measured growth after 48 hrs. We found no significant difference in growth between wild-type, 9F12 Tn, and dUTPase IM in RPMI-LB confirming all three strains can grow in unchelated media. There was no growth for wild-type, 9F12 Tn, and dUTPase IM in c-RPMI-LB indicating all three strains cannot grow in chelated media. As expected, we found that wild-type was the only strain to significantly grow in c-RPMI-LB + Hb, and there was no difference in growth between our dUTPase mutants in c-RPMI-LB and c-RPMI-LB + Hb. This indicates that the *dUTPase* gene is required for nutrient acquisition from hemoglobin (figure 4).

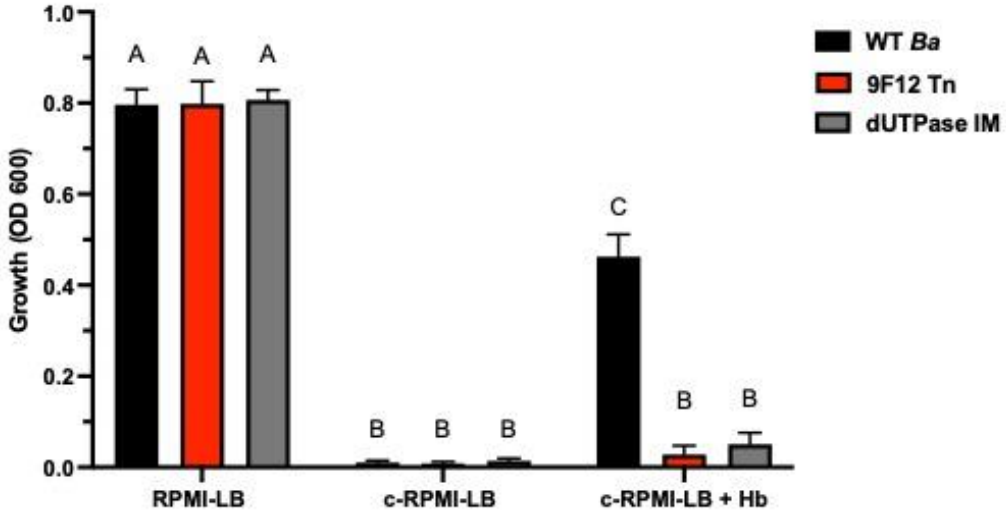

Figure 4***:*** **The *dUTPase* gene is necessary for growth with hemoglobin as a nutrient source.**
Wild-type *B. anthracis* (WT *Ba*), 9F12 Tn, and dUTPase IM were grown in RPMI-LB, c-RPMI-LB, and c-RPMI-LB + Hb. Data is presented as mean $^{+}$/- SEM from 12 independent experiments. Same letters indicate $p > 0.01$, different letters indicate $p < 0.01$ by one-way ANOVA with Tukey's post-hoc test.

However, while iron is abundant in hemoglobin, additional experiments are necessary to conclude that iron is the key nutrient rather than another divalent cation since our chelation protocol would have stripped all divalent cations from the media, not just iron. Therefore, we next wanted to determine if iron was the critical nutrient being acquired from hemoglobin.

To confirm that iron is the critical nutrient being supplied by Hb, we supplemented c-RPMI-LB with all the missing divalent cations ($ZnCl_2$, $CaCl_2$, $MgCl_2$, $MnCl_2$) except for iron creating the media c-RPMI-LB^{2+}. Like c-RPMI, this media should not allow bacterial growth due to the lack of iron. We also supplemented c-RPMI-LB^{2+} with hemoglobin (c-RPMI-LB^{2+}+ Hb), and lastly, as a positive control, we supplemented c-RPMI-LB^{2+} with ferric iron (c-RPMI-LB^{2+}+ $FeCl_3$) expecting full restorative growth for all three strains due to iron being present. To our surprise we saw growth of all three strains in c-RPMI-LB^{2+}, which lacked iron (figure 5). Additionally, we observed no significant increases in growth with iron supplementation (compare c-RPMI-LB^{2+} with c-RPMI-LB^{2+}+ $FeCl_3$), indicating that the c-RPMI-LB^{2+} media had iron present in the media prior to the addition of $FeCl_3$. We speculated that there may be trace amounts of iron in the $ZnCl_2$, $CaCl_2$, $MgCl_2$, or $MnCl_2$ supplements we used to restore the other divalent cations thus inadvertently contaminating our c-RPMI-LB^{2+} media with iron.

