Characterization of the VtlR regulons in *Brucella abortus* and *Agrobacterium tumefaciens*

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Abstract

Brucella abortus and *Agrobacterium tumefaciens* are pathogenic bacteria that infect animals and plants, respectively. These bacteria are genetically similar and are found within the same Class, *Alphaproteobacteria*, and Order, *Rhizobiales*, of the domain *Eubacteria*; however, they survive and replicate in vastly different environmental niches. In Order to adapt to different environments, bacteria utilize several mechanisms of gene regulation to tightly control gene expression. Two of these mechanisms include transcriptional regulators and small regulatory RNAs (sRNAs), which can activate and repress gene expression through various interactions with DNA, mRNA, and proteins. A well-conserved transcriptional regulator among the *Rhizobiales* is VtlR, a virulence-associated transcriptional LysR regulator. The objectives of this dissertation were three fold: 1) characterize the known regulon of VtlR in *B. abortus* with regards to gene regulatory function and virulence, 2) determine the regulon of VtlR in *A. tumefaciens* and define the mechanism by which this regulation occurs, and 3) define the role of an ABC-type transport system indirectly regulated by VtlR in *B. abortus* that putatively imports the non-proteinogenic amino acid gamma-aminobutyric acid (GABA).

VtlR was characterized in *B. abortus* as a virulence-associated transcriptional regulator that directly activates four genes: the sRNA AbcR2, and the three small hypothetical proteins BAB1_0914, BAB2_0512, and BAB2_0574; and deletion of *vtlR* led to a significant defect in the ability of *B. abortus* to cause infection *in vitro* and *in vivo*. Since dysregulation of *abcR2* alone could not account for the defect in virulence, it

was hypothesized that one or all three hypothetical proteins could be responsible for a virulence phenotype observed in Δ*vtlR*. This turned out to not be the case, as a deletion of the entire VtlR regulon displayed no difference in virulence compared to the parental strain. Further characterization of the small hypothetical proteins is outlined in Chapter 2 and the data revealed bona fide translation of each small protein, and the deletion strain of the VtlR regulon displayed a growth defect when grown in the presence of the sugar fucose. This phenotype was subsequently observed in Δ*vtlR* as well. This led to the identification of a putative fucose transport and metabolism locus in *B. abortus* that has yet to be studied.

In *A. tumefaciens*, VtlR is necessary for proper attachment to plant cells and biofilm formation and regulates over 200 genes, significantly more than the four genes VtlR regulates in *B. abortus*. The mechanism by which this occurs was unknown, and the relationship between VtlR and AbcR1 or AbcR2 was uncharacterized. The data in Chapter 3 outline the VtlR network by showing that VtlR regulation of myriad genes in *A. tumefaciens* is primarily indirect via the direct regulation of a few sRNAs. This direct interaction was shown experimentally and a VtlR binding box was identified in the *A. tumefaciens* genome. This project outlines the divergence of a regulatory element between phylogenetically related organisms that occupy different environmental niches.

The AbcR sRNAs are conserved throughout the *Rhizobiales* and regulate numerous ABC-type transport systems within these bacteria. In *A. tumefaciens*, one of these transport systems specifically transports the amino acds proline and GABA. *B. abortus* contains homologs of this system, which led to the hypothesis that the brucellae may also transport GABA but for a yet unknown purpose. The data in Chapter 4 revealed

that *B. abortus* also transports GABA *in vitro* and this transport is under the regulation of AbcR1 and AbcR2. This transport was increased under extreme nutrient limitations and was uninhibited by the presence of other amino acids. Metabolic studies showed GABA is not utilized by *B. abortus* under aerobic conditions, and transcriptomic data revealed increased expression of several loci in the presence of GABA. Altogether, this study uncovers a putative signaling role for the amino acid GABA that has been understudied in bacterial pathogens that infect animal hosts.

Overall, the work presented in this dissertation is focused on further elucidating the biological role of downstream regulatory targets of both VtlR and the sRNAs AbcR1 and AbcR2 in the related organisms *Brucella abortus* and *Agrobacterium tumefaciens*. Findings show that while there are similarities between the two systems, there are also many differences that may be attributed to the vastly different lifestyles of each organism.

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General Audience Abstract

Brucella abortus and *Agrobacterium tumefaciens* are two highly related bacterial pathogens that infect mammals and plants, respectively. Although genetically related, both organisms survive and replicate in vastly different environmental niches with one living in the soil (i.e., *A. tumefaciens*) and the other living within immune cells of the infected host (i.e., *B. abortus*). In Order to quickly adapt to changing environmental conditions, the bacteria must rapidly control gene expression through multiple regulatory mechanisms. The works presented in this dissertation will focus on further characterizing one of these regulatory systems and comparing the homologous systems shared by *B. abortus* and *A. tumefaciens*. This includes uncovering a putative sugar transport and metabolism system, as well as discovering the potential for host-pathogen signaling via the well-studied neurotransmitter GABA.

Dedication

I am dedicating this dissertation to my mother, Kimberly Dalferes. She dedicated her first book to me, so now we are even.

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Table of Contents

List of Figures

List of Tables

Chapter 1: Background and Introduction

The *Alphaproteobacteria* and *Rhizobiales*

The work presented in this dissertation will focus on characterizing genetic systems within two organisms: *Brucella abortus* and *Agrobacterium tumefaciens*. Both of these bacteria belong to the Order *Rhizobiales* within the Class *Alphaproteobacteria*. The organisms found within the Class *Alphaproteobacteria* are very diverse and include pathogens, symbionts, and free living bacteria; plant pathogens and animal pathogens; and organisms with genome sizes that range from 1-10 Megabases (Ettema & Andersson, 2009).

Interestingly, the *Alphaproteobacteria* also have a close relationship to the mitochondrion, a eukaryotic organelle. Since the 1990s, it has been thought that eukaryotes obtained the organelle via an endosymbiotic relationship with a bacterium and scientists have presented evidence suggesting this ancestry (Gray *et al.*, 1999). Further sequence analysis has resulted in several publications designating the mitochondrial ancestry as a lineage of bacteria from the *Alphaproteobacteria* (Wang & Wu, 2015, Fitzpatrick *et al.*, 2006, Thrash *et al.*, 2011). However, now that metagenomic data and sequence analyses have improved, a recent study suggested that mitochondrial origin was not from the *Alphaproteobacteria*, and instead are from a lineage of *Proteobacteria* that diverged prior to the evolution of the *Alphaproteobacteria* (Martijn et al., 2018). Further research is warranted to confirm these results and better understand the phylogenetic origin of mitochondria.

