Characterization of Deoxycholate-Responsive Genes Utilized by *Brucella abortus* 2308 During Oral Infection

Christian Lehman

Thesis submitted to the faculty of the Virginia Polytechnic Institute and State University in partial fulfillment of the requirements for the degree of

Master of Science

In

Biomedical and Veterinary Sciences

Clayton C. Caswell, Chair

Kevin Lahmers

Thomas Inzana

Stephen B. Melville

May 8th, 2017

Blacksburg, VA

Keywords: *Brucella abortus*, deoxycholate, oral infection

Copyright 2017, Christian Lehman

Characterization of Deoxycholate-Responsive Genes Utilized by *Brucella abortus* 2308 During Oral Infection

Christian Lehman

Academic Abstract

Brucellosis is a chronic, recurring disease caused by the bacterium *Brucella abortus*, along with other species of the genus *Brucella*, and is one of the most common bacterial zoonosis worldwide. The bacteria preferentially infect and reside within host macrophages, causing an undulant fever, joint pain, and other flu-like symptoms, in addition to more severe problems like hepatosplenomegaly and endocarditis. *Brucella* infection is most often acquired via inhalation through the respiratory route, or via consumption of unpasteurized dairy products. Although ingestion is a major route of infection, the transcriptional response of *B. abortus* during oral infection remains poorly characterized. In this project, RNA sequencing was used to discover genes with the greatest transcriptional changes in *B. abortus* subjected to deoxycholate, a host bile acid encountered by bacteria during oral infection. Gene deletion strains of *B. abortus* were then created and tested for susceptibility to pH and bile acid stress, along with their ability to invade and replicate within macrophages. If the genes of interest are important for the oral infection process, *B. abortus* strains lacking these genes will likely be more susceptible to pH and deoxycholate stress and may exhibit attenuation in the macrophage infection model. Determination of genes important for the oral infection process would further elucidate the molecular mechanisms by which *B. abortus* invades the host, and could help lead to future treatments and novel therapeutics.

General Audience Abstract

Brucellosis, caused by the bacterium *Brucella abortus*, is a zoonotic disease, meaning that humans can acquire the illness from animals. Once infected, sufferers of brucellosis experience a chronic, recurring fever that repeatedly rises and falls. Additionally, the disease can cause enlargement of the spleen and liver, and can sometimes cause inflammation of the valves within the heart. Although *B. abortus* can infect a host through many routes of entry (inhalation, accidental injection, etc), patients are often infected through the consumption of contaminated, unpasteurized dairy products. The genes utilized by *B. abortus* during oral infection have not been well characterized, so it is not well known what mechanisms *B. abortus* uses to survive the pH and bile acid stresses it faces in the host stomach and intestines. This research examines which genes are increasingly or decreasingly utilized by *B. abortus* when it is subjected to deoxycholate—a bile acid stress used to simulate the host small intestine. Genes that exhibited the largest change in expression upon deoxycholate exposure were then chosen for further study: new strains of *B. abortus* lacking these genes of interest were created to determine if the gene deletion decreased the bacteria's ability to survive acid and deoxycholate stress, along with its ability to infect host macrophages, a type of white blood cell. If deletion of these genes weakens the ability of *B. abortus* to survive and infect, then these genes likely have a role during the oral infection process. By further elucidating which genes are used by *B. abortus* to survive host defenses and infect via the oral route, one could then create new medicines that are more effective at inhibiting the mechanisms needed by *B. abortus* for successful infection and persistence within the host.

Acknowledgments

I would like to thank my advisor Dr. Clay Caswell for all of his guidance, patience, and unwavering support that made this project possible.

I would like to thank my committee, Dr. Tom Inzana, Dr. Kevin Lahmers, and Dr. Stephen Melville for their insight and their direction.

I would like to thank Becky Jones and Susan Rosebrough for all of their help and guidance in the program.

Finally, I would like to thank my wonderful lab mates, Lauren Sheehan and James Budnick, for all of their support and guidance, for challenging me, for working alongside me, and for helping me with my countless questions—I truly could not have done this without them.

Dedications

I would like to dedicate this Master's Thesis to:

To my parents for their love and unwavering support throughout this project.

To my brother, Michael, for inspiring me with our countless discussions of the limitless possibilities of science, whether they be outlandish and silly, or beneficial and practical.

To my Biology and Chemistry professors at Hampden-Sydney College, and particularly my undergraduate advisor, Dr. Mike Wolyniak, for introducing me to the world of scientific research.

To my friends and family who have continuously supported me.

And to my wonderful girlfriend, Molly, who has ceaselessly encouraged and reassured me throughout this project; I could not have done this without your support.

Table of Contents

List of Figures

List of Tables

Chapter 1: Literature Review

Overview of *Brucella abortus*

The brucellae are Gram-negative facultative intracellular pathogens; they are very small coccobacilli—typically 0.6-1.5 µm long and 0.5-0.8 µm wide, and they are facultative anaerobes. *Brucella* spp. belong to the class Alphaproteobacteria, and are closely related to *Sinorhizobium meliloti* and *Agrobacterium tumefaciens*—plant symbionts and plant pathogens, respectively, and they are also closely related genus *Bartonella*, another intracellular parasite (Guerra 2007). There are numerous nomenspecies within the genus *Brucella*, and they typically derive their specific names from their preferred hosts, such as *B. suis* and *B. canis* which preferentially infect swine and dogs. The brucellae are capable of infecting a diverse array of mammalian species, from *B. ovis* which infects rams, to *B. pinnipedialis* and *B. ceti*, which can infect seals and sealions, and whales and porpoises, respectively. The brucellae were briefly classified to be monophyletic, meaning that all of the species are actually different biovars of the same species—*Brucella melitensis* (Gándara 2001). However, it was later agreed upon that, because the biovars preferentially infected different species, the species designations were descriptive enough that they were reinstated (Osterman 2003). Together, the brucellae are responsible for the disease brucellosis, also called Malta fever or Bang's disease in humans, which is one of the most common bacterial zoonoses, and infects an estimated 500,000 humans annually (Alturi 2011).

Prevalence of Brucellosis

Although there are an estimated 500,000 human cases of brucellosis reported annually worldwide, the majority of these cases are reported from regions where *Brucella* species are considered to be endemic pathogens. Many developed countries in the West have had success in eliminating brucellosis due to an increase in vaccination of livestock and increased regulations about the pasteurization of milk and dairy products. In addition, improvements in diagnostic techniques and national incidence monitoring have essentially eliminated brucellosis in the United States, Canada, and western Europe. In fact, Switzerland, Sweden, Norway, Finland, Denmark, Germany, and many countries in central Europe are considered to be brucellosis-free, with fewer than two cases per 1,000,000 population annually.

In contrast, the regions with the highest incidence of brucellosis are the Mediterranean Basin, the Middle East, and central Asia. In the Mediterranean basin, Tunisia, Algeria, Turkey, and countries of the Balkans (particularly Albania and Macedonia) have the highest occurrence of brucellosis, with an average of between 21 and 262 human cases per million annually. Even Mediterranean countries less affected, such as Spain, France, Italy, and Portugal still have an increased occurrence compared with more northern regions of Europe, and these affected regions are almost all areas that are proximal to the Mediterranean (Pappas 2006). The Middle East is also heavily affected with brucellosis, especially Saudi Arabia, Iraq, and Iran, which have occurrences between 214 and 248 cases per million annually, and Syria, which has had an explosive outbreak, with a staggering 1603 cases per million annually. The high prevalence of brucellosis extends through central Asia, heavily affecting Mongolia

and many countries of the former Soviet Union, including Kazakhstan, Tajikistan, Kyrgyzstan, and Turkmenistan. The incidence of brucellosis ranges between 52 and 212 annual cases per million in this region, with the exception of Kyrgyzstan and Mongolia, which have an annual average of 362 and 606 cases per million, respectively (Pappas 2006).

Brucellosis is still endemic in Mexico and Central and South America, but the number of cases have been declining, with incidences ranging between 0.6 and 16 cases per million annually. Peru and Mexico remain the countries in the region with the highest rates of brucellosis, with an annual 35 and 29 cases per million, respectively. While the number of cases is declining in these regions, there are large areas of the world where *Brucella* species are endemic, but lack effective eradication programs, or there is not national monitoring of the disease, so there is little accurate data available. *Brucella* is considered to be endemic to India and Pakistan; screening in India found that between 2-6% of the general population tested seropositive for brucellosis, with about 6% of subjects who had frequent animal contact testing positive. The prevalence in field veterinarians is about 17% being seropositive, strongly suggesting that *Brucella* was endemic to the country, and that many cases go unreported (Thakur 2002). The elevated rate of seropositive individuals likely results from their exposure to domesticated animals, which also exhibit a high rate of brucellosis: screening of domesticated animals found that cattle, sheep, and goats were 5%, 8% and 2% seropositive for brucellosis (Renukaradhya 2002), respectively. These studies indicate that brucellosis likely affects a vast number of people in the country, but there is no national tracking of cases to give an official number of cases annually. Similarly, there

are not national data available for many countries in Africa, but studies have shown that *Brucella* is endemic to many regions in sub-Saharan Africa. A study examining cattle in Ethiopia found that the number of *Brucella* seropositive cattle was low (2.4%), but that roughly 46% of herds contained one or more animals that tested positive. These data suggest that brucellosis is widely prevalent at a baseline level in Ethiopia and many sub-Saharan countries (McDermott 2002).

While some regions of the world are more disposed to having an endemic prevalence of brucellosis, the combination of government regulations concerning the pasteurization of dairy products, along with an eradication program that aims to monitor the occurrence of brucellosis and reduce the susceptible cattle populations through strategic vaccination can help to reduce or eliminate the pervasiveness of *Brucella* in a region. The effectiveness of an eradication program is evident from the comparison of geographically neighboring countries that have dramatically different occurrences of the disease. A prime example is the declining frequency of brucellosis in Israel, despite being surrounded by countries with significantly higher prevalence, such as Jordan, Lebanon, and particularly, Saudi Arabia and Syria. Similarly, France and the United States have effectively eradicated the disease, while their neighbors, Spain and Mexico, respectively, have comparatively high incidence rates (Pappas 2006). Despite the eradication of endemic *Brucella* in these countries, the disease can be reintroduced to an area if one or more of the eradication strategies are not adhered to. The large majority of US brucellosis cases occur in the southern regions of Texas and California (Doyle 2000, Fosgate 2002), where it is easier for consumers to acquire and ingest

unpasteurized—and contaminated—soft cheeses from across the border (Young 1975, Thapar 1986, Taylor 1989).

Such noncompliance to food safety laws can predispose populations to a resurgence of brucellosis, but many countries experience high prevalence due to lack of public will to make eradication a priority, or lack of infrastructure, networking, or funds to do so. As a result, brucellosis is a disease in which its prevalence correlates strongly with the socioeconomic status of the countries in question. Within the European Union (EU), countries with GDPs of 80% or less than the median EU GDP show significantly higher rates of brucellosis (Pappas 2006). Similarly, central Asia has a high endemic prevalence of the disease partially due to lack of funding, health networking, and regulations. When the Soviet Union collapsed, many of its composite countries in central Asia lost the funding for controlling the spread of *Brucella* as the economy switched from socialism to the free-market. Furthermore, many laws and regulations were abandoned as the singular Soviet government fractured into multiple autonomous governments (Manseki 1993). Without the infrastructure, funding, and regulations to help control the occurrence of *Brucella* in these regions, people will continue to be infected, as the perceived risk of brucellosis does not outweigh their reliance upon their livestock, which for many is their livelihood. Finally, many regions of sub-Saharan Africa lack the infrastructure to implement large-scale vaccinations and health programs to prevent brucellosis. The lack of sufficient health networking results in many patients being misdiagnosed as contracting malaria, and treated accordingly. Even when brucellosis is correctly diagnosed, there are many other endemic diseases (like malaria) that are given priority if there is any funding for health programs (McDermott 2002). For

effective suppression and eradication of human brucellosis, countries must have a multifaceted approach that encompasses mass vaccination of livestock, increased government regulation of food handling, better education on the transmission of the disease and diagnostic techniques, more extensive health networking, and ultimately the funding to make all of that possible.

Symptoms and Treatment of Brucellosis

In cattle, *Brucella abortus* infection can cause spontaneous abortion of fetuses, which facilitates the spread of the bacteria, as other cattle can become infected through licking or inhaling brucellae from the fetus. Brucellosis can also be spread sexually between animals, or can be spread from a mother cow to her calf via contaminated milk. In human brucellosis, the disease cannot be spread from human to human, but people typically acquire the disease through aerosol, laboratory exposure, or through ingestion of unpasteurized dairy products. Brucellosis causes an undulant fever in which the sufferer repeatedly oscillates between a high and low fever. The disease causes flu-like symptoms such as headache, weight loss, malaise, joint pain, and more serious symptoms like hepatosplenomegaly; in rare cases, neurological symptoms and endocarditis can occur (APHIS 2016). Once contracted, brucellosis can be chronic and difficult to treat, usually requiring a six-week combination course of doxycycline and rifampin, or doxycycline and streptomycin, as treatment with a single therapeutic more often results in relapse (Alavi 2009, Skalsky 2008). Even with treatment, the patient can often relapse and require treatment again, and there is currently no human vaccine available. Relapse rate is affected by multiple risk factors, with aging populations and immune compromised individuals at higher risk of relapse. Delay in treatment after

onset of symptoms also increases risk of relapse, and a previous study found that men were more prone to relapse; however, they attributed this to men in their sample population being more likely to work in a setting that exposes them to *Brucella* more often. While many factors influence the risk of relapse after treatment for brucellosis, but the overall likelihood appears to be 18-35% in previous studies (Alavi 2009, Nimri 2003).

Trafficking in Macrophages

During infection, *B. abortus* preferentially infects macrophages, where it both replicates and evades the host immune system. *B. abortus* lacks many traditional virulence factors like fimbriae, toxins, or capsule, but utilizes a Type-4 Secretion System (T4SS) to secrete effector proteins into the host cytosol, and prevents full fusion of the phagosome with the lysosome. The brucellae remain within the *Brucella*-Containing Vacuole (BCV) which associates with the host endoplasmic reticulum, where the bacteria replicate and proliferate (Ke 2015, Celli 2003). Brucellae need to actively utilize the T4SS to evade degradation by the host, as one study showed that *B. abortus* 2308 and heat-treated *B. abortus* 2308 (dead cells) trafficked through the early endosome identically. In both cases, the early endosomes are characterized by high levels of LAMP-1, cathepsin B, and an acidic pH. For dead brucellae within the BCV, the endosome remained this way and the cells are eventually degraded; however, 2 hours after infection, the live brucellae induced a change in the BCV, causing a reduction in cathepsin B and neutralization of the acidic pH. Once the acidity was neutralized to an acceptable pH, the brucellae then start to divide and replicate, having successfully escaped full phagosome-lysosome fusion and destruction. In live brucellae

lacking the *Hfq* or *bacA* genes (which encode a master regulator of pathogenicity and stress resistance, and a protein that modifies fatty acids and is necessary for invasion, respectively (Cui 2013, Ferguson 2004)) the brucellae are unable to neutralize the acidity of the BCV, and are unable to replicate within the host, further demonstrating the *B. abortus* must play an active role in endosome trafficking to survive the later stages, but not during initial internalization (Bellaire 2005).