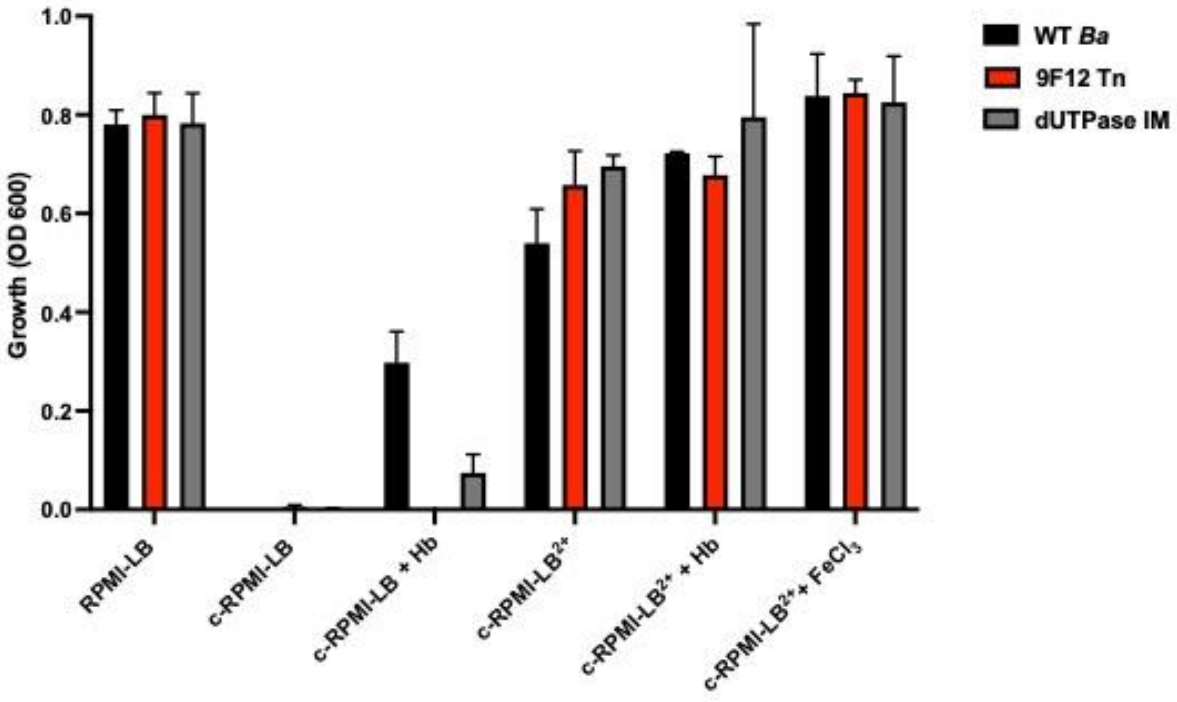

Figure 5: Supplementation with iron-free cations restores full growth. Wild-type *B. anthracis* (WT *Ba*), 9F12 Tn, and dUTPase IM were grown in RPMI-LB, chelated RPMI-LB (c-RPMI-LB), or c-RPMI-LB supplemented with iron-free divalent cations (c-RPMI-LB^{2+}). The addition of hemoglobin (+Hb) or 10µM iron (+$FeCl_3$) to the media is indicated. Data is presented as mean $^{+}$/- SEM from 3 independent experiments. Same letters indicate $p > 0.01$, different letters indicate $p < 0.01$ by one-way ANOVA followed by Tukey's post-hoc test.

After reviewing literature surrounding *B. anthracis* and iron-acquisition, we discovered the use of iron-chelator compound 2,2'-bipyridyl, which has been commonly used when studying iron acquisition and can chelate low levels of iron contamination (Maresso et al., 2006). To test the effectiveness of 2,2'-bipyridyl, we grew wild-type, 9F12 Tn, and dUTPase IM in c-RPMI-LB^{2+} and c-RPMI-LB^{2+} supplemented with 500 µM 2,2'-bipyridyl and measured growth after 48 hrs. We found no growth in c-$RPMI^{2+}$ with 2,2'-bipyridyl (figure 6). Therefore, the use of 2,2'-bipyridyl successfully chelated trace iron contamination.