Within the Class *Alphaproteobacteria* is the Order *Rhizobiales*. Many bacteria within the Order *Rhizobiales* interact with host cells, but have varied effects on host physiology. *Sinorhizobium meliloti* and *Bradyrhizobium* spp. are symbionts that can live

within root nodules of the host plants and provide a means to fix nitrogen for the host. *A. tumefaciens*, however, is a pathogen that induces uncontrollable plant cell proliferation, which provides the bacterium with unique nutrient sources for growth. *Brucella* spp. and *Bartonella* spp. are intracellular mammalian pathogens that are responsible for prevalent zoonosis worldwide (Gupta & Mok, 2007). While these organisms have very different relationships with their hosts, they share genetic similarities that organize them within the same Order of life. This dissertation will focus on characterizing regulator systems within *Brucella abortus* and *Agrobacterium tumefaciens*, outlining similarities and differences between these related bacteria.

Brucella spp.

History.

Brucellosis is considered an ancient disease and several medical accounts give indirect evidence of *Brucella* infection throughout history. Hippocrates described brucellosis in his notes in 450 BC, and in the Bible, Luke noted a long-term fever in Paul the Apostle who had visited the Isle of Malta (Ficht, 2009, Akpinar, 2016). More direct evidence of the presence of *Brucella* spp. in close proximity to humans was the identification of *Brucella* specific protein signatures in the oldest known cheese, approximately 3200 years old (Greco *et al.*, 2018).

While brucellosis is an ancient disease, the identification of the causative agent and epidemiological study of the organism came to light during the Crimean War of the mid-1800s. During the war, British soldiers in Crimea presented with a febrile disease that led to incapacitation and sometimes death. Interestingly, one person who traveled to Malta to help injured soldiers was Florence Nightingale, considered the founder of

modern nursing. At some point during her time there, she contracted *Brucella* and became chronically infected (Young, 1995). A group of scientists and doctors were assembled to identify the causative agent of the disease and determine a plan to combat its spread on the island; this group was known as the Mediterranean Fever Commission. The Scottish physician Sir David Bruce identified the causative agent of this fever, when he identified small Gram-negative coccobacilli organisms from the spleen and liver of British soldiers who had succumbed to fever on the Isle of Malta (Akpinar, 2016, Moreno, 2014). In 1906, another member of this group, Themistocles Zammit, identified that goats could be carriers of *Brucella* and that the bacterium could be found in the milk of infected goats (Wyatt, 2005). These results led the group to attribute the prevalence of disease on the Island of Malta to goats on the island that were found to be infected with *Brucella* (Wyatt, 2005). The Commission suggested disallowing the use of unpasteurized milk on the island and canned milk was imported from the U.K. This caused a rapid decreased in infection within military personnel. However, milk vendors on the island were furious and protested this ban (Vassallo, 1992).

In 1914, *Brucella* was isolated from prematurely aborted pigs in the United States and several years later, in 1918, American microbiologist Alice Evans made several ground breaking discoveries in *Brucella* transmission (Akpinar, 2016). One of her biggest contributions to *Brucella* research was that organisms isolated from goats, pigs, and cattle had antigenic differences, however, the organisms isolated from infected hosts were no different in morphological, cultural, or biochemical tests performed. These different organisms were dissimilar in host specificity, but were identical in all other aspects tested; thus she grouped them within the same genus, *Brucella* (Akpinar, 2016). She is

also credited with attributing raw cow milk as a major risk for the spread of human brucellosis and urged the pasteurization of milk to protect human health (Colwell, 1999). Soon after these major discoveries, eradication efforts were pursued in the U.S. and a strict eradication program significantly decreased the spread of human brucellosis.

Eradication of bovine brucellosis in the United States.

While the eradication of disease is an easy concept to accept, it is an extremely difficult and costly task to accomplish. Eradication includes an understanding of the transmission of the disease, creation of a diagnostic measure of disease within a specific location, removal of infected individuals if possible, development of a vaccine and administration to uninfected individuals, and surveillance and continued administration of the vaccine following eradication (Moreno, 2014). Altogether, eradication produces a high financial burden on any state attempting this process. Eradication efforts in the United States began in the 1930s with the launch of the Federal-State Cooperative Brucellosis Eradication program included in the Jones-Connally bill (Wise, 1980). This was a time when a predicted 11% of cattle and 45% of dairy herds contained infected individuals and economical loss of lowered milk production was $400 million in 1956. Unfortunately, the overarching event of World War II slowed progress, but significant strides were made in the eradication of the disease via surveillance and vaccination after the war was over. There was significant pressure on the dairy industry by health boards nationwide that set minimum standards for dairy products to begin implementing policies of only accepting dairy products for human consumption from brucellosis-free herds. This continued and the National Brucellosis Eradication Program still exists today in the United States. The

department of agriculture states that there are less than 10 affected herds remain in the United States today (Service, 2018).

Current prevalence of human brucellosis.

The last major epidemiological study of worldwide brucellosis showed that brucellosis caused over 500,000 newly acquired infections annually, however, this study was conducted in 2006 and over a decade separates this study and the time this dissertation was written (Pappas *et al.*, 2006). As stated earlier, a major route of infection for *Brucella* spp. by non-health related individuals is through the consumption of raw dairy products. Keeping this in mind, Dadar et al. utilized a metadata approach to understand the worldwide prevalence of brucellosis by identifying reported cases of both human brucellosis infection and detection of contaminated dairy products (Dadar *et al.*, 2018). The majority of reported contaminated dairy cases were from Africa, the Middle East, and Southeast Asia and a majority of the human brucellosis cases were also from these regions (Dadar *et al.*, 2018). Few cases were reported from countries with strict control and eradication programs, like the United States and Spain. One major conclusion of the study was that the rates of infection and contamination across the world are highly variable due to different control strategies utilized by different countries. While this analysis was conducted recently and increases understanding on the spread of brucellosis via contaminated dairy products worldwide, a broader epidemiological study needs to be performed to understand human brucellosis via all routes of infection.

Recently, there have been several confirmed cases of human brucellosis in the United States. Each case was attributed to the consumption of raw dairy products and the infectious agent was the live attenuated *Brucella abortus* bovine vaccine strain RB51

(Cossaboom *et al.*, 2018, Agriculture, 2018, CDC, 2017). The most recent potential exposure in the U.S. was attributed to milk from a farm in Pennsylvania that included 19 states and hundreds of potential exposures to contaminated milk products (Network, 2019). These cases are of interest because they highlight a major health concern to the growing support for the "raw food movement" (Salzberg, 2016).

Hosts and disease presentation.

In animal hosts, brucellosis primarily affects the reproductive tract, causing abortions in pregnant females and sterility of males, and chronic infection can lead to complications with the liver and spleen (von Bargen *et al.*, 2012). In humans, however, brucellosis initially presents as flu-like symptoms, including fever, fatigue, anorexia, etc. in infected individuals (de Figueiredo *et al.*, 2015). Chronic infection in humans can cause increased damage to organs throughout the body, leading to arthritis, orchitis, endocarditis, and neurological issues (Dean *et al.*, 2012).