Because *B. abortus* lacks many traditional virulence factors, it relies primarily on passive evasion of the host immune system. It does not actively secrete an immunosuppressive effector to hamper innate immune cell function and recruitment, rather, it simply lacks highly recognizable PAMPs (Pathogen-Associated Molecular Patterns), so the host does not identify the threat. Because the surface antigens of *B. abortus* are poorly recognized by the host, there is little induction of proinflammatory cytokines, and thus little recruitment of host leukocytes to the site of infection. This passive evasion of the host immunes system is evidenced by the absence of any significant proinflammatory response when *B. abortus* cell lysate is injected into test animals—signifying that there is no active process required to suppress immune activation. Additionally, the lack of PAMP recognition in *B. abortus* is further reinforced by the fact that artificial depletion neutrophils in the host does not significantly change the infection course or outcome. Host neutrophils simply are largely incapable of recognizing *B. abortus* as an invader through typical PRRs (Pattern Recognition Receptors). For example, TLR4 and TLR2 gene knockouts in mice do not cause the host to be more susceptible to infection by the bacteria (Barquero-Calvo 2007).

Trafficking in Oral Infection

Oral ingestion is the most common route of exposure for *B. abortus*, and like all other intestinal pathogens, it must overcome numerous host barriers before it can establish an infection. One of the earliest encountered and most effective barriers associated with the oral route is the stomach. Ingested pathogens require a method to withstand the highly acidic gastric acid of the stomach, where the pH can drop to as low as 0.5 to 1.0. Many species of bacteria, including *Klebsiella pneumoniae*, *Yersinia enterocolitica*, and *Helicobacter pylori* neutralize the low pH via production of the urease enzyme, which catalyzes the conversion of urea to CO_2 and ammonia. The generated ammonia raises the ambient pH enough that the bacteria can survive passage through the stomach. Likewise, *B. abortus* has been shown to produce urease, and the enzyme is essential for its survival in both *in vitro* and *in vivo* models. Urease-deficient *B. abortus* exhibited a significant decrease in survival versus the wild type (*B. abortus* 2308) at pH 2 when supplemented with various concentrations of urea. This phenotype was confirmed in the mouse oral infection model, as there was a 95% reduction in the presence of *B. abortus* in mice infected with urease-deficient mutants compared to mice infected with strain 2308. Thus, urease function is critical for successful passage of *B. abortus* by the oral route of infection (Sangari 2006).

Bacteria that persist through the stomach encounter another barrier: bile acid. Bile acids in the duodenum have detergent-like properties, and allow the body to emulsify ingested fat. As a result, bile acids also have antimicrobial properties, disrupting the lipid membranes of relevant microbes in the gut (Villarreal 2014, Gueguen 2013). Intestinal pathogens require mechanisms to eliminate bile acid stress, and do so

by utilizing enzymes like CGH (Chologlycine Hydrolase). CGH activity is essential for *B. abortus* survival under bile acid stress, as a *Δcgh* strain exhibited a significant decrease in growth under different concentrations of bile. The utilization of CGH to survive bile acid stress is evident in *B. abortus*, along with other genera of bacteria frequently found in the host microflora, such as *Lactobacillus, Bifidobacterium, Clostridium,* and *Bacteroides*. The *cgh* gene has also been identified in the pathogens *Francisella tularensis* and *Bordetella pertussis,* indicating the mechanisms to degrade host bile acids are an important survival strategy for many ingested microbes (Delpino 2006).

B. abortus cells that successfully reaches the host ileum are then selectively internalized by microfold cells (M cells) within the Peyer's patches. M cells are a component of the GALT (Gut-Associated Lymphoid Tissue) and specialize in antigen uptake, internalizing sample antigens from the gut lumen, transporting the antigens through the cell via transcytosis, and presenting them to a proximal macrophage or dendritic cell. However, many pathogens, such as *Salmonella enterica* and *Yersinia enterocolitica* exploit this M cell function by facilitating their own uptake. *B. abortus* triggers its uptake by using Hsp60 (Heat Shock Protein 60) to bind the M cell PrP^C (Cellular Prion Protein) or GP2 receptor, traffic out of the gut lumen via transcytosis, and is delivered to the macrophage, its preferred host. *B. abortus* relies on this invasion strategy to reach its replicative niche, as *B. abortus* was significantly less able to invade the M cells of PrP^C deficient mice (Nakoto 2012). Although the methods by which *B. abortus* surpasses the host barriers during oral infection are generally understood, many of the genes and regulatory mechanisms used in this route of infection are poorly characterized.

Chapter 2: Determination of Deoxycholate-Responsive Genes

Introduction

In this section of the project, RNA sequencing was used to determine the transcriptional response of *B. abortus* 2308 treated with deoxycholate, a bile acid found in the gut. Genes that exhibited the highest-fold activation in response to deoxycholate were chosen for further study. RNA sequencing was chosen to reveal these deoxycholate-responsive genes because of its numerous advantage over previous systems like microarrays. While microarrays can be limited by requisite knowledge of existing genome sequence and annotation, and have a limited ability to accurately quantify fold-transcriptional changes due to high background and signal saturation, RNA sequencing has much greater resolution. Existing gene annotations are not necessarily required for detection of mRNA transcripts, and extreme transcriptional changes can be quantified due to the drastically reduced background and no risk of signal saturation. Previous RNA sequencing studies have revealed transcriptional changes as great as 9000-fold in *Saccharomyces cerevisiae* (Wang 2009), but many RNA sequencing experiments look at changes of 4-, 2-, or even 1.5-fold (Loomis 2011). For the data set in this experiment, only genes with 2-fold or greater changes were considered for examination, and the genes with the most dramatic fold changes were chosen.

The five genes with the highest-fold activation chosen were *bab1_2138*, *bab_rs17350*, *bab1_1534*, *bab1_0418*, and *bab1_0420*. Bioinformatic methods such as NCBI BLAST were used to determine the potential functions of these genes that have not been previously characterized in *Brucella*. For genes where bioinformatic methods predicted a putative gene function, previous literature describing them in other

organisms was reviewed. Through the use of bioinformatic methods and literature review, the potential functions of these uncharacterized genes can be partially elucidated.

bab1_2138

bab1_2138 (also designated as *bab_rs26115*) is a 357-nucleotide open reading frame (ORF) that NCBI BLAST predicts is the gene *rlpA*, or “Rare Lipoprotein A”. In previous studies, it has been demonstrated that *rlpA* encodes a lytic transglycosylase, a conserved outer membrane protein that plays a role in peptidoglycan degradation. Peptidoglycan provides rigidity and support to the bacterial cell wall, and is very important for maintenance of turgor pressure and resistance to osmotic stress in hypotonic environments. However, because peptidoglycan is rigid, its subunits must be removed or inserted in order for the cell to grow or divide (Vollmer 2008a). There are multiple types of proteins that are responsible for peptidoglycan deconstruction, such as endopeptidases, amidases, carboxypeptidases, and lytic transglycosylases—all of which cleave different segments of peptidoglycan. Lytic transglycosylases, such as RlpA, cleave the glycan backbone, meaning the bond between repeating units of NAG and NAM (*N*-acetlyglucosamine and *N*-acetylmuramic acid) within peptidoglycan. The other protein types are responsible for cleaving cross-links between adjacent peptides, severing peptide branches from the glycan backbone, or cleaving individual amino acids from the ends of peptides, respectively (Vollmer 2008b, van Heijenoort 2011). Because stem peptides and peptide cross-links protruding from the glycan backbone create steric hindrance, RlpA can only effectively cleave the peptidoglycan backbone if the strand is free of such peptides and cross-links. It has been experimentally demonstrated that

RlpA will only carry out its enzymatic function if the peptidoglycan strands have already been partially degraded by the other protein classes, leaving only the naked glycan backbone (Jorgensen 2014).

RlpA has been shown to have two major domains in its protein structure: a C-terminal SPOR domain and a Double Psi Beta Barrel (DPBB) domain in the center of the peptide sequence. The DPBB domain is the actual enzymatic portion of RlpA, where the protein hydrolyzes naked glycan strands between the NAG and NAM subunits (Punta 2012). The cleavage by RlpA targets the middle of glycan backbones, leading previous researchers to describe it as an “endo” lytic transglycosylase, as opposed to some other “exo” lytic transglycosylases which cleave NAG-NAM pairs off of the termini of peptidoglycan chains (Morlot 2010). The C-terminal SPOR domain—called such because it was first identified in an amidase responsible for degrading peptidoglycan to liberate a spore from its mother cell (Duncan 2013)—is a conserved, roughly 75 amino acid sequence found not only in RlpA, but also found in at least 7,000 proteins across more than 2,000 bacterial species (Punta 2012). The SPOR domain is responsible for localization of RlpA to the lateral wall and septal ring of the cell during division (Gerding 2009, Arends 2010). When a *ΔSPOR mCherry-rlpA* translational fusion was created in *Pseudomonas aeruginosa*, it was observed that RlpA failed to localize to the lateral wall and septal ring. In the same study, a full deletion of *rlpA* resulted in mutants that were susceptible to hypotonic stress, as low NaCl in the growth agar caused the cells to adopt a more coccoid shape, and they often failed to separate fully during division, forming chains of cells. However, *ΔSPOR* mutants do not exhibit the characteristic morphological defects shown by *ΔrlpA* mutants, as there is no cell

chaining or loss of the cell rod shape in *P. aeruginosa* (Jorgensen 2014). This puzzling relationship has been observed in previously tested species, and it has been proposed that, although the RlpA cannot specifically localize and bind to the peptidoglycan via the SPOR domain, the active site within the DPBB domain has high specificity to naked glycan backbones. This high specificity and affinity allows at least some of the proteins to bind and cleave a sufficient amount of peptidoglycan to rescue the phenotype, even without a SPOR domain to facilitate initial binding. (Ursinus 2004, Möll 2009, Gerding 2009).

bab_rs17350

bab_rs17350 is a 96-nucleotide ORF within the *ure1* operon of *B. abortus*. The gene is a new annotation and thus lacks a name under the previous "*bab#_#*" nomenclature. NCBI BLAST does not predict the potential function of the gene, only that it encodes a hypothetical protein 31 amino acids in length. The ORF is located downstream of the *ure1A* gene and upstream of the *ure1B* gene, and the segment is highly conserved across all of the *Brucella* species. Both NCBI BLAST and protein SWISS-MODEL were unable to predict the function or homology to other genes or proteins. Additionally, NCBI Conserved Domain Predictor, EMBL-EBI InterPro, and ExPASy Prosite were unable to identify any domains within the peptide sequence.

bab1_1534

The gene *bab1_1534* (also called *bab_rs23225* under the new nomenclature) is a 657-nucleotide open-reading frame that encodes a putative glyoxylase II (GloB or GlxII) or the similar YcbL protein, predicted by NCBI BLAST. GloB, also sometimes

called hydroxyacylglutathione hydrolase, belongs to the MBL-fold metallohydrolase superfamily of proteins, which carry out a plethora of functions—mostly as hydrolytic enzymes. Most of the proteins in this family contain two Zn^{2+} ions to carry out hydrolysis, but sometimes bind only one. The family is also described as "β-lactamase-like" as many of the members enzymatically cleave β-lactam antibiotics. In the case of glyoxylase II, it hydrolyzes S-d-lactoylglutathione (SLG) to d-lactate and glutathione (GSH) as part of the two-step glyoxylase system. An analysis of *bab1_1534* by the NCBI Conserved Domain tool also found that the protein encoded by gene had YcbL-like MBL (Metallo β-Lactamase) fold, and was likely a member of the metallo-hydrolase-like MBL-fold superfamily, as the gene was similar to the *ycbL* in *Salmonella enterica* serovar *typhimurium*. Similarly, analysis of *bab1_1534* on Microbes Online predicted that the gene was β-lactamase-like, and likely encoded glyoxylase II. Additionally, the gene is well conserved across all the *Brucella* species, and shared homology with species of *Ochrobactrum anthropic*, *Mesorhizobium*, *Bradyrhizobium*, and *Agrobacterium*.

YcbL, a potential protein encoded by *bab1_1534*, has been previously characterized in other species, and it has been found to function very similarly to glyoxylase II. It also belongs to the metallo-hydrolase superfamily (Daiyasu 2001), and contains a single or double Zn^{2+} binding site, which binds zinc using a HxHxH (triple histidine) motif (Bebrone 2007). Crystallography of YcbL shows that the protein contains a characteristic αβ/βα fold, which consists of an enzymatic active site at the periphery of two central β sheets, surrounded by external, hydrophilic α helices (Carfi 1995, Concha 1996). YcbL requires metal ions for enzymatic function, but previous

studies have shown that there can be variations in metal sequestration: the binding pockets preferentially bind Zn^{2+}, but can also bind iron and manganese ions (Gomes 2002). Although YcbL belongs to the metallohydrolase family, it was found in *S. enterica* that YcbL has no activity on β-lactams, but actually has glyoxylase II activity. The glyoxylase II activity, when tested *in vitro* was stimulated with the exogenous addition of Zn^{2+}, Mn^{2+}, and Fe^{2+}, but not Fe^{3+} (Stamp 2010). It was also demonstrated that YcbL could bind up to two metal ions, but only one is needed, as the protein retained enzymatic activity when 1:1 molar ratios of Zn^{2+} and YcbL were mixed (Limphong 2009, Schilling 2003).