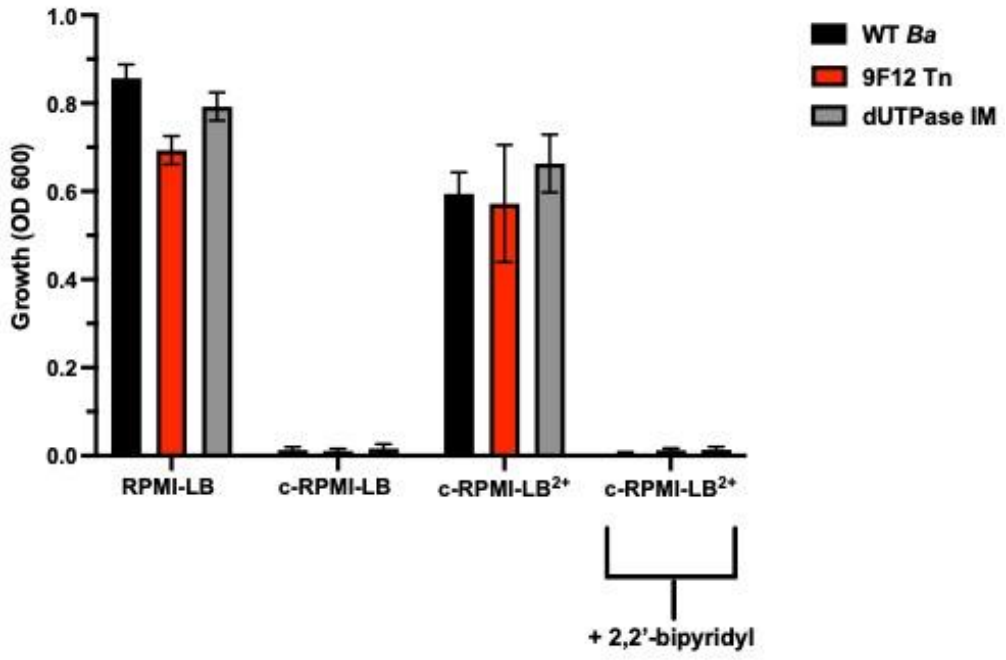

Figure 6: **2,2'-bipyridyl chelates trace iron contamination**
Wild-type *B. antrhacis* (WT *Ba*), 9F12 Tn, dUTPase IM were grown in RPMI-LB, c-RPMI-LB, c-RPMI-LB^{2+}, or c-RPMI-LB^{2+} with the addition if 2,2'-bipyridyl to the media indication c-RPMI-LB^{2+} c-RPMI-LB^{2+} with supplemented 2,2'-bipyridyl. Data is represented as mean $^{+}$/- SEM from 4 independent experiments.

To finalize that iron was being acquired from hemoglobin, we supplemented all medias with 2,2'-bipyridyl and grew wild-type, 9F12 Tn, and dUTPase IM in c-RPMI-LB^{2+} and c-RPMI-LB^{2+}+ Hb and measured growth after 48 hrs. We found that wild-type, 9F12 Tn, and dUTPase IM were able to grow in RPMI-LB reconfirming that our mutants are not different from wild-type in their ability to grow in normal conditions. We next found that all three strains were unable to grow in c-RPMI-LB^{2+}, which indicates that in the absence of iron, *B. anthracis* is unable to grow. Lastly, wild-type was the only one to grow in c-RPMI-LB^{2+}+ Hb, whereas 9F12 Tn and dUTPase IM did not (figure 7). This indicates that the loss of the *dUTPase* gene leads to the inability to acquire iron from hemoglobin. We also grew wild-type, 9F12 Tn, and dUTPase IM in c-RPMI-LB^{2+}supplemented with a high

concentration of iron (c-RPMI-LB^{2+} + $FeCl_3$) and measured growth after 48 hrs. As expected, we found that wild-type, 9F12 Tn, and dUTPase IM in c-RPMI-LB^{2+} + $FeCl_3$ were able to grow when iron was present indicating that the inability to grow was not due to a defect in acquiring iron from the environment but rather acquiring iron from hemoglobin (figure 7). Therefore, we conclude that the *dUTPase* gene is required for iron acquisition from hemoglobin in *B. anthracis*.

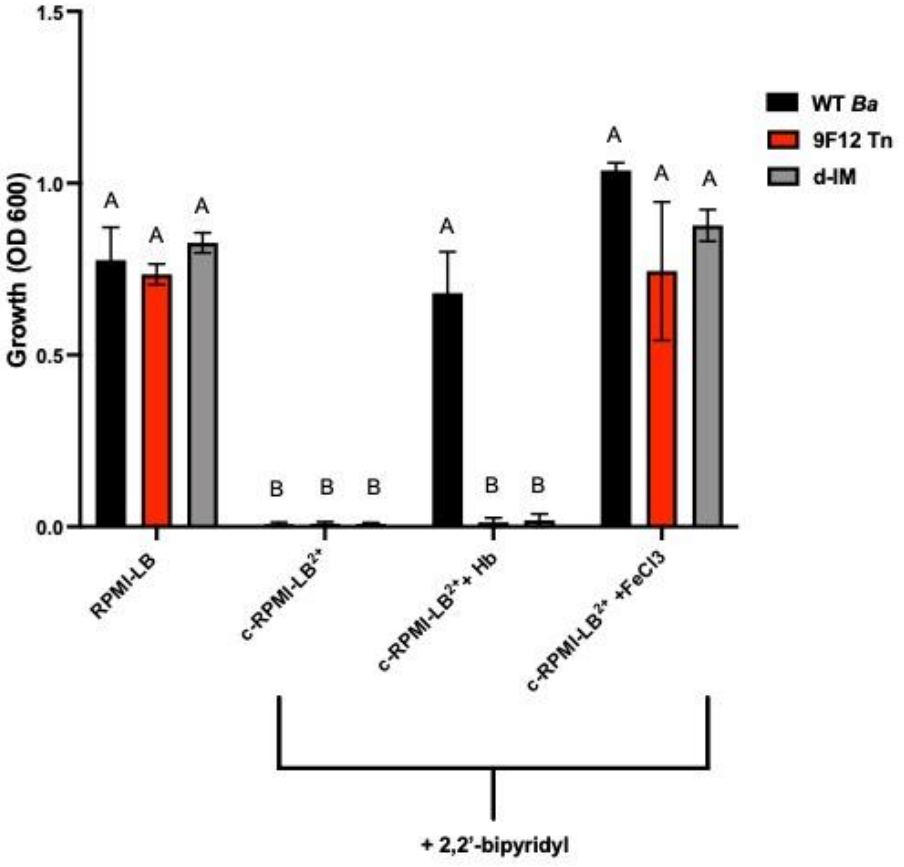

Figure 7: **The *dUTPase* gene is necessary for growth with hemoglobin as an iron source.**
Wild-type *B. anthracis* (WT *Ba*), 9F12 Tn, and dUTPase IM were grown in RPMI-LB, or c-RPMI-LB supplemented with iron-free divalent cations (c-RPMI-LB^{2+}). The addition of 2,2'-bipyridyl, hemoglobin (+Hb) or 100 µM iron (+$FeCl_3$) to the media is indicated. Data is presented as mean $^{+}$/- SEM from 4 independent experiments. Same letters indicate $p > 0.01$, different letters indicate $p < 0.01$ by one-way ANOVA followed by Tukey's post-hoc test.