Brucella spp. can infect a variety of hosts and *Brucella* spp. have been described as biovars, named for the host species they most commonly infect in nature. There are currently 13 species of *Brucella* referenced throughout the literature, including: *melitensis, suis, abortus, ovis, canis, neotomae, microti, inopinata, papionis, vulpis, ceti*, and *pinnipedialis*. While several of these spp. have the capacity to infect more than one species of host, they most commonly infect goats and sheep, swine, cattle, sheep, dogs, desert rats, voles, humans, baboons, red foxes, and marine mammals respectively (Hull & Schumaker, 2018). There is an additional yet to be named species of *Brucella* isolated from amphibians (Al Dahouk *et al.*, 2017). While each species is commonly found in a

specific host in nature, many *Brucella* spp. can also infect other host spp. including humans.

In fact, brucellosis is one of the most common zoonoses worldwide, but not all species of *Brucella* are zoonotic (Pappas *et al.*, 2006). There is extensive evidence to show that *B. melitensis* (goats and sheep), *B. abortus* (cattle), *B. canis* (dogs), and *B. suis* (swine) are zoonotic and cause infection in humans (Moreno, 2014). However, recent insights into human brucellosis show that marine mammal-associated species (*B. ceti* and *B. pinnipidialis*) and *B. neotomae* (desert rat) have zoonotic potential as well, but cases are rare (Whatmore *et al.*, 2008, Suarez-Esquivel *et al.*, 2017).

While most of the current *Brucella* species are associated with domesticated animals, wildlife populations can also become infected with *Brucella*. This presents a challenge to eradication as many of these wildlife populations are protected by law to decrease extinction rates, but also spread brucellosis via contact with uninfected domesticated populations. Examples of potential wildlife carriers of *Brucella* are bison and elk of North America and the ibex of Europe (Rhyan *et al.*, 2013, Mick *et al.*, 2014). The challenge is managing the spread of brucellosis from wildlife populations to domesticated animal populations by either limiting interactions between these groups or by vaccination of wildlife animals, if a suitable vaccine is available.

Pathogenesis.

There are several routes of infection for *Brucella* spp. to enter the host. In animals, *Brucella* can be transmitted sexually through the reproductive tract as well as by coming into contact with contaminated materials, such as an aborted fetus (Moreno, 2014). It is uncommon for *Brucella* to be transmitted sexually in humans and documentation of this

is very limited (Tuon *et al.*, 2017). The most common routes for human infection are inhalation of aerosolized particles and ingestion of contaminated meat or dairy products (Godfroid, 2017). However, contaminated animal products used for human consumption that undergo sterilization processes, such as cooking and pasteurization, do not pose a significant threat to human health. Medical professionals are also at risk of becoming infected and brucellosis is still a frequently reported laboratory acquired infection. Exposure happens by direct contact with mucous membranes, and needle stick injection while working with *Brucella* spp. in a laboratory setting or by occupational accident via lack of awareness of contaminated samples in a medical environment (Coelho & Garcia Diez, 2015).

Upon entering the host, *Brucella* spp. enter cells via two different processes. The first includes being opsonized and phagocytized by professional phagocytes (M cells, macrophages, and neutrophils) and another includes invasion of cells via interactions between the O-chain of lipopolysaccharide (LPS) on the bacterial surface and lipid rafts on the surface of host cells (Porte *et al.*, 2003).

While much of the biomedical research reported focuses on host-pathogen interactions in these cell types, the ability of *Brucella* spp. to infect a wide variety of non-phagocytic cell types has been shown *in vitro*, including HeLa cells, fibroblast Vero cells, and brain microvascular endothelial cells (Pizarro-Cerda *et al.*, 1998, Detilleux *et al.*, 1990, Miraglia *et al.*, 2018).

Once internalized, *Brucella* spp. trafficking can be separated into three different phases: endosomal, replicative, and autophagy-related (Celli, 2015). Early trafficking has been shown to be highly similar to the normal endocytic pathway with the foreign

bacterium encompassed within a membrane-bound compartment. This vacuole is termed the endosomal *Brucella* containing vacuole (eBCV). Once the endosome matures, a fusion event is initiated between the endosome and the lysosome within 12 hours of endocytosis (Starr *et al.*, 2008). Upon fusion, the vacuole undergoes acidification to a pH of ~4-4.5, which induces the expression of a type IV secretion system. Effector proteins are then delivered into the host cell to modulate maturation of the eBCV to the replicative *Brucella* containing vacuole (rBCV) and influence trafficking of the vacuole toward the endoplasmic reticulum of the cell (Kohler *et al.*, 2003). Although *Brucella* infection causes a significant zoonosis worldwide, following infection, less than 10% of the organisms will subvert this fusion event and over 90% will be killed (Kohler *et al.*, 2003).

Brucellae that effectively mature to the rBCV are subsequently trafficked to the endoplasmic reticulum of the cell where components of this organelle will actually fuse with the membrane of the rBCV. Until this point, bacterial division is postponed, but bacterial division and replication will commence upon maturation of the eBCV to the rBCV (Deghelt *et al.*, 2014).

Egress of *Brucella* and cell-cell transmission is less understood but recent advances provide insight into this process. Approximately 48 hours post-infection and after replication has occurred, the rBCV will undergo engulfment by autophagosomal-like structures. Therefore, *Brucella* autophagosomal interactions are currently the most compelling evidence for autophagosomal formation as a means of cell-cell spread by *Brucella* (Starr *et al.*, 2012).

Virulence Factors.

As stated before, *Brucella* spp. are primarily stealth pathogens that persist during infection via evasion of host immunity and *Brucella* do not express "Classic" virulence factors, such as capsule, motility, exotoxins, plasmids, or antigenic variation. However, several virulence factors have been well characterized with regards to *Brucella* pathogenesis and virulence, specifically in the ability to adapt to the harsh intracellular environment where the cells will encounter reactive oxygen and nitrogen species, low pH, nutrient deprivation, anaerobic conditions, and exposure to lytic peptides from fusion of the lysosome with the endosome (i.e., the eBCV) (Roop *et al.*, 2009). These virulence factors include a type IV secretion system which plays a significant role in maturation of the BCV, two component regulatory systems, quorum sensing, urease, superoxide dismutase, and small regulatory RNAs (He, 2012, Roop *et al.*, 2009).