YcbL (sometimes called GloC) has been shown to have glyoxylase II activity, but is a separate enzyme from GloB (glyoxylase II). Together, they complete the second step of the glyoxylase system, which is the conversion of SLG to d-lactate and GSH. The first step is carried out by glyoxylase I (GloA), which creates SLG from hemithioacetal (HTA), a product that forms spontaneously from methylglyoxyl and GSH (Reiger 2015, O'Young 2007, Sukdeo 2008). Together, these enzymes remove methylglyoxyl, which is a cytotoxic ketoaldehyde that forms naturally as a metabolic byproduct (Carrington 1986). When the cell accumulates too many phosphorylated intermediates from glycolysis, it can run low on available phosphate ions; the methylglyoxal synthase enzyme then generates more available phosphate by dephosphorylating dihydroxyacetone phosphate (Green 1968, Totemeyer 1998). This increases the availability of phosphates, but methylglyoxyl is a very reactive electrophile that can damage DNA and proteins within the system, so excess amounts can become cytotoxic (Russell 1993, Kang 2003). When *gloB* and *gloC* were deleted in *E. coli* the

mutants had an increased sensitivity to exogenous methylglyoxyl, resulting in cell death and methylglyoxyl degradation at different timepoints. When the researchers conducted enzyme assays with purified GloB and GloC, they confirmed that the reactions yielded d-lactate and GSH from SLG. They also found that the reactions proceeded even without Zn^{2+} supplementation, but indicated that the proteins had strong binding affinity and retained Zn^{2+} through the purification process; even the addition of a chelator like EDTA could not negate enzyme activity (Reiger 2015, O'Young 2007). Researchers have suggested the glyoxylase system is important for microbes within the human gut, as the host's ingestion of different methylglyoxyl-containing foods and drinks could cause fluctuation of methylglyoxyl within the gut environment (Griffith 1989, Majtan 2012).

bab1_0418 **and** ***bab1_0420***

bab1_0418 and *bab1_0420* (called *bab_rs17925* and *bab_rs17930*, respectively, under the new nomenclature) are two open-reading frames of 237- and 228 nucleotides in length. Analysis with NCBI BLAST shows only that they are both conserved genes in the *Brucella* genus, and both encode hypothetical, uncharacterized proteins. Neither NCBI Protein BLAST nor protein SWISS-MODEL were able to predict their function, or their homology to other proteins. Additionally, NCBI Conserved Domain Predictor and ExPASy Prosite were unable to identify any conserved, recognizable domains within the peptide sequences. EMBL-EBI InterPro was able to identify BAB1_0420 as part of the DUF680 family, which explains only that it is a protein of unknown function that appears to belong to a family of uncharacterized proteins found in the species *Rhizobium loti*. Currently the only enlightening information known about these genes is that they are

very similar in size, sequence, and proximity. The start codon of *bab1_0418* is only 183 nucleotides downstream from the stop codon of *bab1_0420*. Additionally, an alignment of the two nucleotide sequences by EMBL-EBI EMBOSS Needle shows 66.7% identity and similarity between the sequences, with 17.6% gaps. A peptide sequence alignment showed 67.1% identity and 74.4% similarity between the two amino acid sequences, with 13.4% gaps in the alignment, indicating high similarity between the two uncharacterized genes.

Materials and Methods

RNA Sequencing

To determine which genes were up- or down-regulated in response to deoxycholate, *B. abortus* 2308 was inoculated into Brucella broth at 10^6 CFU/mL, and the culture was incubated in a 37 °C shaker for 24 hours. Cultures were then split equally into six tubes, and deoxycholate was added to three of the tubes to a final concentration of 0.5% deoxycholate, and the other three tubes served as untreated controls. To assess the early transcriptional response to deoxycholate, tubes were incubated for 20 minutes to allow time for the initial transcriptional response before being treated with an equal volume of 1:1 ethanol-acetone and frozen at -80 °C. RNA was purified as described previously (Caswell 2012a); samples were thawed, pelleted by centrifugation at 14,000 × *g* for 2 minutes, and RNA was extracted via TRIzol reagent (Invitrogen, Carlsbad, CA). RNA was then precipitated via ethanol, treated with DNase to remove genomic DNA, and was sent to the Bioinformatics Institute (Blacksburg, VA) for library preparation and RNA-sequencing.

RNA samples were treated with the Illumina Ribo-Zero kit for Gram-negative bacteria to deplete rRNA. mRNA transcripts were ligated to the Illumina sequencing adapters, reverse transcribed to form cDNA, and sequenced by the Illumina HiSeq 2500 system. Analysis of the RNA sequencing data for changes in relative expression between untreated and deoxycholate-treated samples was done by Dr. Heath Damron at West Virginia University, as described previously by Damron *et al.* (2016). Briefly, genes showing transcriptional changes with a $P < 0.001$ were aligned to their genomic sequence with KEGG (Kyoto Encyclopedia of Genes and Genomes) Mapper v.2.5, and data were normalized with CLC Genomic Workbench 7.5.1 (Damron 2016). Gene results were then annotated using the current *B. abortus* gene designation system (RefSeq NC_007618.1 and NC_007624.1 for chromosomes I and II, respectively), but will be discussed using their gene designations from the previous system (Chain 2005). Genes for this project were chosen from the list of up-regulated genes with some of the highest increase in relative transcript quantity versus the untreated control (Figure 2.1).

Results

RNA Sequencing

From the RNA sequencing data, it was found that there were 241 genes that had gene expression changes with a significance of $P < 0.001$, and constituting about 5.3 million total gene reads. From these data, the five genes that showed some of the highest-fold upregulation in mRNA transcript level were chosen for further study. The five genes of interest were *bab1_2138* (*bab_rs26115)*, *bab1_1534* (*bab_rs23225*), *bab1_0418* (*bab_rs17925*), *bab1_0420* (*bab_rs17930*), and *bab_rs17350* (this gene was not previously annotated under the previous nomenclature system, so it will be

referred to using the new nomenclature for the remainder of the study). As shown in Figure 2.1, all of the genes of interest showed a dramatic upregulation of mRNA transcripts in response to deoxycholate, with *bab1_1534* having the lowest fold increase of 2.32, and *bab_rs17350* having the most dramatic fold increase of 4.98. All of the genes of interest had a statistically significant increase in expression versus the wild-type—all with p-values less than 3.55×10^{-9}—far smaller than the p-value cutoff of 0.05.

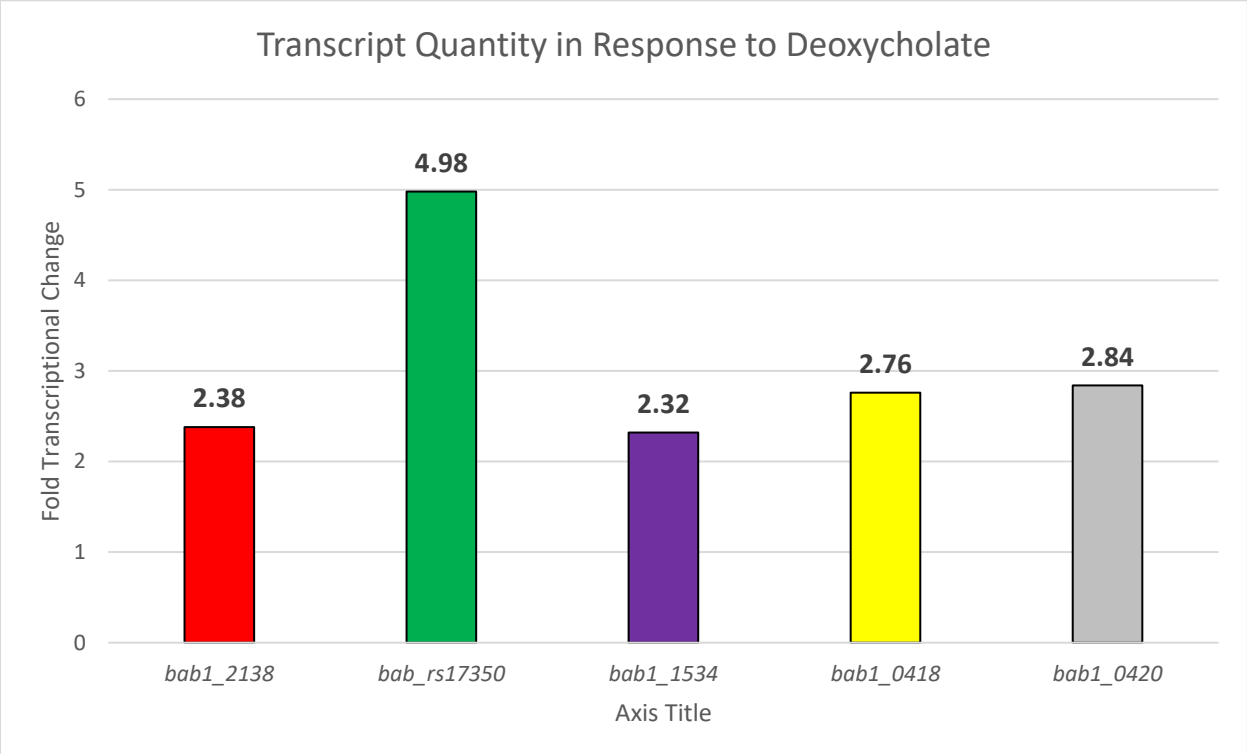


Figure 2.1- RNA sequencing results showing the relative fold increase in transcription of the genes of interest from *B. abortus* 2308 after 20 minute incubation in Brucella broth plus 0.5% deoxycholate. Fold increases are relative to the transcription levels observed in the untreated control. RNA sequencing of untreated and deoxycholate treated were done in triplicate samples.

Chapter 3: Construction and Characterization of Gene Deletion Strains

Introduction

In this module of the project, traditional cloning was used in order to make in-frame, markerless gene deletion strains of *B. abortus* for all five of the genes of interest. This method of cloning was used to prevent the introduction of frameshift mutations or the interruption of operons, which can potentially cause polar effects and disruption of other genes downstream from the genes of interest. These genes were deleted and confirmed via PCR amplification to verify that the novel strains had successful removal of their respective genes of interest; clean deletion of the genes would allow for further evaluation to determine the potential functions for each of the genes. Additionally, basic phenotypic data for each deletion strain was assessed to determine if the strains were affected by any spurious mutations that could result in growth defects or alterations to the lipopolysaccharide (LPS). Basic phenotypic characterizations are essential to ensure that potential phenotypes exhibited by the strains are not simply due to an undesired mutation resulting in stunted growth rate, or creation of a “rough” strain, which causes attenuation of strains during later infection models (Mancilla 2015). Such assessments were necessary to ensure that the deletion strains could be confidently used for future experimentation.

Materials and Methods

Bacterial Growth Conditions

In general, *B. abortus* 2308 and the created strains were grown on Schaedler agar with 5% defibrinated bovine blood (SBA) for plates, or in Brucella broth for liquid culture. Tryptic soy agar plates were also routinely used for disk diffusion and crystal violet assays. For *E. coli* growth, LB (Luria-Bertani) broth was used for liquid culture, and TKIX plates, which consist of tryptic soy agar with 45 µg/mL kanamycin, 0.2 mM isopropyl β-D-1 thiogalactopyranoside (IPTG), and 100 µg/mL 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal). Both *B. abortus* and *E. coli* were incubated at 37 °C and kanamycin was used, when appropriate, at a concentration of 45 µg/mL.

Cloning of Constructs

Plasmid constructs to introduce the desired gene deletion or reconstruction were created via traditional cloning. Deletion constructs were generated via PCR using the gene specific primers (Table 3.1) “Up-Forward” and “Down-Reverse” which annealed roughly 1000 bp up- or downstream of the ORF of interest, and featured BamH1 and Pst1 restriction sites, respectively. These primers were paired with “Up-Reverse” and “Down-Forward” primers that each annealed two to three codons into the ORF in order to generate markerless in-frame gene deletions with roughly 1 kb DNA fragments flanking the gene. All PCR for cloning was conducted using *B. abortus* 2308 genomic DNA as a template, and Phusion® High-Fidelity DNA Polymerase (NEB). The upstream and downstream fragments were digested with BamH1 and Pst1, respectively, then treated with T4 PNK (Polynucleotide Kinase) to phosphorylate the blunt ends of the fragments. The two fragments were then ligated together using T4 DNA ligase into the

pNPTS-138 plasmid, which contains a kanamycin-resistance marker, the *lacZ* gene for blue-white screening, and the *sacB* gene for later counter-selection on sucrose (Jacobs 1999). The plasmids used and constructs created are listed in Table 3.3.

The resulting ligated constructs were then transformed into Mon5 DH5α *E. coli* and plated on TKIX. White colonies were chosen from blue-white screening, and colony screening was used to determine candidates likely to be carrying the correct construct. Plasmid constructs were isolated using a GeneJET Plasmid Miniprep Kit (Thermo-Scientific) from 5 mL overnight cultures of the candidates, and were sequenced with plasmid specific and gene specific primers (Table 3.2) to confirm the sequence fidelity of the construct.

Table 3.1: Primers for Cloning Plasmid Constructs

Primer Name	5'- Sequence -3'	T_m °C
17350 Up For	TAGGATCCAAACCATTCTGTTCA	53.9
17350 Up Rev	CTTCATTTTCATACCTTTCAA	46.3
17350 Dn For	GGGTAAAGGATAGAACTCATG	50
17350 Dn Rev	TACTGCAGAGTTTCAGGCCG	57.2
1_1534 Up For	AGGGATCCATAATTTTCGATAAACGTTCGCA	60
1_1534 Up Rev	CCCCATGTTCTTTGCTCCA	55.3
1_1534 Dn For	GCCTGATTTGCAATGAAAAA	50
1_1534 Dn Rev	TACTGCAGTGATCGTTTACGTAAGAA	56
1_2138 Up For	TAGGATCCAGGTTTGTTGCAAAT	54.9
1_2138 Up Rev	GTTCATACCAGATCCACT	48.2
1_2138 Dn For	CTTTAGGCAAAGCGGCGTTCT	59.1
1_2138 Dn Rev	TACTGCAGGTTGGCGGCCTGG	64.8
1_0418 Up For	TAGGATCCTGTTCCGCGCGCTCA	64.6
1_0418 Up Rev	AGCATTCTAACGAATGACCCGTC	57.1
1_0418 Dn For	GTCAGTAAAGTGGGCTAATGC	53.7
1_0418 Dn Rev	TACTGCAGAATATCGGGAGAATCATT	55.7
1_0420 Up For	TAGGATCCCTGCGCCAGCATG	61.9
1_0420 Up Rev	GAAAGTCATTCAAGTGGATCGAC	53.8
1_0420 Dn For	AACTAAGGCCGTTTCAGACT	53.8
1_0420 Dn Rev	TACTGCAGATGAACGGGCATGTTG	59.6

Table 3.2: Primers for Screening		
Primer Name	5'- Sequence -3'	T_m °C
17350 Con For	CTCGTCAGCGATTTTGTGGT	55.6
17350 Con Rev	GTCACATCGCGTTCCTGCCC	61.3
1_1534 Con For	GGCCAGTTGGCGGGCGG	66.2
1_1534 Con Rev	TACTCAGCCAGCCACGCACGAC	63.9
1_2138 Con For	TCAATTCCTAACGAATCATAAAC	48.9
1_2138 Con Rev	AACAGTTGACGGCGCC	56.4
1_0418 Con For	CCAGAAGCTCGACTATAC	48.9
1_0418 Con Rev	TGGAAATATCAGGAAAATATT	44.6
1_0420 Con For	ATTGTACAATAAACCCATATACA	47.6
1_0420 Con Rev	ATAGGGGTTTTCGGCATAGG	54.5
M13 For	GTTTTCCCAGTCAGCAC	50.6
M13 Rev	CAGGAAACAGCTATGAC	47.0

Creation of Strains

The gene deletion or reconstruction plasmid constructs were introduced into *B. abortus* 2308 (or the respective deletion strain for reconstruction) by electroporation, and transformants were selected by plating on SBA plus 45 µg/mL kanamycin. A transformant colony was then grown in 1 mL Brucella broth for 7 hours, and then plated on SBA plus 10% sucrose for counter-selection. Candidates were then restreaked on SBA plus 10% sucrose and SBA plus kanamycin to confirm loss of the plasmid, and colony screening with gene specific flanking primers was used to confirm deletion or reconstruction of the gene of interest. To further confirm the deletion or reconstruction of the gene of interest, genomic DNA was isolated from cultures of the strains, and presence or absence of the gene was confirmed via PCR with gene specific primers.