The *dUTPase* gene is required for virulence of *B. anthracis* in *G. mellonella*

To determine whether dUTPase IM had attenuated virulence *in vivo* we selected *G. mellonella* as our live infection model due to their ability to be incubated at 37°C, which is the optimal temperature for bacterial pathogens, and their parallels with the human innate immune system (Tsai et al., 2016). Previous studies have also validated that *G. mellonella* as an ideal infection model for *B. anthracis* (Malmquist et al., 2018). Larvae were injected with a non-lethal saline buffer solution (PBS), to ensure trauma from injection was not the causative agent of larval death, wild-type, 9F12 Tn, and dUTPase IM. Larval death was recorded every 24 hrs for a total of 72 hrs. As predicted larvae injected with PBS exhibited an average survival rate of 80% whereas the larvae injected with wild-type exhibited survival rates less than 20%. The larvae injected with 9F12 Tn and dUTPase IM had survival rates that resembled PBS and were significantly different than wild-type in their ability to kill the infected host (figure 8). Therefore, we conclude that the *dUTPase* gene is necessary for virulence of *B. anthracis.*

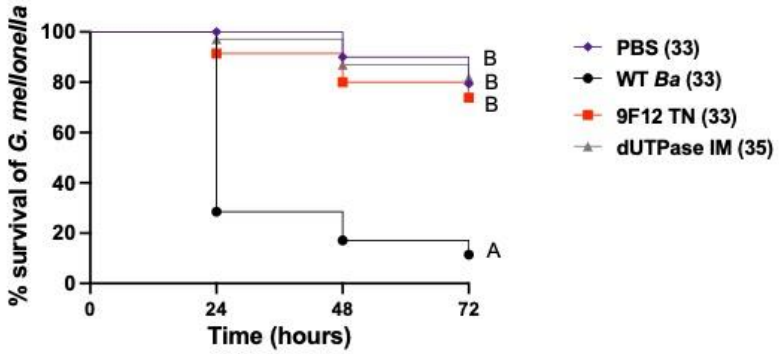

Figure 8: The *dUTPase* gene is necessary for *B. anthracis virulence.*
Percent survival of *G. mellonella* injected with PBS, wild-type *B. anthracis* (WT *Ba*), 9F12 Tn, dUTPase IM at 24, 48, and 72 hours. Each infection was repeated 3 independent experiments with the total number of worms for each condition in parentheses. Same letters indicate $p > 0.01$, different letters indicate $p < 0.01$ using the log-rank test.

Discussion

This study has demonstrated that the *dUTPase* gene *in B. anthracis* is necessary for acquisition of iron from hemoglobin. To our knowledge dUTPase does not have any direct correlation with iron acquisition from hemoglobin with the majority of literature stating that it is a pronounced regulator of uracil in DNA (Kerepesi et al., 2016). However, recent studies have demonstrated that dUTPases may act as previously unrecognized signaling molecules in a variety of cells from prokaryotic to eukaryotic (Penadés et al., 2013). Therefore, it is possible that loss of *dUTPase* is causing misexpression of other genes which may be associated with iron acquisition from hemoglobin. Future studies would be needed to determine whether loss of dUTPase results in a dysregulation of multiple genes in *B. anthracis*. There is also the possibility that the gene disrupted in 9F12 Tn is not a *dUTPase*. This gene is annotated as a dUTPase based on homology to known dUTPases, however, no functional studies have been carried out confirming this role. Interestingly, studies have shown that a decrease in or lack of dUTPase activity results in chromosome fragmentation and cell death of gram-positive bacteria (Pecsi et al., 2012). However, we did not see a negative impact on our 9F12 Tn and dUTPase IM mutants in normal growing conditions (figure 2). Addtionally, dUTPases are not found in the closely related species *B. cereus* (Moroz et al., 2004). Therefore, further analysis of *dUTPase* gene in 9F12 Tn would be needed to confirm that it actually functions as a dUTPase.

The *dUTPase* and *ysdC* genes are located next to each other in the genome and are likely regulated as an operon. Therefore, a large disruption, such as the transposon, in the *dUTPase* gene, may have also disrupted the *ysdC* gene, which resulted in the phenotypes observed. Conversely, the plasmid used to disrupt the d*UTPase* gene in our dUTPase IM has an internal

promoter (figure 2), which should allow transcription of the *ysdC* gene in our mutant but not at native levels of control. Given that both mutants have a similar phenotype and the uncertainty about the function of *dUTPase* in iron acquisition, it is important to determine the role of *ysdC* in this system. We will perform RT PCR to determine if *ysdC* is being transcribed in our mutants. If we see do not see amplification or faint amplification of the *ysdC* gene in our mutants, this would indicate that *ysdC* is also disrupted and could also be playing a role in the acquisition of iron from hemoglobin. One way to determine whether *dUTPase,* the entire operon (*dUTPase/ysdC*), or just *ysdC* is responsible for this phenotype is through complementation, which is the reintroduction of the gene on a plasmid in order to restore a phenotype. If we saw a greater restorative phenotype with *dUTPase* and not *dUTPase/ysdC* or *ysdC,* this would indicate that the *dUTPase* is necessary for the acquisition of iron from hemoglobin. If we saw the greatest phenotype with *dUTPase/ysdC*, this would indicate the whole operon is responsible for the acquisition of iron from hemoglobin. In contrary, if we saw the greatest restorative phenotype with just *ysdC,* this would indicate only *ysdC* is responsible for iron-acquisition from hemoglobin, and further analysis of the relationship between aminopeptidases and iron acquisition from hemoglobin would need to be completed.