Moreover, several virulence factors characterized in *Brucella* spp. have unique characteristics not observed by many other organisms. Two specific virulence factors with unique and less understood functions in *Brucella* spp. are flagellum synthesis and quorum sensing. *Brucella* spp., with the exception of the new amphibian species, are non-motile; however, transmission electron microscopy revealed the existence of a sheathed flagellum protruding from *Brucella melitensis* (Fretin et al., 2005). Even more astounding is the fact that expression of flagellum components was induced during infection and deletion of these genes led to attenuation of *B. melitensis* in a mouse model of infection. This is perplexing and a question has risen as to why *B. melitensis* and perhaps other *Brucella* spp. would expend the energy to produce this appendage.

Quorum sensing, a means of cell-cell communication among bacteria via small molecule signals, in *Brucella* is also unique in function when compared to other bacteria

(Camilli & Bassler, 2006). In 2002, Taminiau et al. identified the small communication molecule produced by *B. melitensis* and it was identified as C12-HSL, a homoserine lactone (Taminiau *et al.*, 2002). An interesting observation was that *Brucella* spp. produced a lower concentration of C12-HSL when grown *in vitro* compared to other bacteria. An interesting hypothesis for this is that *Brucella* are enclosed within their own BCV throughout intracellular trafficking and low levels of signaling molecule may be for "self-sensing" of the intracellular vacuolar environment. Instead of utilizing quorum sensing to communicate with a large population of bacteria, *Brucella* may utilize quorum sensing to communicate with itself in the confined space of the BCV (Taminiau *et al.*, 2002).

Vaccines and Therapeutics.

The intracellular lifestyle of *Brucella* spp. presents a challenge for treatment, and current treatment includes the long-term use of antibiotics for a matter of weeks. The World Health Organization recommends the use of doxycycline, a ribosome inhibitor, and rifampin, an RNA polymerase inhibitor, for six weeks (Khan & Zahoor, 2018). This treatment can be problematic since the vaccine strain RB51, a bovine vaccine that is pathogenic to humans, is resistant to rifampin. Animals are not commonly treated for infection and are generally quarantined from uninfected animals and/or euthanized.

There are several vaccines currently used on domesticated animals to combat *Brucella* infection including two widely used vaccines for bovine brucellosis, RB51 and S19. There is currently no human vaccine, but without transmission from human to human and transmission being zoonotic in nature, a focus has been to develop successful animal vaccines. S19 is a live attenuated strain that was isolated from cow milk in the

1920s and was used to start mass vaccination in the U.S. in the 1940s eradication efforts (Dorneles *et al.*, 2015). RB51 is a rough, missing the O-chain of the LPS, strain of *B. abortus* isolated in the 1980s and it is widely used today. Both S19 and RB51 are pathogenic to humans so animal workers must use precaution while administering these vaccines (Dorneles *et al.*, 2015).

Agrobacterium tumefaciens

History.

Interestingly, the causative agent of crown gall in plants, *Agrobacterium tumefaciens*, was isolated around the same time *Brucella* was isolated from soldiers on the Island of Malta in the early 1900s. It is disputed, however, who first isolated *Agrobacterium tumefaciens* as the causative agent of crown gall disease. Fridiano Cavara published a description of the disease from grapevines in Italy and isolated a bacterium that caused similar disease in young grapevines in 1887; George Hedgcock also isolated *A. tumefaciens* from grapevine galls as a part of a USDA study in 1904 (Kado, 2014); and in 1907, C.O. Townsend and Erwin F. Smith also reported isolating the organism from plant tumors. However, Townsend and Smith are usually credited with being the first to isolate *A. tumefaciens* (Smith & Townsend, 1907).

Regardless of who isolated *A. tumefaciens* first from crown gall, there was an outpouring of research following this discovery to then elucidate the mechanism by which *A. tumefaciens* caused these tumors. The next major breakthrough came in the mid-1900s when several studies identified the presence of phytohormones (auxins) that caused tumor growth and showed that tumor growth was due to genetic changes in the eukaryotic cell. First, several publications were released showing that the addition of auxins to plants in the absence of *Agrobacterium* produced highly similar tumor formation, and the addition of these compounds could rescue virulence of an attenuated strain of *A. tumefaciens*. These results lead to the hypothesis that auxins were the causative molecules driving tumor formation. Several studies published by Braun and White showed that secondary tumors, derived from the initial tumor cell, were devoid of

bacteria and that these tumors grew well in the absence of added phytohormones (White & Braun, 1942). These data indicated that these cells were genetically altered to sustain tumor growth in the absence of the bacterium. Another indication of genetic alteration was the overabundance of opines produced by plants. Opines are a unique source of carbon and nitrogen utilized by specific microbes; and while they are produced in trace amounts in tobacco and other plants, they are overproduced in *Agrobacterium* infected plants (Kado, 2014), indirectly indicating genetic manipulation of the host cell.

In the 1970s, there was a strong focus on answering how bacterial DNA was transported and incorporated into the host. This was identified in 1974, when Zaenen et al. described the presence of a large plasmid isolated solely from virulent strains of *Agrobacterium* and loss of this plasmid converted these strains to an avirulent state (Zaenen *et al.*, 1974). The presence of the plasmid was confirmed by detection of the plasmid DNA in crown gall cells themselves, giving direct evidence of host genetic manipulation (Chilton *et al.*, 1977).

Once identification of the virulence-associated extrachromosomal element, later termed the Ti plasmid, occurred, *Agrobacterium* research focused on characterizing the genetic elements of this plasmid. The genetic elements of this plasmid will be discussed later but one major element is the T-DNA that was shown to be incorporated directly into the host genome. Biomedical research was changed forever in the discovery that tumor-inducing genes could be removed from the T-DNA region and replaced with foreign DNA, and this newly assembled T-DNA had no effect on the ability of the region to be transferred into the plant cell (Caplan *et al.*, 1983, Fraley *et al.*, 1985).

Biomedical applications.

Due to the natural ability of *A. tumefaciens* to transform eukaryotic cells, the bacterium itself has been transformed from a pathogen and pest to a unique biomedical tool. After transformation in plants was shown, the ability of *A. tumefaciens* to transform other organisms, including fungi, bacteria, algae, and several other eukaryotic cell lines, was also discovered (Hwang *et al.*, 2017). A major advantage to the utilization of transgenic, genetically modified, plants is the possibility to introduce genes that give better crop yield and confer resistance to threats of plant health, like pests or climate, and could significantly improve human health in affected areas (Valentine, 2003).

Hosts and disease presentation.

Infected plants can present growth tumors on their trunks or roots called crown galls, which can be caused by several different species of *Agrobacterium* (Cleene, 1976). These galls can be physically taxing on the plant and can cause disintegration and wilt, although, several plant species are asymptomatic. Treatment for crown gall includes removal of the infected plant and separation from healthy plants to prevent spread of the bacterium.