Table 3.3: Plasmids and Strains		
Name	Description	References
Mon5 DH5α *E. coli*	Chemically competent DH5α *E. coli* (Monserate Biotechnology Group)	Monserate
B. abortus 2308	Wild-type *Brucella abortus* strain	
B. abortus CL009	*B. abortus* 2308 with markerless, in-frame deletion of *bab1_2138*	This Study
B. abortus CL010	*B. abortus* 2308 with markerless, in-frame deletion of *bab_rs17350*	This Study
B. abortus CL011	*B. abortus* 2308 with markerless, in-frame deletion of *bab1_1534*	This Study
B. abortus CL012	*B. abortus* 2308 with markerless, in-frame deletion of *bab1_0418*	This Study
B. abortus CL013	*B. abortus* 2308 with markerless, in-frame deletion of *bab1_0420*	This Study
pNPTS-138	Cloning vector containing *sacB* and Kan^R Marker	Jacobs 1999
pCL009	pNPTS-138 containing in-frame deletion of *bab1_2138* and the flanking 1 kb upstream and downstream of the gene	This Study
pCL010	pNPTS-138 containing in-frame deletion of *bab_rs17350* and the flanking 1 kb upstream and downstream of the gene	This Study
pCL011	pNPTS-138 containing in-frame deletion of *bab1_1534* and the flanking 1 kb upstream and downstream of the gene	This Study
pCL012	pNPTS-138 containing in-frame deletion of *bab1_0418* and the flanking 1 kb upstream and downstream of the gene	This Study
pCL013	pNPTS-138 containing in-frame deletion of *bab1_0420* and the flanking 1 kb upstream and downstream of the gene	This Study
pCL014	pNPTS-138 containing reconstruction of *bab1_2138* and the flanking 1 kb upstream and downstream of the gene	This Study
pCL015	pNPTS-138 containing reconstruction of *bab_rs17350* and the flanking 1 kb upstream and downstream of the gene	This Study
pCL016	pNPTS-138 containing reconstruction of *bab1_1534* and the flanking 1 kb upstream and downstream of the gene	This Study
pCL017	pNPTS-138 containing reconstruction of *bab1_0418* and the flanking 1 kb upstream and downstream of the gene	This Study
pCL018	pNPTS-138 containing reconstruction of *bab1_0420* and the flanking 1 kb upstream and downstream of the gene	This Study

Crystal Violet Assays

Crystal violet assays were used to confirm that the brucellae transformed with the gene deletion or reconstruction plasmids did not have a spurious mutation that caused the strain to become a “rough” strain. A rough strain is a strain in which the cells create LPS that lacks the O-polysaccharide; this deficiency causes the cells to be more susceptible to destruction by macrophages and the host immune system, and causes them to readily take up the stain crystal violet (Mancilla 2015). The deletion or reconstruction strains were plated on TSA plates and grown for 48 hours at 37 °C. TSA plates were then flooded with crystal violet for 10 seconds, after which the stain was poured off, and the cells were washed with 95% ethanol. The candidates were then visually inspected on the plate for uptake of the crystal violet stain, signifying that the strain was rough, or rejection of the purple stain, indicating that the strain was smooth (Turse 2011). *B. abortus* 2308 and RB51 were used as smooth and rough controls, respectively.

Growth Curve

To assess if the gene deletion strains had a growth rate defect, colonies were taken from an SBA plate, inoculated into 20 mL flasks of Brucella broth to a final concentration of 5 x 10^3 CFU/mL, and the cultures were grown in a 37 °C shaking incubator. Growth of the cultures was monitored every 12 hours spectrophotometrically at an absorbance of 600 nm, and by diluting and plating aliquots of the cultures on SBA. Colony counts were then taken after 48 hours of incubation at 37 °C.

Results

Creation of Strains

Sequencing of all of the gene deletion and reconstruction plasmid constructs showed perfect sequence fidelity in all of the constructs. No unwanted mutations were observed in the genes of interest or their promoter regions, and the gene deletions were in-frame, resulting in the start codon, stop codon, and two to six codons between, as intended, resulting in an abridged ORF of four to six codons in length. Introduction of the gene deletions via homologous recombination was confirmed by PCR with gene-specific primers that flanked the gene of interest by 150-200 base pairs on either side, and by using genomic DNA from each deletion strain as a template. As shown in Figure 3.1, DNA bands generated via PCR from the deletion stains were shorter than bands generated from wild-type 2308 template by an amount proportional to the loss of the gene of interest; this indicates that the gene deletion was properly introduced, and the wild-type allele was successfully removed from the deletion strains.

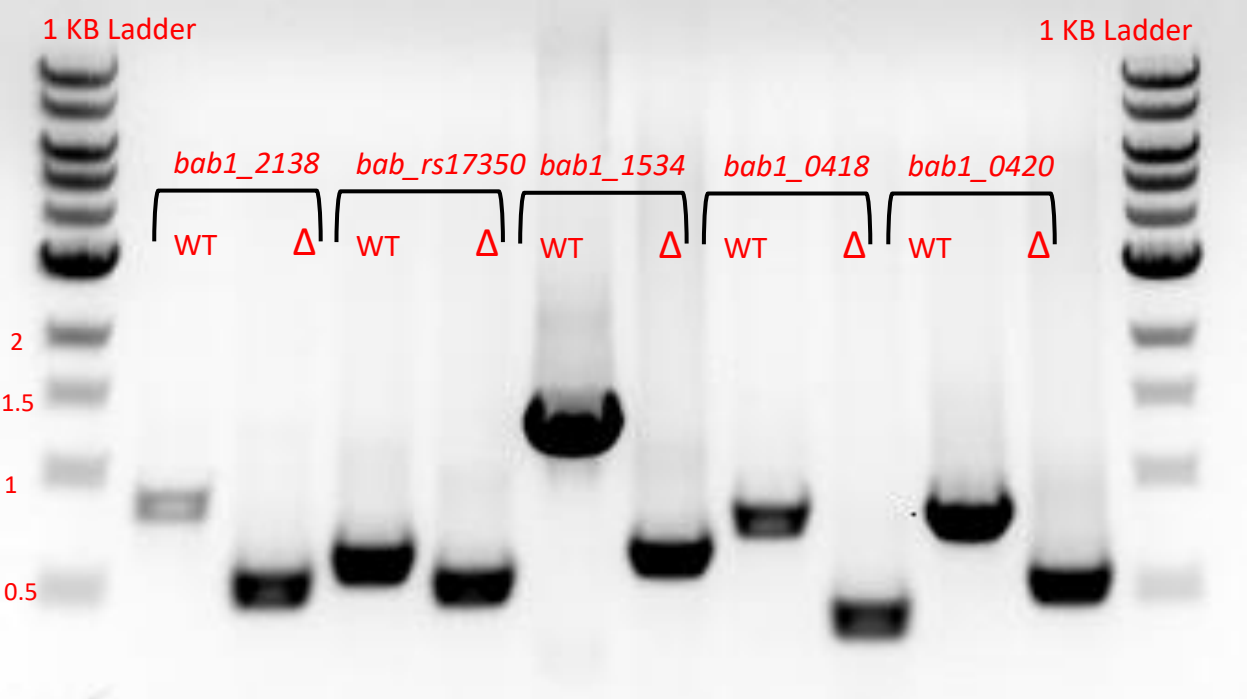


Figure 3.1- Ethidium bromide gel depicting the band sizes from amplification of wild type and deletion strains. Gene specific confirmation primers were used for PCR confirmation that the genes of interest were successfully deleted. PCR utilized genomic DNA from the wild type 2308 as the control and genomic DNA from the deletion strain of interest as the template DNA. Band sizes were measured via comparison with 1 kb DNA ladder (units in kilobases).

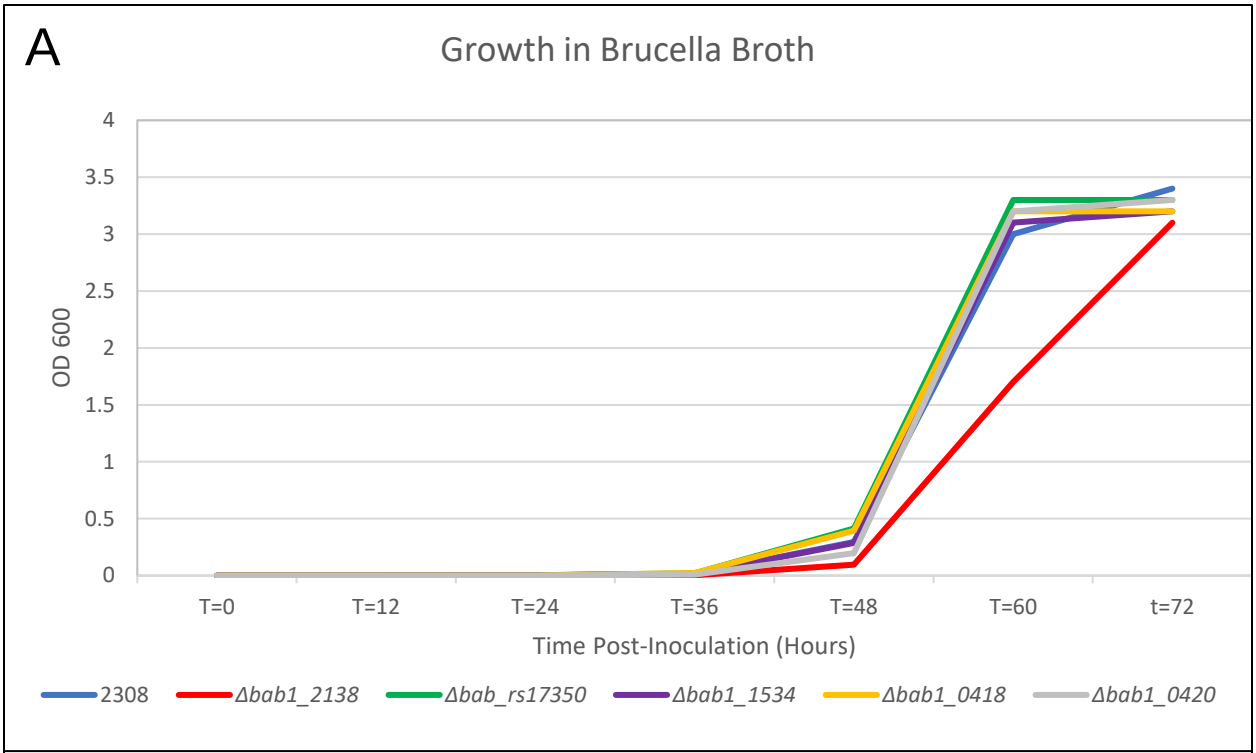
Crystal Violet Assays

Crystal violet staining of the gene deletion strains indicated that there was no phenotypic change of their LPS structure, as all of the strains rejected the crystal violet stain and remained white, and RB51, the rough control, took up the stain, becoming purple (Data not shown).

Growth Curve

Colony counts of the gene deletion strains at the different time points indicated that, although the strains did exhibit different quantities of cells as the incubation progressed, these differences were likely due to variations in the starting quantity

inoculated into the cultures at T=0. The rate at which the cells divided was roughly identical between each time point, indicating that none of the strains exhibited any kind of deficiency in its ability to grow. The parallel growth rates of the deletion strains were further evidenced by similarity their absorbance readings at 600 nm during each time point (Figure 3.2a). However, the Δbab*1_2138* strain exhibited a drastically different optical density at the 60-hour time point, despite ultimately resulting in a CFU count comparable to the other strains; this suggests that *Δbab1_2138* may have a morphological defect that causes the same number of CFU to exhibit a comparatively reduced absorbance reading (Figure 3.2b).



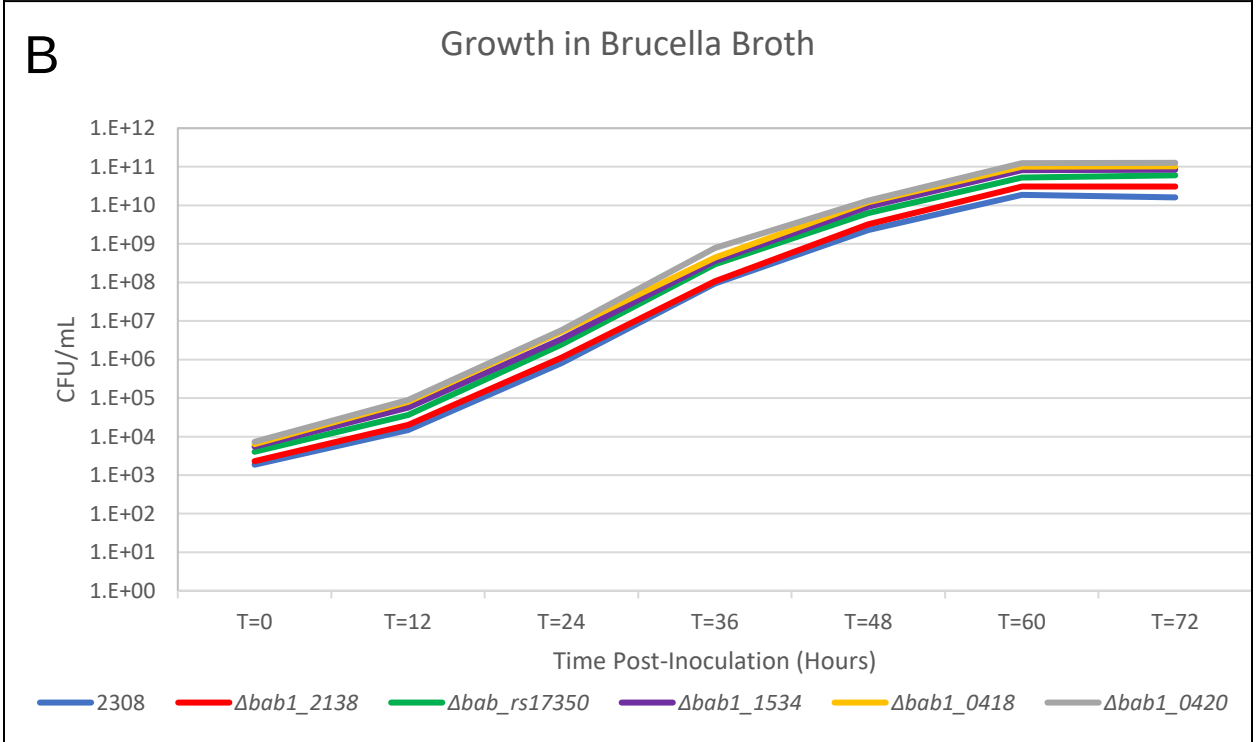


Figure 3.2- Rate of growth of the *B. abortus* gene deletion strains across 72 hours, measured by spectrophotometer at an absorbance of 600 nm (A) and by dilution and plating to determine CFU counts (B). All cultures were started at a concentration of 5 × 10^3 CFU/mL, and measurements were taken at 12 hour timepoints for 72 hours. Graphs represent one sample per strain.