We also observed that the loss of the *dUTPase* gene resulted in attenuated virulence in our live-infection model *G. mellonella* (figure 8). There is no evidence in the literature that *G. mellonella* contains iron-containing molecules with homology to hemoglobin. Instead, the larvae rely on the iron-binding proteins transferrin and apoferritin found in hemolymph for homeostasis and antimicrobial resistance (Dunphy et al., 2002). Therefore, there is no clear relationship between *B. anthracis'* ability to acquire iron from hemoglobin and survival in

the larvae. Nonetheless, there is also the possibility that there is a hemoglobin-like structure in *G. mellonella* that has yet to be discovered that could account for loss of virulence in the waxworm. As mentioned earlier, dUTPases may act as a signaling molecule that could regulate a variety of cellular processes (Penadés et al., 2013). It is possible that the inability to acquire iron from hemolobin is only one of several possible virulence-associated phenotypes that are as yet undiscovered. We attempted to test the susceptibility to hydrogen peroxide (H_2O_2), a common reactive oxygen species (ROS) found in our innate immune system as well as *G. mellonella*; however, due to large variance in our results prevented us from reaching a conclusion either way. We will continue to look at other virulence-associated phenotypes that may be associated with loss of the dUTPase and/or aminopeptidase genes from *B. anthracis*. Interestingly, there are studies that show that aminopeptidases are tied to *B. anthracis'* lethal toxins (Wickliffe et al., 2008), with one study finding that the use of the aminopeptidase inhibitor bestatin protected against lethal toxin (Klimpel et al., 1994). While these studies focused primarily on eukaryotic aminopeptidases, there is the possibility that the aminopeptidase gene found in the same putative operon as dUTPase could play a role.

The knowledge learned from studying novel factors, such as *dUTPase*, has provided for a greater understanding of *B. anthracis* virulence. To our knowledge, this is the first study that linked a dUTPase to nutrient acquisition from the host. Future studies will help confirm the relative role dUTPase/aminopeptidase and more specifically focus on their mechanisms of action. This has the potential to open up new understanding of virulence mechanisms that could apply to bacterial pathogens beyond just *B. anthracis*. This in turn may open up

additional drug targets. This is crucial due to the decrease in antibiotic development and the increase in antibiotic resistant bacteria.

References

1. Balderas, M. A., Nobles, C. L., Honsa, E. S., Alicki, E. R., & Maresso, A. W. (2012). Hal is a bacillus anthracis heme acquisition protein. *Journal of Bacteriology*, *194*(20), 5513–5521. https://doi.org/10.1128/jb.00685-12
2. Bower, W. A., Schiffer, J., Atmar, R. L., Keitel, W. A., Friedlander, A. M., Liu, L., Yu, Y., Stephens, D. S., Quinn, C. P., & Hendricks, K. (2019). Use of anthrax vaccine in the United States: Recommendations of the Advisory Committee on Immunization Practices, 2019. *MMWR. Recommendations and Reports*, *68*(4), 1–14. https://doi.org/10.15585/mmwr.rr6804a1
3. Carlson, C.J., Kracalik, I.T., Ross, N. *et al.* The global distribution of *Bacillus anthracis* and associated anthrax risk to humans, livestock and wildlife. *Nat Microbiol* **4**, 1337–1343 (2019). https://doi.org/10.1038/s41564-019-0435-4
4. Callegan, M. C., Jett, B. D., Hancock, L. E., & Gilmore, M. S. (1999). Role of hemolysin BL in the pathogenesis of extraintestinal *bacillus cereus* infection assessed in an endophthalmitis model. *Infection and Immunity*, *67*(7), 3357–3366. https://doi.org/10.1128/iai.67.7.3357-3366.1999
5. Cheng, C., Wang, X., Dong, Z., Shao, C., Yang, Y., Fang, W., Fang, C., Wang, H., Yang, M., Jiang, L., Zhou, X., & Song, H. (2015). Aminopeptidase T of M29 family acts as a novel intracellular virulence factor for listeria monocytogenes infection. *Scientific Reports*, *5*(1). https://doi.org/10.1038/srep17370
6. Chun, J.-H., Hong, K.-J., Cha, S. H., Cho, M.-H., Lee, K. J., Jeong, D. H., Yoo, C.-K., & Rhie, G.-eun. (2012). Complete genome sequence of bacillus anthracis H9401,

an isolate from a Korean patient with anthrax. *Journal of Bacteriology*, *194*(15), 4116–4117. https://doi.org/10.1128/jb.00159-12