Crown gall is commonly found on plants belonging to the families *Rosaceae* (rose), *Vitaceae* (grape), and *Juglandaceae* (walnut), as well as woody plants like stone fruit trees (Kado, 2014). However, a wide variety of plant spp. are susceptible to infection via bacteria within the genus *Agrobacterium*, including over 400 plant species (Pulawska, 2010). There is evidence, however, suggesting host specificity by different strains of *Agrobacterium* is linked to the Ti plasmid (Loper & Kado, 1979, Thomashow *et al.*,

1980), and a specific strain of *A. tumefaciens* pathogenic to one plant, may have no effect on a different plant (Pulawska, 2010).

Pathogenesis.

In the rhizosphere, the area directly surrounding the plant's roots, plants exude amino acids, sugars, proteins, polysaccharides and other metabolites into the environment (Brencic & Winans, 2005). Numerous microorganisms in this environment can utilize these metabolites. *A. tumefaciens* will sense some of these metabolites, an example of a chemoattractant, and trigger the bacterium to swim towards this root, a process known as chemotaxis (Subramoni *et al.*, 2014, Brencic & Winans, 2005). These root exudates will also signal proximity to the host and induce expression of genes for attachment to the host.

One major finding in *A. tumefaciens* pathogenesis was that unlike *Brucella* spp., *A. tumefaciens* is not an intracellular organism and instead relies on attachment to host cells during pathogenesis (Kado, 2014). Upon sensing of chemoattractants, *A. tumefaciens* will express several factors to attach to the host surface. One of these mechanisms includes the secretion of cyclic glucans that potentially act as adhesins between the microbe and host polysaccharides or lectins (Cangelosi *et al.*, 1989, Brencic & Winans, 2005). A second mechanism of attachment is via the synthesis of cellulose by *A. tumefaciens* that allows for the formation of bacterial clusters on the surface of the plant cell (Matthysse, 1983).

The presence of phenolic compounds, sugars, and low pH in the rhizosphere will induce the expression of *vir* genes on the Ti plasmid (Stachel *et al.*, 1986, Brencic & Winans, 2005, Subramoni *et al.*, 2014). As mentioned above, the Ti plasmid may play an

important role in host specificity, but the Ti plasmid also contains the T-DNA, which will be incorporated into the host genome as well as genes for the successful conjugation of the T-DNA. There are 6 *vir* operons on the Ti plasmid, each with a distinct function in the regulation (*virA*), processing (*virC*), transport (*virB*) via a type IV secretion system, and incorporation (*virD* and *virE*) of the T-DNA into the plant genome (Zupan *et al.*, 2000).

T-DNA contains genes encoding for phytohormone and opine synthesis and these genes are induced once incorporated into the plant genome. As mentioned previously, phytohormones are messenger molecules that can induce cell growth, in this case, causing increased cell proliferation into tumors (2010). Opines are a form of carbon and nitrogen that can be catabolized by a subset of microbes, including *Agrobacterium* spp. Secretion of opines by infected plant cells provides an advantage for *Agrobacterium* spp. in nature over other organisms that cannot catabolize these metabolites. Genes responsible for opine catabolism are also located on the Ti plasmid. The importance of the Ti plasmid in *A. tumefaciens* virulence cannot be understated. Interestingly, an opine transport system located on this plasmid is under the regulation of the highly conserved sRNA AbcR1, described in chapter 3.

AbcR1/AbcR2

AbcR1 and AbcR2 are two small regulatory RNAs that are found throughout the *Rhizobiales* (Sheehan & Caswell, 2018). Small regulatory RNAs (sRNAs) are transcripts of 50-500 nucleotides in length and regulate gene expression by interactions with either target mRNA or target proteins, leading to the activation or repression of target gene expression (Waters & Storz, 2009). In *A. tumefaciens*, AbcR1and AbcR2 are genetically

and structurally similar, however, AbcR1 is more significant in gene regulation than AbcR2 (Wilms *et al.*, 2011). Proteomic analysis revealed that AbcR1 primarily regulates the expression of ABC transport systems; this is further established via transcriptomic data described in Chapter 3 of this dissertation (Overloper *et al.*, 2014).

Contradictory to *A. tumefaciens*, AbcR1 and AbcR2 are functionally redundant sRNAs in *B. abortus* and regulate the same subset of genes (Caswell *et al.*, 2014, Sheehan & Caswell, 2017). Both AbcR1 and AbcR2 also contribute to *B. abortus* virulence equally and an isogenic deletion of *abcR1* or *abcR2* has no effect on virulence, while a strain containing a deletion of both *abcR1* and *abcR2* is attenuated in a macrophage and mouse model of infection compared to the parental strain (Caswell *et al.*, 2012). Proteomic and transcriptomic analysis revealed that a majority of the proteins and transcripts dysregulated in a *abcR1* and *abcR2* deletion strain are ABC-type transporters, similar to those found in *A. tumefaciens* (Caswell *et al.*, 2012). One of these ABC-type transport systems will be the focus of Chapter 4 in this dissertation.

While the regulatory function of AbcR1 and AbcR2 has been described previously in *B. abortus* and *A. tumefaciens*, it was not understood how the expression of *abcR1* and *abcR2* were regulated in these bacteria until recently. Our group has shown that *abcR2* is directly regulated in *B. abortus* by the transcriptional regulator VtlR (Sheehan *et al.*, 2015).

VtlR

VtlR is a LysR-type transcriptional regulator that is highly conserved among the Order *Rhizobiales* and LysR-type transcriptional regulators are the most common type of transcriptional regulator in prokaryotes (Maddocks & Oyston, 2008). In *Sinorhizobium*

meliloti, a plant symbiont, *smc01225* encodes a LysR-type transcriptional regulator homologous to VtlR that has been shown to be necessary for proper nodulation of the host and is named LsrB for LysR-type symbiosis regulator (Luo *et al.*, 2005). While the full regulon of LsrB in *S. meliloti* is unknown, several genes have been shown to be regulated by LsrB. Genes regulated by LsrB include those involved in glutathione biosynthesis, lipopolysaccharide biosynthesis, and an oxidative stress response regulator (Lu *et al.*, 2013, Tang *et al.*, 2014). Interestingly, the function of LsrB homologs in *B. abortus* and *A. tumefaciens* are drastically different from *S. meliloti*.