Discussion

In this module, where gene-deletion strains were created and checked for basic phenotypic problems, each strain was correctly made via insertion of a plasmid featuring the 1 kb flanking regions up- and downstream of the genes of interest, the subsequent homologous recombination to remove the intact genes from the *B. abortus* genome, and counter-selection to ensure disposal of the cloning vector. The creation of these deletion strains was verified by isolation of each strain's genomic DNA, and PCR amplification to ensure that the genes of interest were successfully removed. With the gene removals successfully verified, growth curves and crystal violet assays were used to certify that each strain did not exhibit reduced growth rate or altered LPS, which could complicate future experimentation.

In-frame, markerless gene deletions were successfully created for each gene of interest, as verified by sequencing and PCR amplification. Crystal violet assays indicated that each deletion strain had intact LPS, certifying that any potential attenuation during future infection models would not be due to increased immune recognition as a result of the loss of the O-polysaccharide. Additionally, assessment of CFU count at different timepoints during a growth curve indicated that each deletion strain grew at the same rate, indicating that the gene deletions did not result in impedance of growth. However, the strain *Δbab1_2138* did exhibit a discrepancy at the 60-hour timepoint. Quantification of CFU showed no difference with the wild-type strain, but the OD_{600} suggested that there were roughly half as many CFU for the *Δbab1_2138* strain. This incongruity could be explained by potential morphological defects in the strain: because *BAB1_2138* encodes a putative lytic transglycosylase, the cells could

be forming chains, as exhibited in similar deletion strains in *P. aeruginosa* (Jergensen 2014). Brucellae organized in chains could be growing at the same rate as the wild-type, but their increased relative density could cause their CFU quantity to be underestimated by spectrophotometer. The possibility of a morphological defect is further supported by the oddities in colony color and consistency. The *Δbab1_2138* strain was slightly, but noticeably grayer in hue than the traditionally pale, yellow-brown colonies exhibited by wild type. Additionally, when scraping large quantities of the *Δbab1_2138* strain off of plates, the brucellae have a dry, "frosting-like" consistency that is dramatically different from *B. abortus* 2308. This altered consistency makes the brucellae prone to clumping, which makes them more difficult to resuspend after pelleting, and causes them to quickly sediment at the bottom of their broth culture tube if left undisturbed for too long (about half an hour). This rapid aggregation and sedimentation could contribute to the discrepancy between their actual CFU count and the reported OD_{600} absorbance reading, as the cells could start settling on the bottom before absorbance readings are taken. However, cultures were always thoroughly resuspended by pipetting immediately before absorbance readings or dilutions, so it is more likely that the cells could exist as chains. The peculiar appearance and consistency exhibited by the *Δbab1_2138* strain, in conjunction with previous research indicating its propensity for chaining, would make the strain an ideal candidate for future visualization with electron microscopy to assess the morphology of the brucellae.

Despite the peculiarity of *Δbab1_2138*, every strain showed successful deletion of its respective gene of interest, and no growth rate or LPS irregularities were observed during the growth curve or crystal violet staining. With the creation and certification of

each strain complete, the exploration and characterization of potential phenotypes could begin.

Chapter 4: Phenotypic Analysis of Gene Deletion Strains

Introduction

In this segment of the project, each gene deletion strain was tested to determine if it exhibited increased sensitivity to various stresses, including chemical and acid stresses, and to determine if any strain was less able to survive and colonize murine peritoneal macrophages. By subjecting gene deletion strains to different types of stresses, one is able to infer the potential function of the gene in question. Because the genes of interest were chosen based on their relative upregulation in response to the gut bile acid stress, deoxycholate, stresses were chosen based on the types of barriers that *B. abortus* faces during oral infection, such as deoxycholate and acid stress. Sodium dodecyl sulfate (SDS) was also chosen to be tested alongside deoxycholate, due to its similar, detergent properties. Polymyxin B, which is able to bind LPS and disrupt membrane integrity, was also chosen for testing due to its detergent-like and antibacterial properties (Zavascki 2007). Polymyxin B is similar to naturally occurring bactericidal cationic peptides, and disrupts membranes by binding the lipid A portion of LPS. However, *B. abortus* is naturally more resistant than other Gram-negative to polymyxin B due to its LPS structure and hydrophobic surface (Jensen 2010). This natural resistance likely arises from the *Brucella* O-polysaccharide in LPS, and cationic ornithine lipids in the outer membrane, both of which help to shield the polymyxin B binding site of LPS (Martínez de Tejada 1995). Sodium chloride was used as a stress to determine if any of the gene deletions resulted in a sensitivity to general osmotic stress. Another stress, hydrogen peroxide was chosen to determine if the genes had any role in resisting such reactive oxygen species (ROS), as the brucellae may

encounter within a macrophage. Finally, by using the deletion strains to infect isolated peritoneal macrophages, one could infer if any of the genes had a role in initial macrophage invasion, prevention of phagosomal-lysosomal fusion, persistence, or replication. Because none of these genes have been previously characterized, a screening of different stresses associated with oral infection and macrophage survival is essential and valuable for determining the potential roles of these genes.

Materials and Methods

Disk Diffusion Assays

Sensitivities of the deletion strains to different chemical stresses were determined via disk diffusion assays as described previously by Sheehan *et al.* (2015). Each *B. abortus* strain was taken from an SBA plate, suspended in PBS (pH 7.4), diluted to an OD_{600} of 0.15 (equating to 10^9 CFU/mL), and then inoculated into LB broth + 0.6% agar to a final concentration of 2.5×10^7 CFU/mL. Four mL of the suspension were pipetted onto each TSA plate for a final quantity of 10^8 CFU/plate, and the agar was allowed to solidify. A sterile Whatman paper disk 7 mm in diameter was placed at the center of each plate, and 7 µL of either 10% deoxycholate, 20% SDS, 30% H_2O_2, 1 M polymyxin B, or 5 M NaCl were pipetted onto the disks. In molar quantities, each disk contained 1.78 µmol deoxycholate, 4.85 µmol SDS, 68.39 µmol H_2O_2, 7 µmol polymyxin B, or 25 µmol NaCl. Plates were allowed to incubate at 37 °C for two days, after which the zones of inhibition (ZOI) were measured with a ruler. All disk diffusions were performed in triplicate (Sheehan 2015).

Acid Stress Assay

To determine if the *B. abortus* gene deletion strains had an increased susceptibility to acid stress versus the wild type 2308, all strains were taken from SBA plates and suspended in 1 mL aliquots of phosphate-buffered saline (PBS, pH 7.4) at a concentration of 10^9 CFU/mL, when aliquots were removed for serial diluting and plating on SBA to get pretreatment CFU quantities. Cells were then pelleted by centrifugation at 14,000 RPM, and resuspended to maintain 10^9 CFU/mL in citric acid of pH 2. Cells were then incubated at 37 °C for 30 minutes, after which they were pelleted and resuspended in the same volume of PBS (pH 7.4). Finally, surviving cells were serially diluted and plated on SBA, where they were then incubated at 37 °C, and colony counts were determined after 48 hours (Krishnamurthy 1998). A ratio was then used to compare the survival rate of each strain before (in pH 7.4) and after acid stress in pH 2, by dividing the calculated CFU/mL of the survivor quantity by the starting quantity. Tests were performed in triplicate, and results are reported in number of survivors per million starting CFU.

Macrophage Infection

The abilities of the deletion strains to survive and replicate within macrophages was assessed as described previously by Sheehan *et al.* (2015). Primary murine macrophages were isolated from the peritoneal cavities of BALB/c mice via lavage with 8 mL of Dulbecco's minimal essential medium (DMEM) plus 5% fetal bovine serum (FBS). The quantity of viable macrophages were then determined by hemocytometer with trypan blue staining, and macrophages were seeded into a 96-well plate at a concentration of 1.5×10^5 cells per well to incubate overnight at 37 °C with 5% CO_2. *B.*

abortus deletion strains were then taken from an SBA plate and opsonized for 30 minutes before being inoculated into each macrophage well at a multiplicity of infection (MOI) of 50 brucellae to 1 macrophage. The macrophages were incubated with the bacteria for 2 hours to enable phagocytosis, and then each well was washed with DMEM plus 5% FBS. The DMEM plus 5% FBS and 50 µg/mL gentamycin was added to each well to kill any bacteria not internalized by the macrophages. After a 2 hour incubation, media was removed from the wells, macrophages were lysed in PBS plus 0.1% deoxycholate, and the brucellae in the lysate were serially diluted and plated on SBA. Macrophages at the 24 and 48 hour time points were similarly lysed, but were maintained in DMEM plus 5% FBS and 10 µg/mL gentamycin after the initial 2 hour treatment with the higher concentration of gentamycin. The experiment was done once with *Δbab1_2138*, then repeated identically with the other four deletion strains. In each experiment, the strains were tested in triplicate (Gee 2005).

Statistical Analysis

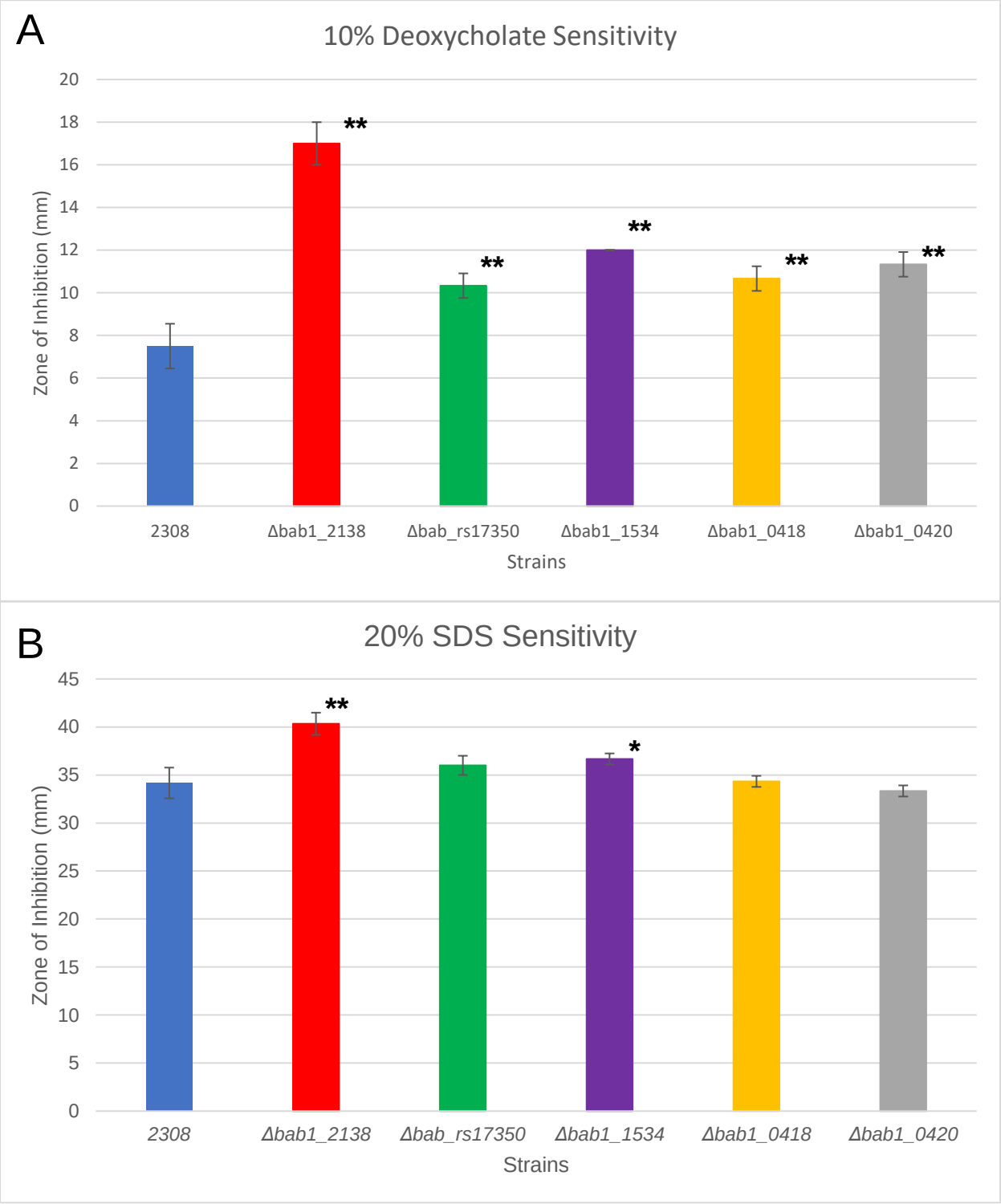
Statistically significant differences between strain 2308 and the deletion mutants in were determined via one-way ANOVA Dunnett multiple comparisons tests for each assay. For the comparison of 2308 and *Δbab_2138* in the macrophage experiment at each timepoint, a Welch unpaired *t* test was used to determine a significant difference between the two groups. For disk diffusion assays with deoxycholate, SDS, and hydrogen peroxide, a separate, but an identically conducted experiment was used to assay the sensitivities of *Δbab1_0418* and *Δbab1_0420*. The data for the wild-type controls from the later experiment were combined with the wild-type control from the earlier experiment so that all deletion strains could be compared against a consistent

control for each respective stress. All statistical analyses were done in the program GraphPad InStat 3, and in all tests, statistical significance was established as $P < 0.05$.

Results

Disk Diffusion Assays

When the zones of inhibition in the disk assays were measured for the different gene deletion strains, it was found that every strain had a statistically significant (all with $P < 0.01$) increase in sensitivity to 10% deoxycholate versus the wild-type control (Figure 4.1a). This indicates the all of the genes of interest play an important role in resisting, whether directly or indirectly, the detergent-like properties of deoxycholate and protecting the cell against lysis. However, the increased sensitivity is not necessarily a general sensitivity to detergents, as the deletion strains responded differently to SDS. Δ*bab1_2138* ($P < 0.01$) and *Δbab1_1534* ($P < 0.05$) were similarly sensitive to SDS, but the other strains were not (Figure 4.1b). In response to 30% hydrogen peroxide stress, none of the strains exhibited an altered level of sensitivity versus the wild type except for Δ*bab1_2138* ($P < 0.05$), which was significantly more sensitive to the stress (Figure 4.1c). When stressed with the antibiotic polymyxin B, the strains Δ*bab1_2138* ($P < 0.01$) and Δ*bab1_0420* ($P < 0.05$) exhibited a statistically significant increase in sensitivity to the stress; Δ*bab1_0418* did not show statistical significance, but it appears to trend towards being sensitive (Figure 4.1d). When 5 M NaCl was used to test if the deletion strains had sensitivities to osmotic stress, it was found that none of the strains exhibited any inhibition of growth in the area around the disk (data not shown).