7. Clark, J., Terwilliger, A., Nguyen, C., Green, S., Nobles, C., & Maresso, A. (2019). Heme catabolism in the causative agent of anthrax. *Molecular Microbiology*, *112*(2), 515-531. https://doi.org/10.1111/mmi.14270
8. Driks, A. (2009). The bacillus anthracis spore. *Molecular Aspects of Medicine, 30*(6), 368–373. https://doi.org/10.1016/j.mam.2009.08.001
9. Dunphy, G. B., Niven, D. F., & Chadwick, J. S. (2002). Iron contributes to the antibacterial functions of the haemolymph of Galleria Mellonella. *Journal of Insect Physiology*, *48*(9), 903–914. https://doi.org/10.1016/s0022-1910(02)00159-2
10. Franks, S. E., Ebrahimi, C., Hollands, A., Okumura, C. Y., Aroian, R. V., Nizet, V., & McGillivray, S. M. (2013). Novel role for the ycegh tellurite resistance genes in the pathogenesis of bacillus anthracis. *Infection and Immunity, 82*(3), 1132-1140. https://doi.org/10.11.28/iai.01614-13
11. Friebe, S., van der Goot, F., & Burgi, J. (2016). The Ins and Outs of Anthrax Toxin. *Toxins*, 8(3), 69. Hhttps://doi.org/10.3390/toxins8030069
12. Goel, A. K. (2015). Anthrax: A disease of biowarfare and public health importance. *World Journal of Clinical Cases*, *3*(1), 20. https://doi.org/10.12998/wjcc.v3.i1.20
13. Ellis-Guardiola, K., Mahoney, B. J., & Clubb, R. T. (2021). Near transporter (NEAT) domains: Unique surface displayed heme chaperones that enable gram-positive bacteria to capture heme-iron from hemoglobin. *Frontiers in Microbiology*, *11*. https://doi.org/10.3389/fmicb.2020.607679

14. Hizi, A., & Herzig, E. (2015). DUTPase: The frequently overlooked enzyme encoded by many retroviruses. *Retrovirology*, *12*(1). https://doi.org/10.1186/s12977-015-0198-9

15. Honsa, E. S., & Maresso, A. W. (2011). Mechanisms of iron import in anthrax. *BioMetals*, *24*(3), 533–545. https://doi.org/10.1007/s10534-011-9413-x

16. Hotta, K., Kim, C.-Y., Fox, D. T., & Koppisch, A. T. (2010). Siderophore-mediated iron acquisition in bacillus anthracis and related strains. *Microbiology*, *156*(7), 1918–1925. https://doi.org/10.1099/mic.0.039404-0

17. Jernigan, D. B., Raghunathan, P. L., Bell, B. P., Brechner, R., Bresnitz, E. A., Butler, J. C., Cetron, M., Cohen, M., Doyle, T., Fischer, M., Greene, C., Griffith, K. S., Guarner, J., Hadler, J. L., Hayslett, J. A., Meyer, R., Petersen, L. R., Phillips, M., Pinner, R., … Gerberding, J. L. (2002). Investigation of bioterrorism-related anthrax, United States, 2001: Epidemiologic findings. *Emerging Infectious Diseases*, *8*(10), 1019–1028. https://doi.org/10.3201/eid0810.020353

18. Kerepesi, C., Szabó, J. E., Papp-Kádár, V., Dobay, O., Szabó, D., Grolmusz, V., & Vértessy, B. G. (2016). Life without dutpase. *Frontiers in Microbiology*, *7*. https://doi.org/10.3389/fmicb.2016.01768

19. Klimpel, K. R., Arora, N., & Leppla, S. H. (1994). Anthrax toxin lethal factor contains a zinc metalloprotease consensus sequence which is required for lethal toxin activity. *Molecular microbiology*, *13*(6), 1093–1100. https://doi.org/10.1111/j.1365-2958.1994.tb00500.x

20. Ladner R. D. (2001). The role of dUTPase and uracil-DNA repair in cancer chemotherapy. *Current protein & peptide science*, *2*(4), 361–370. https://doi.org/10.2174/1389203013380991
21. Lee, J. Y., Passalacqua, K. D., Hanna, P. C., & Sherman, D. H. (2011). Regulation of petrobactin and bacillibactin biosynthesis in Bacillus anthracis under iron and oxygen variation. *PloS one*, *6*(6), e20777. https://doi.org/10.1371/journal.pone.0020777
22. Malmquist, J. (2018) Development and use of A.G. Mellonella Infection Model to discover novel virulence mutants in *B. anthracis.*
23. Maresso, A. W., Chapa, T. J., & Schneewind, O. (2006). Surface protein ISDC and sortase B are required for heme-iron scavenging of *bacillus anthracis*. *Journal of Bacteriology*, *188*(23), 8145–8152. https://doi.org/10.1128/jb.01011-06
24. Maresso, A. W., Garufi, G., & Schneewind, O. (2008). Bacillus anthracis secretes proteins that mediate heme acquisition from hemoglobin. *PLoS pathogens*, *4*(8), e1000132. https://doi.org/10.1371/journal.ppat.1000132
25. Mcgillivray, S. M., Ebrahimi, C. M., Fisher, N., Sabet, M., Zhang, D. X., Chen, Y., Haste, N. M., Aroian, R. V., Gallo, R. I., Guiney, D. G., Friedlander, A. M., Koehler, T. M., & Nizet, V. (2009). CLPX contributes to innate defense peptide resistance and virulence phenotypes of *bacillus anthracis*. *Journal of Innate Immunity*, *1*(5), 494-506. https://doi.org/10.1159/000225955
26. Moayeri, M., Leppla, S. H., Vrentas, C., Pomerantsev, A. P., & Liu, S. (2015). Anthrax pathogenesis. *Annual Review of Microbiology*, *69*(1), 185-208. https://doi.org/10.1146/annurev-micro-091014-104523