Sheehan et al. described the function of VtlR, an ortholog of LsrB, in *B. abortus* as a transcriptional regulator necessary for wild-type virulence of *B. abortus* in mice. VtlR binds directly to the promoters and activates the transcription of four genes; *abcR2*, *bab1_0914*, *bab2_0512*, and *bab2_0574* (Sheehan *et al.*, 2015). The expression of AbcR1 was unchanged in the absence of *vtlR.* Thus, the regulation of *abcR1* is still unknown in *B. abortus.* Deletion of either *abcR1* or *abcR2* had no effect the virulence of *B. abortus* because AbcR1 and AbcR2 are functionally redundant, thus the dysregulation of *abcR2* in a *vtlR* deletion strain could not account for the decrease in virulence in a *vtlR* deletion strain. This led the authors to hypothesize that *bab1_0914*, *bab2_0512*, and/or *bab2_0574* could contribute to virulence and the absence of their expression in the *vtlR* deletion strain accounts for the decrease in virulence observed in the *vtlR* deletion strain. The function and role in virulence of *bab1_0914*, *bab2_0512*, and *bab2_0574* will be addressed in Chapter 2 of this dissertation (Figure 1.1).

VtlR has recently been phenotypically characterized in *A. tumefaciens* (Tang *et al.*, 2018). *In vitro*, *A. tumefaciens*::Δ*vtlR* displayed decreased exopolysaccharide and

biofilm production and this deletion strain was more sensitive to oxidative stress and iron toxicity. *In planta*, deletion of *vtlR* in *A. tumefaciens* led to decreased attachment and transformation to *Arabidopsis* roots and decreased infiltration of tobacco leaves (Tang *et al.*, 2018). Dissimilar from what was shown in *B. abortus*, dysregulation of gene expression in *A. tumefaciens*::Δ*vtlR* is robust with over 400 genes dysregulated in this strain. An interesting aspect of this study was their ability to show heterologous complementation of *lsrB*/*vtlR* between *S. meliloti*, *B. abortus*, and *A. tumefaciens*. In-trans complementation of *vtlR* from *B. abortus* or *A. tumefaciens* was able to restore *in planta* phenotypes observed in by *S. meliloti::*Δ*lsrB*. They were also able to show that in-trans complementation of *vtlR* or *lsrB* from *B. abortus* or *S. meliloti*, respectively, was able to restore the phenotypes of *A. tumefaciens*::Δ*vtlR* adding to the hypothesis that these regulators serve similar functions among the three related organisms (Tang *et al.*, 2018).

Chapter 3 will focus on characterizing the molecular mechanism of VtlR regulation in *A. tumefaciens* (Figure 1.1).

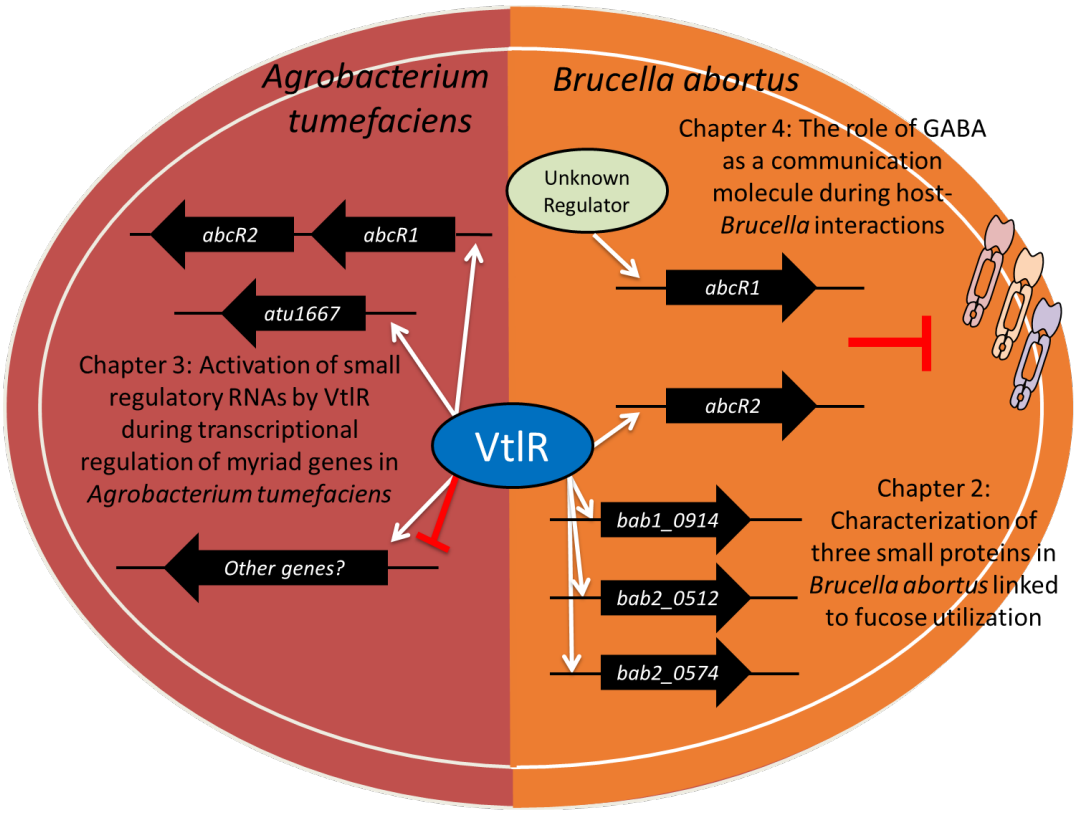

Figure 1.1: Overview of dissertation chapters.

Small Proteins

Chapter 2 will describe the function of three small hypothetical proteins regulated by VtlR in *B. abortus*. The term "small protein" is universal for any protein with an open reading frame less than 100 codons and does not undergo post-translational processing; and small proteins are found in all taxons of life. In bacteria, small proteins have been shown to influence the function of kinases, transport systems, cell division, sporulation, signaling, etc. (Hobbs *et al.*, 2011, Storz *et al.*, 2014). However, determining the function of a small protein can be tedious because most previously described small proteins have no enzymatic function and instead act as chaperones for larger proteins and genetic systems. Thus, bacterial phenotypes resulting from the deletion of small proteins can be subtle if observable at all. Identifying small proteins can be difficult due to genome annotation limitations. GenBank, the sequence database of the National Institutes of

Health, will not accept sequence submissions less than 200 nucleotides in length, which corresponds to a protein of about 66 amino acids. While this cut off discounts numerous small proteins proven to be translated, it may serve as a failsafe from overloading the database. An article published in 1997 sought to illustrate the magnitude of small open reading frames within genomes. They calculated that there are ~260,000 open reading frames with lengths between 2 and 99 codons in *Saccharomyces cerevisiae* (Basrai et al., 1997). Determining which open reading frames encode translated small proteins presents a primary challenge to small protein research. A very successful, but tedious method includes epitope tagging each open reading frame and performing western blot analysis for the expression of the small protein (Hemm *et al.*, 2010). A limitation observed in this method and in our own studies is isolating the small proteins under optimal bacterial growth conditions, which can vary greatly from protein to protein.