A
10% Deoxycholate Sensitivity
Zone of Inhibition (mm)
20
18
16
14
12
10
8
6
4
2
0
**
**
**
**
**
2308
Δbab1_2138
Δbab_rs17350
Δbab1_1534
Δbab1_0418
Δbab1_0420
Strains
B
20% SDS Sensitivity
Zone of Inhibition (mm)
45
40
35
30
25
20
15
10
5
0
**
*
2308
Δbab1_2138
Δbab_rs17350
Δbab1_1534
Δbab1_0418
Δbab1_0420
Strains

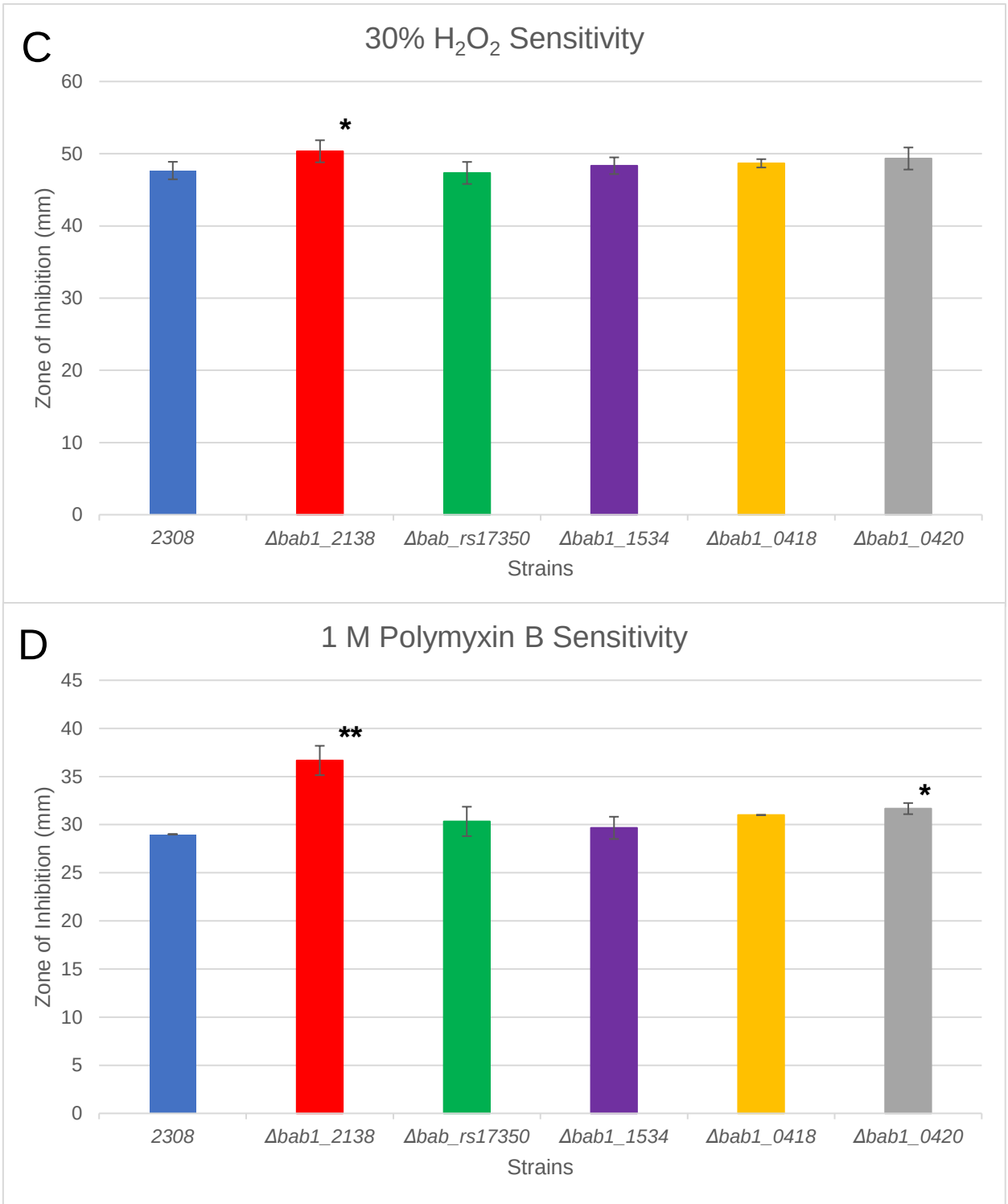


Figure 4.1- Disk Diffusion Assays- Strains were plated in soft agar to a quantity of 10^8 CFU/plate. A whatman paper disk with 7 µL of 10% deoxycholate (A), 20% SDS (B), 30% hydrogen peroxide (C), and 1 M polymyxin B (D) was placed on each plate, and they were incubated 48 hours at 37° C. ZOIs were measured in millimeters by ruler. Statistical significance for each strain compared to the control was determined by one-way ANOVA (*, $P < 0.05$; **, $P < 0.01$)

Acid Stress Assay

When the gene deletion strains were assessed for sensitivity to pH stress, it was found that *Δbab1_2138* ($P < 0.05$) exhibited significantly fewer survivors per million versus wild type after 30 minute incubation in pH 2 citric acid, with only 0.7 survivors per million starting CFU. *Δbab1_1534* and *Δbab1_0418* appeared to trend towards more sensitive and less sensitive, respectively, to acid stress, but were not dramatic enough for statistical significance. *Δbab1_0420* actually exhibited a statistically significant ($P < 0.05$) increase in its ability to survive in acid stress (Fig 4.2).

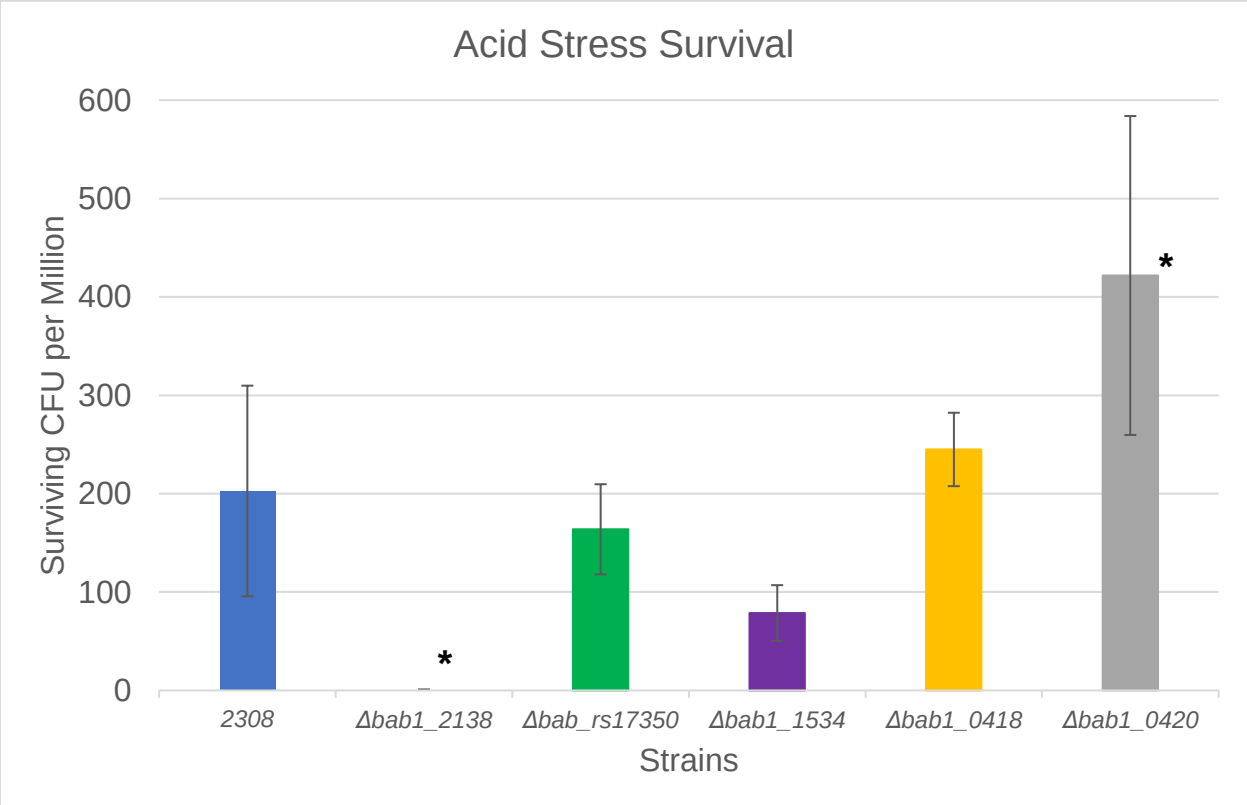


Figure 4.2- Strains were suspended at 10^9 CFU/mL in PBS (pH 7.4), and serially diluted for plating to quantify starting CFU. The cultures were then pelleted and resuspended in citric acid (pH 2) for 30 minutes at room temperature. The cultures were then pelleted and resuspended in PBS (pH 7.4) prior to serial dilution and plating to quantify surviving CFU. Results are represented as a ratio of surviving CFU per million initial CFU. Tests were done in triplicate, and statistical significance was determined by one-way ANOVA (*, $P < 0.05$).

Macrophage Infection

When the gene deletion strains were assessed for attenuation in primary, peritoneal murine macrophages, it was found that Δ*bab1_2138* was phagocytized in quantities roughly identical to the wild type 2308, but the survival rates at every ensuing timepoint were dramatically and statistically significantly decreased ($P < 0.05$ at 2 and 24 hour timepoints, and $P < 0.01$ at the 48 hour timepoint), with nearly a 3500-fold reduction in surviving brucellae at the 48 hour timepoint compared to 2308 (Figure 4.3a). Furthermore, unlike the wild type 2308, which begins to recover and replicate after the 24 hour timepoint, Δ*bab1_2138* never begins to recover. These data indicate that Δ*bab1_2138* is phagocytized as efficiently as the wild type, but is significantly less able to survive and replicate within macrophages.

In a separate, but identically performed macrophage infection experiment, the other deletion strains were tested for their ability to invade, survive, and replicate within macrophages. Although it was found that the Δ*bab1_0420* and Δ*bab_rs17350* strains had a statistically significant (both with $P < 0.01$) reduction in surviving CFU at the 24 hour timepoint, the two strains were not significantly different than wild-type at any other timepoint, including at the final 48 hour timepoint (Fig 4.3b). These data indicate that, if the deletion of these genes do cause slight attenuation of the *Brucellae* at the 24 hour timepoint, it is likely not biologically relevant due to the return to wild-type CFU quantity at the 48 hour mark. Thus, none of the genes deleted from these strains appears important for survival and replication within macrophages.

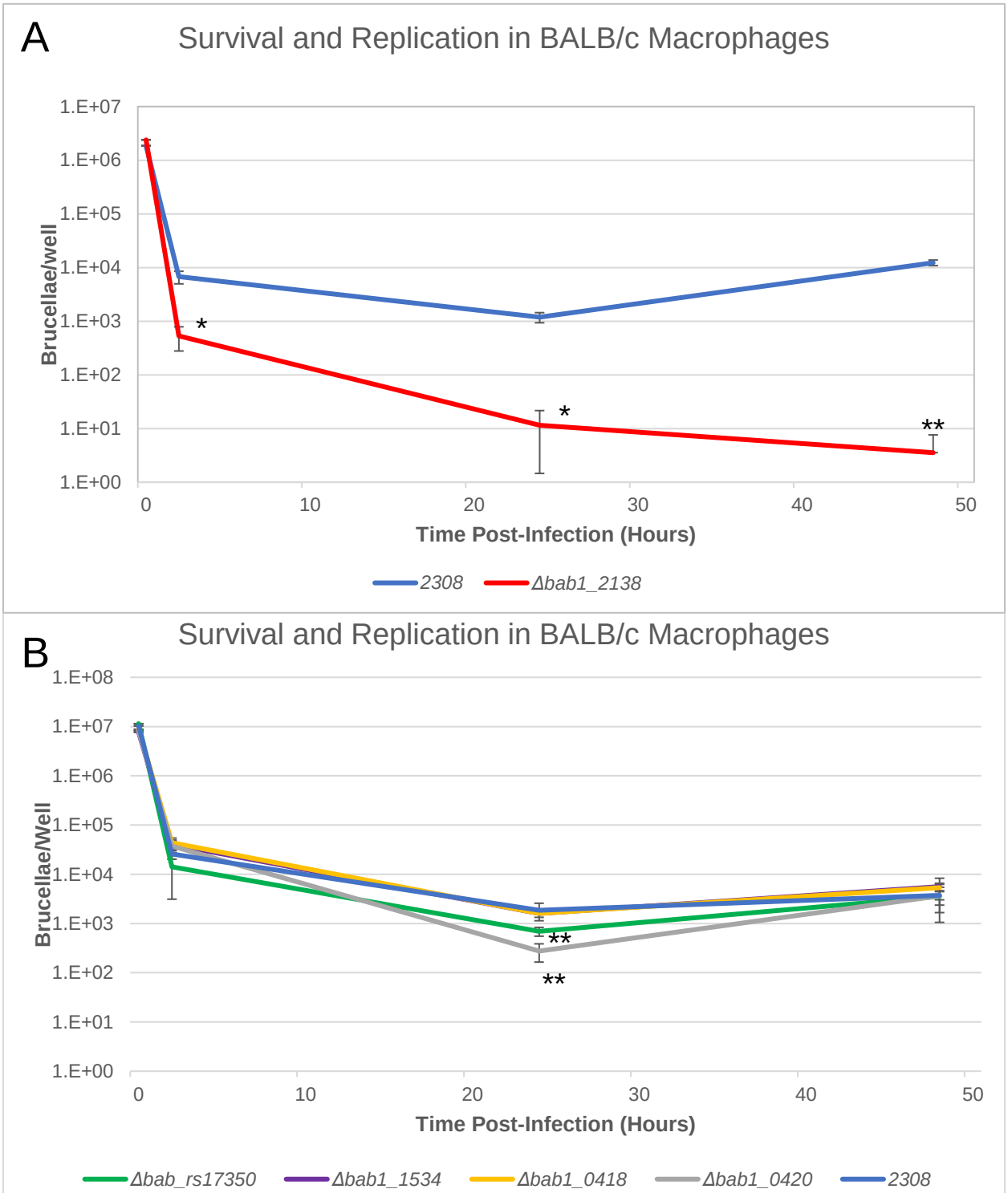


Figure 4.3- Depicts *B. abortus* 2308 and deletion strain survival and replication in primary peritoneal murine macrophages at 0, 2, 24, and 48 hour time points. All strains were used in triplicate, and testing with Δ*bab1_2138* (A) was conducted in a separate experiment from the other deletion strains (B). Statistical significance was determined between each strain and 2308 at each timepoint by Welch unpaired *t* test (A) or one-way ANOVA (B) (*, $P < 0.05$; **, $P < 0.01$)

Discussion

Through the phenotypic analysis of the five gene deletion strains, each strain was determined to be sensitive or attenuated to at least one of the tested stresses. Interestingly, it was found that all of the tested strains were significantly more sensitive to deoxycholate stress during disk diffusion assays. This indicates that all of the genes of interest directly or indirectly play a role in resistance to deoxycholate stress in the gut. However, the strains' sensitivity to deoxycholate was not necessarily a general sensitivity to detergent-like properties, as only the *Δbab1_2138* and *Δbab1_1534* strains were sensitive to a similar detergent, SDS (however, *Δbab_rs17350* was very close to statistically significant sensitivity). To polymyxin B stress, *Δbab1_0420* and *Δbab1_2138* were significantly more sensitive when compared to wild type, and it appeared that *Δbab1_0418* trended towards sensitivity, but was not statistically significant. When tested for sensitivity to osmotic stress, it was found that none of the strains exhibited any growth inhibition whatsoever from the 5M NaCl diffusing from the disk. When the strains were assessed for sensitivity to hydrogen peroxide stress and attenuation in macrophages, it was found that *Δbab1_2138* was the only strain with significant sensitivity in the tests. In the assessment of sensitivity to acid stress, *Δbab1_2138* was the only strain significantly more sensitive; however, *Δbab1_1534* trended towards more sensitive. Surprisingly, *Δbab1_0420* had significantly more surviving brucellae after being subjected to acid stress, and while *Δbab1_0418* was not significantly different, it did show a trend towards acid resistance.