27. Mogridge, J. (2004). Anthrax and bioterrorism: Are we prepared? *The Lancet*, *364*(9432), 393-395. https://doi.org/10.1016/s0140-6736(04)16779-2

28. Moroz, O. V., Harkiolaki, M., Galperin, M. Y., Vagin, A. A., González-Pacanowska, D., & Wilson, K. S. (2004). The crystal structure of a complex of campylobacter jejuni dUTPase with substrate analogue sheds light on the mechanism and suggests the "basic module" for dimeric D(C/u)tpases. *Journal of Molecular Biology*, *342*(5), 1583–1597. https://doi.org/10.1016/j.jmb.2004.07.050

29. Mustafi, D., Bekesi, A., Vertessy, B. G., & Makinen, M. W. (2003). Catalytic and structural role of the metal ion in dutp pyrophosphatase. *Proceedings of the National Academy of Sciences*, *100*(10), 5670–5675. https://doi.org/10.1073/pnas.1031504100

30. Nusca, T. D., Kim, Y., Maltseva, N., Lee, J. Y., Eschenfeldt, W., Stols, L., Schofield, M. M., Scaglione, J. B., Dixon, S. D., Oves-Costales, D., Challis, G. L., Hanna, P. C., Pfleger, B. F., Joachimiak, A., & Sherman, D. H. (2012). Functional and structural analysis of the siderophore synthetase asbb through reconstitution of the petrobactin biosynthetic pathway from bacillus anthracis. *Journal of Biological Chemistry*, *287*(19), 16058–16072. https://doi.org/10.1074/jbc.m112.359349

31. Pecsi, I., Hirmondo, R., Brown, A. C., Lopata, A., Parish, T., Vertessy, B. G., & Tóth, J. (2012). The dUTPase enzyme is essential in mycobacterium smegmatis. *PLoS ONE*, *7*(5). https://doi.org/10.1371/journal.pone.0037461

32. Penadés, J. R., Donderis, J., García-Caballer, M., Tormo-Más, M. Á., & Marina, A. (2013). dUTPases, the unexplored family of signalling molecules. *Current opinion in microbiology*, *16*(2), 163–170. https://doi.org/10.1016/j.mib.2013.02.005

33. Perry, M. R., Ionin, B., Barnewall, R. E., Vassar, M. L., Reece, J. J., Park, S., Lemiale, L., Skiadopoulos, M. H., Shearer, J. D., & Savransky, V. (2020). Development of a guinea pig inhalational anthrax model for evaluation of post-exposure prophylaxis efficacy of anthrax vaccines. *Vaccine*, *38*(10), 2307–2314. https://doi.org/10.1016/j.vaccine.2020.01.068

34. Pishchany, G., & Skaar, E. P. (2012). Taste for blood: hemoglobin as a nutrient source for pathogens. *PLoS pathogens*, *8*(3), e1002535. https://doi.org/10.1371/journal.ppat.1002535

35. Read, T. D., Peterson, S. N., Tourasse, N., Baillie, L. W., Paulsen, I. T., Nelson, K. E., Tettelin, H., Fouts, D. E., Eisen, J. A., Gill, S. R., Holtzapple, E. K., Økstad, O. A., Helgason, E., Rilstone, J., Wu, M., Kolonay, J. F., Beanan, M. J., Dodson, R. J., Brinkac, L. M., … Fraser, C. M. (2003). The genome sequence of Bacillus Anthracis Ames and comparison to closely related bacteria. *Nature*, *423*(6935), 81–86. https://doi.org/10.1038/nature01586

36. Romero-Alvarez, D., Peterson, A. T., Salzer, J. S., Pittiglio, C., Shadomy, S., Traxler, R., Vieira, A. R., Bower, W. A., Walke, H., & Campbell, L. P. (2020). Potential distributions of bacillus anthracis and bacillus cereus biovar anthracis causing anthrax in Africa. *PLOS Neglected Tropical Diseases*, *14*(3). https://doi.org/10.1371/journal.pntd.0008131

37. Sachdeva, S., Palur, R. V., Sudhakar, K. U., & Rathinavelan, T. (2017). E. Coli Group 1 capsular polysaccharide exportation nanomachinary as a plausible antivirulence target in the perspective of emerging antimicrobial resistance. *Frontiers in Microbiology*, *8*. https://doi.org/10.3389/fmicb.2017.00070

38. Shafazand, S., Doyle, R., Ruoss, S., Weinacker, A., & Raffin, T. A. (1999). Inhalational anthrax. *Chest*, *116*(5), 1369–1376. https://doi.org/10.1378/chest.116.5.1369