Several computational methods have been developed to predict small proteins within eukaryotes and prokaryotes, but similarly to sRNA prediction software, they often overestimate the number of predicted proteins and further verification is needed to confirm the existence of these small hypothetical proteins (Friedman *et al.*, 2017, Yang *et al.*, 2011).

Another challenge to studying small proteins is differentiating the function of the small protein from the small transcript (sRNA) also encoded by the gene. Several characterized sRNAs have later been proven to encode a small protein as well as a functional sRNA (Raina *et al.*, 2018). In fact, Friedman et al. predicted that about 10% of all bacterial sRNAs are also translated to small proteins (Friedman *et al.*, 2017).

The small proteins characterized in this dissertation are putatively involved in metabolism and/or transport of a sugar, and there are several examples of small proteins throughout bacteria that influence transport and metabolism.

AcrZ.

AcrZ is a 49 amino acid protein that associates with a major multidrug efflux pump, AcrAB-TolC in *E. coli*, and is conserved among several enterobacteria (Hobbs *et al.*, 2012). Deletion of *acrZ* led to bacterial sensitivity to a multitude of antimicrobial compounds known to be substrates of the AcrAB-TolC efflux pump. Interestingly, a deletion of *acrZ* was only sensitive to about half of the same substrates as an *acrB* deletion strain leading the authors to hypothesize that *acrZ* could be responsible for substrate specificity or *acrZ* interacts with other mechanisms that contribute to the function of AcrAB-TolC (Hobbs *et al.*, 2012).

SgrT.

SgrT is an example of a well-studied small protein encoded by a gene that encodes both a functional small regulatory RNA, SgrS, and a small protein, SgrT. Interestingly, both SgrT and SgrS function as a means to limit glucose transport in *Escherichia coli* under the stressful condition of high glucose phosphate concentrations (Raina & Storz, 2017). The sRNA SgrS was initially studied in *E. coli* and shown to negatively regulate *ptsG* mRNA concentration, which encodes a glucose transporter. PtsG transcript levels were diminished in the presence of SgrS and increased in the absence of SgrS expression (Vanderpool & Gottesman, 2004). Also, overexpression of SgrS rescued growth of *E. coli* in the presence of a high non-metabolized phosphosugar concentrations, adding to the model that SgrS limits glucose transport in environments with high glucose

concentrations. Since this initial study, it has been shown that SgrS regulates multiple genetic systems in *E. coli* and has a more global role in the regulation of PTS genes (Rice & Vanderpool, 2011, Bobrovskyy & Vanderpool, 2014).

Several years later, Wadler et al. identified an open reading frame (ORF) located within the 5' end of SgrS. This ORF encodes a 43 amino acid protein that showed similar stress phenotypes to those observed in Δ*sgrS*, but careful analysis revealed that these phenotypes are attributed to both SgrS and SgrT independently (Wadler & Vanderpool, 2007). Lloyd et al. further characterized SgrT function by showing that SgrT solely exhibits inhibition of PtsG and not ManXYZ, both of which are under the regulation of SgrS (Lloyd *et al.*, 2017). While there has been limited direct evidence to show direct binding of SgrT to $EIICB^{Glc}$, indirect evidence by Lloyd et al. and Kosfeld et al. supports the hypothesis that SgrT mediated regulation is through direct interactions with $EIICB^{Glc}$ (Kosfeld & Jahreis, 2012, Lloyd *et al.*, 2017).

Small Proteins in *Brucella* spp.

There are few publications that directly characterize "small proteins" in *Brucella* spp. but the findings present some interesting insights into the importance of small proteins in *Brucella* pathogenesis. One of the first studies to elucidate the function of small proteins in *Brucella* was conducted by making targeted deletions of small proteins (<15 kDa) that were conserved among *B. abortus* 2308, *B. suis* 1330, and *B. melitensis* 16M that had no predicted function and were not present in *Agrobacterium tumefaciens* C58 (Sun *et al.*, 2012). Of the 30 putative small proteins targeted, three mutants were less fit to infect the macrophage-like cell line J774, and four mutants were less fit to infect the animal model of brucellosis (including the three shown to have decreased virulence in a cell line). The

authors focused on characterizing one of these small proteins, CydX, and showed that this small protein contributes to the function of cytochrome bd oxidase (Sun *et al.*, 2012).

Another small hypothetical protein necessary for the virulence of *B. abortus* is VhpA (Tian *et al.*, 2018). Tian et al. observed that deletion of *vhpA* decreased the ability of the bacterium to cause infection in a mouse model, but had no effect on bactericidal effects or adhesion, invasion, and survival *in vitro*. Remarkably, almost 400 genes were dysregulated in the *vhpA* deletion strain when compared to the parental strain (Tian *et al.*, 2018). This phenotype is more indicative of a small RNA and more experiments are necessary to validate the translation of the small protein. These two studies encompass the current literature on small proteins in *Brucella* spp. and warrants further investigation into studying the role of other small proteins in *Brucella* biology.

γ-aminobutyric acid and bacterial pathogenesis

Chapter 4 in this dissertation focuses on characterizing γ–amino butyric acid (GABA) transport in *B. abortus,* which is shown to be regulated by AbcR1 and AbcR2. While GABA is notorious as the most abundant inhibitory neurotransmitter in the adult human brain, this molecule serves many physiological functions in animals, plants, and bacteria.

The role of GABA in plant development and immunity.

There are numerous studies characterizing the role of GABA as a neurotransmitter in vertebrates, but this molecule also plays an important role in metabolism, developmental growth, and immunology in plants (Palanivelu *et al.*, 2003, Bown & Shelp, 2016). GABA is a four carbon amino acid that can be metabolized to succinate and then fed into the TCA cycle for energy production, a process known as the GABA shunt. This shunt is well conserved among eukaryotes and prokaryotes. GABA can feed into this shunt

through the import of exogenous GABA or by the conversion of GABA from glutamate via the glutamate decarboxylase system (GAD) (Shelp *et al.*, 1999). The GAD system is an important method for both eukaryotic and prokaryotic cells to increase the pH of the intracellular environment. To do this, glutamate is transported into the cytoplasm via the antiporter GadC, where GadB decarboxylates glutamate to GABA with the addition of a proton. The GABA and proton are then exported out of the cell via GadC, which increases the pH of the bacterial cytoplasm (De Biase & Pennacchietti, 2012). With regards to plant development, it has been shown that an overactive GAD system leads to high concentrations of intracellular GABA and significant growth defects of the plant (Baum *et al.*, 1996).