From these data, it can be inferred that the *bab_rs17350* does not play a role in the resistance to many of the stresses encountered within the gut, as it was only

sensitive to deoxycholate stress. The lack of any observable phenotype to pH stress indicates that the gene does not have a role in acid resistance. Despite the gene's locus within the *ure1* operon, its removal did not inhibit the strain's ability to survive in acid. The strain's sensitivity to deoxycholate indicates that the gene likely does help *B. abortus* survive passage through the small intestine, but it does not help in the resistance to general detergent stress, like SDS or polymyxin B. Additionally, the gene does not appear to be necessary for infiltration and replication within macrophages, and it does not appear to have a role in resistance to ROS like hydrogen peroxide.

Interestingly, while almost nothing is known about the genes *Δbab1_0418* and *Δbab1_0420*, the deletion strains seemed to mimic each other closely in the phenotypes they exhibited. Both gene deletions resulted in significant sensitivity to deoxycholate, and while *Δbab1_0420* showed a significant sensitivity to polymyxin B and resistance to acid stress, *Δbab1_0418* trended similarly to both stresses. Across every phenotypic test, the two deletion strains showed almost the same set of sensitivities and resistances. These near-identical test results, in conjunction with the high nucleotide similarity between the two genes, suggest that *bab1_0418* and *bab1_0420* have very similar functions in *B. abortus*. The lack of a phenotype in macrophages and to hydrogen peroxide indicate that the genes do not have a role during survival, trafficking, and replication within macrophages, but may play a role in maintaining membrane stability. Because these two deletion strains were sensitive to deoxycholate and polymyxin B, two stresses known to destabilize cell walls and membranes (Gueguen 2013, Zavascki 2007), it is likely that these genes play a role in stabilizing the membrane against emulsification by deoxycholate or inhibit polymyxin B binding to LPS

within the cell wall. The lack of a phenotype to SDS indicates that the genes do not confer resistance to all detergents; however, SDS is an artificially manufactured surfactant (Dunn 1970) that is not naturally found within the gut, so the preservation of the genes would still help with resistance to deoxycholate, a natural bile acid that the brucellae would realistically encounter.

The *Δbab1_1534* deletion strain demonstrated sensitivity only to deoxycholate and SDS, indicating that the gene may be responsible for conferring general resistance to detergents and surfactants. Because *bab1_1534* purportedly encodes GloB or YcbL, both of which enzymatically convert s-lactoylglutathione (SLG) to lactate and glutathione (GSH), the resistance it confers to detergents is likely an indirect result of its action, rather than a direct system that actively degrades bile acids or similarly protects the cell from detergents. For its potential role during the oral infection process, it has been found that different foods naturally contain different concentrations of methylglyoxal, so food-borne brucellae may utilize the glyoxylase system to protect themselves from ambient methylglyoxal. Because methylglyoxal is a reactive electrophile that can damage proteins and DNA, it has been previously observed that methylglyoxal can disrupt the structure of defensin1, an antimicrobial peptide produced by the innate immune system, and cause the peptide to lose its antibacterial properties (Majtan 2012). Salivary defensins are also secreted in high quantities by epithelial cells of the oral cavity as a means to inhibit growth of bacteria and other microbes (Gürsoy 2016). While excess methylglyoxal is hazardous to bacteria, it also can disrupt host antimicrobial peptides like defensins, so food-borne *B. abortus* may be using the *bab1_1534* gene to detoxify excess methylglyoxal that could damage the brucellae, but

could simultaneously rely on ambient methylglyoxal as protection from host antibacterial peptides.

The *Δbab1_2138* deletion strain was of particular interest because, with the exception of hypertonic stress, the strain was the most sensitive strain to every stress tested, with statistically significant sensitivity to deoxycholate, SDS, Polymyxin B, H_2O_2, and acid stress. Additionally, the strain was exceedingly attenuated in the macrophage infection model, with nearly a 3500-fold reduction in surviving brucellae versus the wild-type at the 48 hour timepoint. The general fragility of this strain to all of the stresses, in conjunction with the odd changes in color and consistency of its colonies, suggests that the deletion of *bab1_1534* results in defects in its cell wall or membrane. The lack of a rough phenotype, as verified by crystal violet assay, shows that the cell wall change and attenuation in macrophages is not due to loss of the O-polysaccharide from the strain's LPS. Additionally, the strain's sensitivity is not due simply to growing slower than the other strains, as its growth rate was comparable to *B. abortus* 2308. Because *bab1_2138* encodes a putative RlpA, the sensitivities it displays are likely the result of errors in the hydrolysis and reformatting of peptidoglycan within the cell wall. It was already observed that a deletion of *rlpA* in *P. aeruginosa* can cause chaining of the cells due to their inability to hydrolyze peptidoglycan normally (Jergensen 2014). Remodeling of peptidoglycan is essential during cell division since peptidoglycan within the cell wall is a rigid network. Normally, the dividing cell forms a Z-ring made from a polymer of FtsZ proteins, and these proteins create an interface between the cytoplasm and the layers of the cell membrane and wall; this interface allows peptidoglycan remodeling proteins to deconstruct existing structures, and compose new peptidoglycan

at the new pole of each daughter cell (Collier 2010). Peptidoglycan remodeling proteins, such as RlpA localize to the septal ring during division to help deconstruct peptidoglycan, so the deletion of a putative *rlpA* gene may inhibit the cells' ability to properly separate. Previous research has also suggested that lytic transglycosylases, like RlpA, help anchor the outer membrane to the peptidoglycan layer; deletion of lytic transglycosylases in *P. aeruginosa* caused the cells to become significantly more sensitive to detergent-like stresses, such as bile acids (Lamers 2015). Because the *Δbab1_2138* deletion strain was significantly more sensitive to every stress that can destabilize membranes, it is likely that the gene does indeed encode a lytic transglycosylase. Loss of that gene likely caused the cells to lose some structural stability in their cell walls due to an inability to properly dismantle peptidoglycan to make room for future assembly. Such a collapse in the deletion strain's ability to resist almost every stress, and its inability to persist within macrophages indicates that *bab1_2138* is critical for successful survival and host infection by *B. abortus*.

Chapter 5: Closing Remarks

Through this project, five deoxycholate-responsive genes have been explored to determine their potential role in oral infection by *B. abortus*. Deletion strains for each gene were created to explore how the genetic loss affected their cell morphology, their growth rate, their ability to resist numerous stresses, and their ability to survive and replicate within macrophages. Through these tests, the potential functions of these five genes have been better elucidated. The data support the idea that *bab1_2138* and *bab1_1534* encode a putative lytic transglycosylases and glyoxylase II, respectively. And the other genes of interest, for which there has been essentially no previous exploration, have been shown to result in sensitivities to multiple stresses, supporting the belief that they are important for the survival of *B. abortus*.

While this project has been valuable in conducting preliminary exploration of these five genes, whether they have predicted functions or have no previously known functions, there are future experiments that would further characterize the functions of them. The exploration of *bab1_2138* could benefit greatly from electron microscopy to assess whether the deletion strain does indeed form chains and fail to divide properly. Further examination of a strain with a BAB1_2138 translational fusion protein connected to a fluorescent protein (such as GFP) could show whether the purported lytic transglycosylase actually localizes to the septal ring during cell division. With the *bab1_1534* gene, it would be valuable to express and purify the putative glyoxylase II protein and conduct *in vitro* enzyme assays. Incubation of the BAB1_1534 protein with S-d-lactoylglutathione to determine if lactate and glutathione is formed would confirm that the protein is acting as a glyoxylase II. The other three genes of unknown function

could be further explored with western blot analysis to determine if a respective protein is actually being created in response to deoxycholate exposure, or if the genes are only producing mRNA transcripts that could perform regulatory functions. Similarly, GFP fusions of each protein could be created to determine if they localize to specific locations within the cell. Finally, due to the apparent similarities between *bab1_0418* and *bab1_0420* in both their sequences and their resulting phenotypes in deletion strains, it would be interesting to create a double-deletion strain deficient in both genes. Phenotypic analysis in such a strain would be valuable in determining if the genes share some redundancy in their functions, and to determine if loss of both genes would result in an additive effect in their sensitivities to different stresses.

Though further experimentation is needed to definitively classify the genes in question, this study has been valuable in laying the groundwork for their future exploration. By further characterizing deoxycholate-responsive genes, it furthers understanding of how *B. abortus* is able to evade host barriers and ultimately survive to cause an infection. More profound understanding of this interaction allows for the exploration of potential targets for therapeutics—and ultimately—for more effective treatments of brucellosis.

References

- APHIS. Facts About Brucellosis. 2016, from https://www.aphis.usda.gov/animal_health/animal_diseases/brucellosis/downloads/bruc-facts.pdf

- Alavi SM, Alavi SMR, Alavi L. Relapsed human brucellosis and related risk factors. *Pak J Med Sci* 2009;25(1):46-50.

- Arends SJ, Williams K, Scott RJ, Rolong S, Popham DL, Weiss DS. Discovery and characterization of three new *Escherichia coli* septal ring proteins that contain a SPOR domain: DamX, DedD, and RlpA. *J Bacteriol*. 2010;192:242–255.

- Atluri VL1, Xavier MN, de Jong MF, den Hartigh AB, Tsolis RM. Interactions of the human pathogenic *Brucella* species with their hosts. *Annu Rev Microbiol*. 2011;65:523-41. doi: 10.1146/annurev-micro-090110-102905.

- Barquero-Calvo E, Chaves-Olarte E, Weiss DS, et al. *Brucella abortus* Uses a Stealthy Strategy to Avoid Activation of the Innate Immune System during the Onset of Infection. Ojcius D, ed. *PLoS ONE*. 2007;2(7):e631. doi:10.1371/journal.pone.0000631.

- Bebrone C. Metallo-beta-lactamases (classification, activity, genetic organization, structure, zinc coordination) and their superfamily. *Biochem Pharmacol*. 2007;74:1686-1701

- Bellaire BH, Roop RM, Cardelli JA. Opsonized Virulent *Brucella abortus* Replicates within Nonacidic, Endoplasmic Reticulum-Negative, LAMP-1-Positive Phagosomes in Human Monocytes . *Infect Immun*. 2005;73(6):3702-3713. doi:10.1128/IAI.73.6.3702-3713.2005.

- Carrington SJ, Douglas KT. The glyoxalase enigma—the biological consequences of this ubiquitous enzyme. *IRCS Med Sci* 1986;11:762–8.

- Caswell CC, Gaines JM, Roop RM. The RNA Chaperone Hfq Independently Coordinates Expression of the VirB Type IV Secretion System and the LuxR-Type Regulator BabR in *Brucella abortus* 2308. *J Bacteriol*. 2012a;194(1):3-14. doi:10.1128/JB.05623-11.

- Caswell CC, Gaines JM, Ciborowski P, et al. Identification of two small regulatory RNAs linked to virulence in *Brucella abortus* 2308. *Mol Microbiol.* 2012b;85(2):345-360. doi:10.1111/j.1365-2958.2012.08117.x.

- Carfi A, Pares S, Duee E, Galleni M, Duez C, Frere JM, Dideberg O. The 3-D structure of a zinc metallo-beta-lactamase from Bacillus cereus reveals a new type of protein fold. *EMBO J*. 1995;14:4914–4921.

- Chain PSG, Comerci DJ, Tolmasky ME, et al. Whole-Genome Analyses of Speciation Events in Pathogenic Brucellae . *Infect Immun*. 2005;73(12):8353-8361. doi:10.1128/IAI.73.12.8353-8361.2005.

- Collier, J. A new factor stimulating peptidoglycan hydrolysis to separate daughter cells in *Caulobacter crescentus*. *Mol Microbiol*. 2010; 77: 11–14. doi:10.1111/j.1365-2958.2010.07225.x

- Concha NO, Rasmussen BA, Bush K, Herzberg O. Crystal structure of the wide-spectrum binuclear zinc beta-lactamase from *Bacteroides fragilis*. *Structure*. 1996;4:823-836

- Cui M, Wang T, Xu J, et al. Impact of Hfq on Global Gene Expression and Intracellular Survival in *Brucella melitensis*. Kwaik YA, ed. *PLoS ONE*. 2013;8(8):e71933. doi:10.1371/journal.pone.0071933.

- Daiyasu H, Osaka K, Ishino Y, Toh H. Expansion of the zinc metallo-hydrolase family of the beta-lactamase fold. *FEBS Lett*. 2001;503:1–6.

- Damron FH, Oglesby-Sherrouse AG, Wilks A, Barbier M. Dual-seq transcriptomics reveals the battle for iron during *Pseudomonas aeruginosa* acute murine pneumonia. *Scientific Reports*. 2016;6:39172. doi:10.1038/srep39172.

- Delpino MV, Marchesini MI, Estein SM, et al. A Bile Salt Hydrolase of *Brucella abortus* Contributes to the Establishment of a Successful Infection through the Oral Route in Mice. *Infect Immun*. 2007;75(1):299-305. doi:10.1128/IAI.00952-06.

- Doyle TJ, Bryan RT. Infectious disease morbidity in the US region bordering Mexico, 1990–1998. *J Infect Dis*. 2000; 182: 1503–10.

- Duncan TR, Yahashiri A, Arends SJR, Popham DL, Weiss DS. Identification of SPOR Domain Amino Acids Important for Septal Localization, Peptidoglycan Binding, and a Disulfide Bond in the Cell Division Protein FtsN. *J Bacteriol*. 2013;195(23):5308-5315. doi:10.1128/JB.00911-13.

- Dunn ER. Process of Making Solid Foams from Polymer Emulsions. 1970; patent US3491033

- Fosgate GT, Carpenter TE, Chomel BB, Case JT, DeBess EE, Reilly KF. Time-space clustering of human brucellosis, California, 1973–1992. *Emerg Infect Dis* 2002; 8: 672–78.