39. Shapiro-Ilan, D. I., Fuxa, J. R., Lacey, L. A., Onstad, D. W., & Kaya, H. K. (2005). Definitions of pathogenicity and virulence in invertebrate pathology. *Journal of Invertebrate Pathology*, *88*(1), 1–7. https://doi.org/10.1016/j.jip.2004.10.003

40. Tsai, C. J., Loh, J. M., & Proft, T. (2016). Galleria mellonella infection models for the study of bacterial diseases and for antimicrobial drug testing. *Virulence*, *7*(3), 214–229. https://doi.org/10.1080/21505594.2015.1135289

41. van Opijnen, T., & Camilli, A. (2013). Transposon insertion sequencing: A new tool for systems-level analysis of microorganisms. *Nature Reviews Microbiology*, *11*(7), 435–442. https://doi.org/10.1038/nrmicro3033

42. Vértessy, B. G., & Tóth, J. (2008). Keeping uracil out of DNA: Physiological role, structure and catalytic mechanism of dutpases. *Accounts of Chemical Research*, *42*(1), 97–106. https://doi.org/10.1021/ar800114w

43. Wickliffe, K. E., Leppla, S. H., & Moayeri, M. (2008). Killing of macrophages by anthrax lethal toxin: Involvement of the N-end rule pathway. *Cellular Microbiology*, *10*(6), 1352–1362. https://doi.org/10.1111/j.1462-5822.2008.01131.x

44. Wooldridge, K. G., & Williams, P. H. (1993). Iron uptake mechanisms of pathogenic bacteria. *FEMS Microbiology Reviews*, *12*(4), 325–348. https://doi.org/10.1111/j.1574-6976.1993.tb00026.x

45. Zawadzka, A. M., Abergel, R. J., Nichiporuk, R., Andersen, U. N., & Raymond, K. N. (2009). Siderophore-mediated iron acquisition systems in bacillus cereus:

Identification of receptors for anthrax virulence-associated petrobactin. *Biochemistry*, *48*(16), 3645–3657. https://doi.org/10.1021/bi8018674

46. Zou, L., Evans, C. R., Do, V. D., Losefsky, Q. P., Ngo, D. Q., & McGillivray, S. M. (2021). Loss of the ClpXP protease leads to decreased resistance to cell-envelope targeting antimicrobials in bacillus anthracis Sterne. *Frontiers in Microbiology*, *12*. https://doi.org/10.3389/fmicb.2021.719548
47. Center for Biological Evaluation and Research. (2018, February 2) *Anthrax*. U.S. Food and Drug Administration. Retrieved November 1, 2021 from https://www.fda.gov/vaccines-blood-biologics/vacines/anthrax
48. *The Threat of an Anthrax Attack*| *CDC* (n.d.). Centers for Disease Control and Prevention. Retrieved November 1, 2021. From https://www.cdc.gov/anthrax/bioterrorism/threat.html

Vita

Kyle Ross Gallegos was born June 15th, 1998 in Grapevine, Texas. He is the son of Ian Gallegos and Lett Gallegos. A graduate of Liberty Christian High School in Argyle, Texas. He received a Bachelor of Arts degree with a major in Biology and minor in Chemistry from The University of North Texas, Denton, in December 2019.

In August of 2021, he continued his education by enrolling in graduate study at Texas Christian University, Fort Worth, where he received his Master of Science in 2023. While earning his Master in Biology, he held a Teaching Assistantship and was a member of the American Society of Microbiologist. After earning his master, Kyle will be continuing his teaching at Texas Christian University through an adjunct position while looking into furthering his graduate education.

Abstract

The Characterization of the Potential Iron-Acquisition Gene dUTPase in *Bacillus anthracis*

By Kyle R. Gallegos, M.S., 2023
Department of Biology
Texas Christian University

Thesis Advisor: Shauna McGillivray Ph.D., Professor of Biology & Associate Department Chair

Bacillus anthracis is the causative agent of the fatal disease anthrax, and its virulence is of great interest due to its potential as a biological weapon. *B. anthracis* causes disease by both escaping immune defenses and acquiring nutrients. A necessary nutrient that pathogens must acquire from its host is iron. To discover novel genes essential for iron acquisition, we screened transposon mutants in iron-deficient media with hemoglobin as the sole source of iron. We further prioritized the mutants discovered in our *in vitro* screen by assessing for attenuated virulence using our *in vivo G. mellonella* infection model. We found one mutant that has a disruption in the first gene of a two-gene operon containing putative *dUTPase* and *aminopeptidase* genes known as 9F12 Tn. Neither of these genes have been previously linked to iron acquisition. To confirm the role of the *dUTPase* gene in the observed 9F12 Tn phenotype, we created an independent insertional mutant in the *dUTPase* gene (dUTPase IM). We found that both of our mutants, 9F12 Tn and dUTPase IM, could not use hemoglobin as a source of iron. We also found that *G. mellonella* injected with 9F12 Tn and dUTPase IM had higher survival rates than those injected with the parent strain. Our results indicate that the *dUTPase* gene is necessary for iron-acquisition and virulence in *B. anthracis*. This study furthers our understanding of iron acquisition in a bacterial pathogen and increases our knowledge of how *B. anthracis* causes disease.