- Ferguson GP, Datta A, Baumgartner J, Roop RM 2nd, Carlson RW, Walker GC. Similarity to peroxisomalmembrane protein family reveals that Sinorhizobium and *Brucella* BacA affect lipid-A fatty acids. Proc Natl Acad Sci USA. 2004 Apr 6;101(14):5012-7. Epub 2004 Mar 24.

- Gándara B, Merino AL, Rogel MA, Martínez-Romero E. Limited Genetic Diversity of *Brucella* spp. *Journal of Clinical Microbiology*. 2001;39(1):235-240. doi:10.1128/JCM.39.1.235-240.2001.

- Gee JM, Valderas MW, Kovach ME, et al. The *Brucella abortus* Cu,Zn Superoxide Dismutase Is Required for Optimal Resistance to Oxidative Killing by Murine Macrophages and Wild-Type Virulence in Experimentally Infected Mice . *Infection and Immunity*. 2005;73(5):2873-2880. doi:10.1128/IAI.73.5.2873-2880.2005.

- Gerding MA, Liu B, Bendezu FO, Hale CA, Bernhardt TG, de Boer PA. Self-enhanced accumulation of FtsN at Division Sites and Roles for Other Proteins with a SPOR domain (DamX, DedD, and RlpA) in *Escherichia coli* cell constriction. *J Bacteriol*. 2009;191:7383–7401.

- Gomes CM, Frazao C, Xavier AV, Legall J, Teixeira M. Functional control of the binuclear metal site in the metallo-beta-lactamase-like fold by subtle amino acid replacements. *Protein Sci.* 2002;11:707–712.

- Green ML, Lewis JB. The oxidation of aminoacetone by a species of Arthrobacter. *Biochem J* 1968;106:267–70.

- Griffith R, Hammond EG. Generation of swiss cheese flavor components by the reaction of aminoacids with carbonylcompounds. *J Dairy Sci* 1989;72:604–13.

- Gueguen E, Durand E, Zhang XY, d' Amalric Q, Journet L, Cascales E. Expression of a *Yersinia pseudotuberculosis* Type VI Secretion System Is Responsive to Envelope Stresses through the OmpR Transcriptional Activator. Beloin C, ed. *PLoS ONE*. 2013;8(6):e66615. doi:10.1371/journal.pone.0066615.

- Guerra H. The *Brucellae* and their success as pathogens. C*rit Rev Microbiol*. 2007;33(4):325-31.

- Gürsoy M, Gürsoy UK, Liukkonen A, Kauko T, Penkkala S, Könönen E. Salivary antimicrobial defensins in pregnancy. *J Clin Periodontol* 2016; 43: 807–815. doi: 10.1111/jcpe.12581.

- Hofmann AF. The Continuing Importance of Bile Acids in Liver and Intestinal Disease. *Arch Intern Med*. 1999;159(22):2647-2658. doi:10.1001/archinte.159.22.2647.

- Jacobs C, Domian IJ, Maddock JR, Shapiro L. Cell cycledependent polar localization of an essential bacterial histidine kinase that controls DNA replication and cell division. *Cell*. 1999 97:111–120

- Jean Celli, Chantal de Chastellier, Don-Marc Franchini, Javier Pizarro-Cerda, Edgardo Moreno, Jean-Pierre Gorvel. *J Exp Med*. 2003 Aug 18; 198(4): 545–556. doi: 10.1084/jem.20030088

- Jensen AE, Halling SM. Effect of polymyxin B and environmental conditions on isolation of Brucella species and the vaccine strain RB51. *Comp Immunol Microbiol Infect Dis*. 2010;33:121-131.

- Jorgenson MA, Chen Y, Yahashiri A, Popham DL, Weiss DS. The Bacterial Septal Ring Protein RlpA is a Lytic Transglycosylase that Contributes to Rod Shape and Daughter Cell Separation in Pseudomonas aeruginosa. *Mol Microbiol*. 2014;93(1):113-128. doi:10.1111/mmi.12643.

- Kang JH. Oxidative damage of DNA by the reaction of amino acid with methylglyoxal in the presence of Fe(III). *Int J Biol Macromol* 2003;33:43–8.

- Ke Y, Wang Y, Li W, Chen Z. Type IV secretion system of *Brucella* spp. and its effectors. *Frontiers in Cellular and Infection Microbiology*. 2015; 5:72. doi:10.3389/fcimb.2015.00072.

- Krishnamurthy P, Parlow M, Zitzer JB, et al. *Helicobacter pylori* Containing Only Cytoplasmic Urease Is Susceptible to Acid. Fischetti VA, ed. *Infect Immun*. 1998;66(11):5060-5066.

- Lamers RP, Nguyen UT, Nguyen Y, Buensuceso RNC, Burrows LL. Loss of membrane-bound lytic transglycosylases increases outer membrane permeability and *β*-lactam sensitivity in *Pseudomonas aeruginosa*. *MicrobiologyOpen*. 2015;4(6):879-895. doi:10.1002/mbo3.286.

- Limphong P, McKinney RM, Adams NE, Bennett B, Makaroff CA, Gunasekera TS, Crowder MW. Human Glx2 contains an Fe(II)Zn(II) center but is active as a mononuclear Zn(II) enzyme. *Biochemistry*. 2009;48:5426–5434.

- Loomis WF, Shaulsky G. Developmental changes in transcriptional profiles. *Dev Growth Differ*. 2011;53: 567–575. doi:10.1111/j.1440-169X.2010.01241.x

- Majtan J, Klaudiny J, Bohova J, et al. Methylglyoxal-induced modifications of significant honeybee proteinous components in manuka honey: Possible therapeutic implications. *Fitoterapia* 2012;83:671–7.

- Manaseki S. Mongolia: a health system in transition. *BMJ* 1993; 307: 1609–11.

- Mancilla M. Smooth to Rough Dissociation in *Brucella*: The Missing Link to Virulence. *Front Cell Infect Microbiol*. 2015; 5:98. doi:10.3389/fcimb.2015.00098.

- Martin FA, Posadas DM, Carrica MC, Cravero SL, O'Callaghan D, Zorreguieta A. Interplay between Two RND Systems Mediating Antimicrobial Resistance in *Brucella suis* . *J Bacteriol*. 2009;191(8):2530-2540. doi:10.1128/JB.01198-08.

- Martínez de Tejada G, Pizarro-Cerdá J, Moreno E, Moriyón I. The outer membranes of Brucella spp. are resistant to bactericidal cationic peptides. *Infect Immun*. 1995;63(8):3054-3061.

- McDermott JJ, Arimi SM. Brucellosis in sub-Saharan Africa: epidemiology, control and impact. *Vet Microbiol* 2002; 90: 111–34.

- Möll A, Thanbichler M. 2009. FtsN-like proteins are conserved components of the cell division machinery in proteobacteria. *Mol Microbiol*. 72:1037–1053

- Morlot C, Uehara T, Marquis KA, Bernhardt TG, Rudner DZ. A highly coordinated cell wall degradation machine governs spore morphogenesis in *Bacillus subtilis*. *Genes Dev*. 2010;24:411–422.

- Nakato G, Hase K, Suzuki M, Kimura M, Ato M, Hanazato M, Tobiume M, Horiuchi M, Atarashi R, Nishida N, Watarai M, Imaoka K, Ohno HJ Cutting Edge: *Brucella abortus* exploits a cellular prion protein on intestinal M cells as an invasive receptor. *Immunol*. 2012 Aug 15;189(4):1540-4. doi: 10.4049/jimmunol.1103332. Epub 2012 Jul 6.

- Nimri LF. Diagnosis of recent and relapsed cases of human brucellosis by PCR assay. *BMC Infect Dis*. 2003;3:5. doi:10.1186/1471-2334-3-5.

- Osterman B, Moriyon I. International committee on systematics of prokaryotes; subcommittee on the taxonomy of *Brucella*: minutes of the meeting, 17 September 2003, Pamplona, Spain. *Int J Syst Evol Microbiol*. 2006;56(5):1173–1175.

- O'Young J, Sukdeo N, Honek JF. Escherichia coli glyoxalase II is a binuclear zinc-dependent metalloenzyme. *Arch Biochem Biophys* 2007;459:20–6.

- Pappas G, Papadimitriou P, Akritidis N, Christou L, Tsianos EV. The New Global Map of Human Brucellosis. *Lancet Infect. Dis.* 2006 Feb;6(2):91-9.

- Punta M, Coggill PC, Eberhardt RY, Mistry J, Tate J, Boursnell C, Pang N, Forslund K, Ceric G, Clements J, Heger A, Holm L, Sonnhammer EL, Eddy SR, Bateman A, Finn RD. The Pfam protein families database. *Nucleic Acids Res*. 2012;40:D290–301.

- Reiger M, Lassak J, Jung K: Deciphering the role of the type II glyoxalase isoenzyme YcbL (GlxII-2) in *Escherichia coli*. *FEMS Microbiol Lett* 2015; 362 (2): 1-7. doi: 10.1093/femsle/fnu014

- Russell JB. Glucose toxicity in Prevotella ruminicola: methylglyoxal accumulation and its effect on membrane physiology. *Appl Environ Microb* 1993;59:2844–50.

- Sangari FJ, Seoane A, Rodríguez MC, Agüero J, García Lobo JM. Characterization of the Urease Operon of *Brucella abortus* and Assessment of Its Role in Virulence of the Bacterium. *Infect Immun*. 2007;75(2):774-780. doi:10.1128/IAI.01244-06.

- Sheehan LM, Budnick JA, Roop RM, Caswell CC. Coordinated Zinc Homeostasis Is Essential for the Wild-Type Virulence of *Brucella abortus*. Christie PJ, ed. *J Bacteriol*. 2015;197(9):1582-1591. doi:10.1128/JB.02543-14.

- Schilling O, Wenzel N, Naylor M, Vogel A, Crowder M, Makaroff C, Meyer-Klaucke W. Flexible metal binding of the metallo-beta-lactamase domaglyoxalase II incorporates iron, manganese, and zinc in vivo. *Biochemistry*. 2003;42:11777–11786.

- Skalsky K, Yahav D, Bishara J, Pitlik S, Leibovici L, Paul M. Treatment of human brucellosis: systematic review and meta-analysis of randomised controlled trials. *BMJ*. 2008;336(7646):701-704. doi:10.1136/bmj.39497.500903.25.

- Sorg JA, Sonenshein AL. Bile salts and glycine as cogerminants for *Clostridium difficile* spores. *J. Bacteriol.* 2008 Apr;190(7):2505-12. Doi: 10.1128/JB.01765-07. Epub 2008 Feb 1.

- Stamp AL, Owen P, Omari KE, et al. Structural and functional characterization of *Salmonella enterica* serovar Typhimurium YcbL: An unusual Type II glyoxalase. *Protein Sci*. 2010;19(10):1897-1905. doi:10.1002/pro.475.

- Sukdeo N, Honek JF. Microbial glyoxalase enzymes: metalloenzymes controlling cellular levels of methylglyoxal. *Drug Metab Drug Interact* 2008;23:29–50.

- Taylor JP, Perdue JN. The changing epidemiology of human brucellosis in Texas, 1977–1986. *Am J Epidemiol* 1989; 130: 160–65.

- Thakur SD, Thapliyal DC. Seroprevalence of brucellosis in man. *J Commun Dis* 2002; 34: 106–09.

- Thapar MK, Young EJ. Urban outbreak of goat cheese brucellosis. *Pediatr Infect Dis* 1986; 5: 640–43.

- Totemeyer S, Booth NA, Nichols WW, et al. From famine to feast: the role of methylglyoxal production in Escherichia coli. *Mol Microbiol* 1998;27:553–62.

- Turse JE, Pei J, Ficht TA. Lipopolysaccharide-Deficient *Brucella* Variants Arise Spontaneously during Infection. *Front Microbiol*. 2011;2:54. doi:10.3389/fmicb.2011.00054.

- Matthias Reiger, Jürgen Lassak, Kirsten Jung; Deciphering the role of the type II glyoxalase isoenzyme YcbL (GlxII-2) in *Escherichia coli*. *FEMS Microbiol Lett* 2015; 362 (2): 1-7. doi: 10.1093/femsle/fnu014

- Renukaradhya GJ, Isloor S, Rajasekhar M. Epidemiology, zoonotic aspects, vaccination and control/eradication of brucellosis in India. *Vet Microbiol* 2002; 90: 183–95

- Theriot CM, Koenigsknecht MJ, Carlson PE Jr, Hatton GE, Nelson AM, Li B, Huffnagle GB, Z Li J, Young VB Antibiotic-induced shifts in the mouse gut microbiome and metabolome increase susceptibility to Clostridium difficile infection. *Nat Commun*. 2014; 5:3114. doi: 10.1038/ncomms4114.

- Ursinus A, van den Ent F, Brechtel S, et al. Murein (Peptidoglycan) Binding Property of the Essential Cell Division Protein FtsN from *Escherichia coli*. *J Bacteriol*. 2004;186(20):6728-6737. doi:10.1128/JB.186.20.6728-6737.2004.

- Van Heijenoort J. Peptidoglycan Hydrolases of Escherichia coli. *Microbiol Mol Biol Rev: MMBR*. 2011;75(4):636-663. doi:10.1128/MMBR.00022-11.

- Villarreal, J. M., Becerra-Lobato, N., Rebollar-Flores, J. E., Medina-Aparicio, L., Carbajal-Gómez, E., Zavala-García, M. L., Vázquez, A., Gutiérrez-Ríos, R. M., Olvera, L., Encarnación, S., Martínez-Batallar, A. G., Calva, E. and Hernández-Lucas, I. The *Salmonella enterica* serovar Typhi ltrR-ompR-ompC-ompF genes are involved in resistance to the bile salt sodium deoxycholate and in bacterial transformation. *Mol Microbiol*. 2014; 92: 1005–1024. doi:10.1111/mmi.12610

- Vollmer W, Blanot D, de Pedro MA. Peptidoglycan structure and architecture. *FEMS Microbiol Rev*. 2008a; 32:149–167

- Vollmer W, Joris B, Charlier P, Foster S. Bacterial peptidoglycan (murein) hydrolases. *FEMS Microbiol Rev*. 2008b; 32:259–286.

- Wang Z, Gerstein M, Snyder M. RNA-Seq: a revolutionary tool for transcriptomics. *Nature reviews Genetics*. 2009;10(1):57-63. doi:10.1038/nrg2484.

- Yasugi M, Okuzaki D, Kuwana R, Takamatsu H, Fujita M, Sarker MR, Miyake M Transcriptional Profile during Deoxycholate-Induced Sporulation in a *Clostridium perfringens* Isolate Causing Foodborne Illness. *Appl Environ Microbiol*. 2016 May 2;82(10):2929-42. doi: 10.1128/AEM.00252-16. Print 2016 May 15.

- Young EJ, Suvannoparrat U. Brucellosis outbreak attributed to ingestion of unpasteurized goat cheese. *Arch Intern Med* 1975; 135: 240–43.

- Zavascki AP, Luciano ZG, Li J, Roger L. Nation; Polymyxin B for the treatment of multidrug-resistant pathogens: a critical review. *J Antimicrob Chemother* 2007; 60 (6): 1206-1215. doi: 10.1093/jac/dkm357