Increased GABA concentrations can also defend the plant from damage by insects, fungi, and bacteria. Biotic and physical stress can lead to damaged plant cells and release of attractants that could attract more biotic threats to the plant. However, when this stress occurs, the intracellular environment becomes acidified, resulting in an increase in the activity of the GAD system, leading to an increase in GABA concentrations around the damaged plant cell (Shelp *et al.*, 1999). In insect larva, an increase in GABA in the larva diet led to significant decreases in growth rate and survival rates of the larvae, and similar effects have been shown in plant associated bacteria (Ramputh & Bown, 1996).

In bacteria, there has been extensive work to describe the negative effects of GABA on *Agrobacterium* spp. and *Pseudomonas* spp. virulence in plants. *P. syringae* is a bacterial pathogen of plants and has been used as a model of plant-microbe interactions since the 1980s due to the bacterium's ability to infect both the natural host, tomato, and

common laboratory model *Arabidopsis* (Xin & He, 2013). *P. syringae* can transport exogenous GABA through the permease GabP. GABA is transported and metabolized in *P. syringae* to be assimilated into the TCA cycle as discussed above, and a deletion of *gabP* led to a growth defect when GABA is provided as a carbon source (McCraw *et al.*, 2016). Deletion of *gabP* had no effect on the virulence of *P. syringae*; however, deletion of the GABA transaminase did have an effect on the virulence of *P. syringae*. A mutant of the GABA transaminase in *P. syringae* led to increased concentrations of intracellular GABA, which was shown to decrease the expression of the type-III secretion system, a virulence factor in *P. syringae* (Park *et al.*, 2010). This mutation and subsequent deregulation of the type-III secretion system accounted for significant differences in infection *in planta* (Park *et al.*, 2010).

GABA transport is partially regulated by AbcR1 in *Agrobacterium tumefaciens*. The *A. tumefaciens* genome contains two transport systems responsible for the transport of GABA, and one of these has been shown to be negatively regulated by AbcR1. In the absence of *abcR1*, ^{14}C-GABA uptake was significantly increased in *A. tumefaciens* (Wilms *et al.*, 2011). Transport of GABA leads to increased expression of the lactonase AttM, which in turn degrades and decreases the concentration of OC8-HSL, a quorum sensing molecule, in *A. tumefaciens* (Chevrot *et al.*, 2006). This is done when exogenous plant-derived GABA is transported via one of the two ABC-type transport systems shown to import GABA in *A. tumefaciens*, GABA is then catabolized to succinate via the GABA shunt as previously stated, and the presence of GABA and its byproducts succinate and succinic semialdehyde induces the expression of the *attKLM* operon, part of which encodes the lactonase AttM (Wang *et al.*, 2006). This increased expression of the

lactonase AttM leads to decreased quorum sensing molecules and decreased expression of virulence related genes. This quorum quenching pathway has been shown to decrease the susceptibly of tobacco plants with increased levels of GABA (via GAD overexpression) to *A. tumefaciens* infection (Chevrot *et al.*, 2006). Interestingly, it has been shown that *A. tumefaciens* will preferentially transport other small chain amino acids and proline over the transport of GABA and that the presence of proline can alleviate the level of quorum quenching discussed above (Haudecoeur *et al.*, 2009, Planamente *et al.*, 2010), although this mechanism is not well understood.

Nonaka et al. showed that T-DNA transferred from *A. tumefaciens* to a tomato plant was increased in an engineered tomato plant that expressed low GABA concentrations by decreasing the expression of the GAD system in the plant. Conversely, a strain of *A. tumefaciens* with increased GABA transaminase activity, and which will degrade GABA at a faster rate than the parental strain, had increased T-DNA transfer and transformation rates in a tomato plant (Nonaka *et al.*, 2017).

Over time though, both *Pseudomonas* spp. and *Agrobacterium* spp. have retained these GABA import systems, despite their negative effects on pathogenesis. This suggests that these systems play important role in bacterial physiology that is not yet well understood.

Human immunity.

GABA has more recently been shown to be an immunomodulator in animals, but this is much less understood than the role of GABA in plant immunity. In 2010, Bhat et al. published an article describing the ability of immune cells to synthesize and catabolize GABA. They showed that both macrophages, DCs, and T cells had the ability to secrete

GABA and treatment with inhibitory GABAergic agents led to decreased concentrations of inflammatory cytokines due to changes in the MAPK signaling pathway (Bhat *et al.*, 2010). This finding led the authors to hypothesize that GABA may be utilized by immune cells as a means of cell-cell communication during periods of inflammation throughout disease.

In 2018, Kim et al. potentially linked this new finding with bacterial infection in immune cells. They showed that autophagy related GABAergic signaling by macrophages led to increased killing of the intracellular bacterial pathogens *Mycobacterium* (Mtb), *Salmonella*, and *Listeria* (Kim *et al.*, 2018). During Mtb infection, they showed that GABA-treated macrophages produced greater fusion of lysosomes and Mtb containing phagosomes, leading the authors to attribute enhanced phagosomal maturation with activation of GABAergic signaling. Conversely, by inhibiting GABAergic signaling, they were able to show that the bacterial load of *M. marinum*, *S. typhimurium*, and *L. monocytogenes*, were increased *in vivo* (Kim et al., 2018).

Studies outlining the effect of GABA in animal immunity are very limited but these studies provide prominent preliminary evidence that GABA signaling is important for immune signaling and response to bacterial infection.

GABA in bacterial physiology.

GABA is an important molecule for bacterial physiology with numerous studies supporting GABA as an important molecule for acid response. Gut microbiota must navigate an acidic environment and several enteric organisms utilize the glutamate decarboxylase (GAD) system to counteract the harmful effects of low pH. Again, the GAD system functions by converting glutamate to GABA with an attached proton, then

transporting GABA out of the cell, in turn increasing the pH of the intracellular environment (De Biase & Pennacchietti, 2012). Interestingly, the Classical species of *Brucella* do not contain a functional GAD system due to genetic mutations, including *B. abortus*, *B. suis*, and *B. melitensis*; but several of the newer species have been shown to utilize this system to manage the acidic environment of the gut (Damiano *et al.*, 2015, Pennacchietti *et al.*, 2018). As for *Helicobacter pylori*, *Brucella* spp. have been shown to utilize urease in Order to passage through the gut during oral infection (Bandara *et al.*, 2007, Sangari *et al.*, 2007).

Studies in *Pseudomonas* spp., a pathogen of both plants and animals, have revealed another less well understood role for GABA in bacterial physiology; enhancement of virulence. In *P. aeruginosa*, pre-exposure to GABA led to an increase in cytotoxicity of *P. aeruginosa* on glial cells and increased virulence in a *C. elegans* model of killing (Dagorn *et al.*, 2013b). *P. aeruginosa* exposed to GABA also showed a decrease in biofilm formation, increase in HCN (non-peptide toxin) production, differences in protein expression, and increased production of the quorum sensing molecule 3oxoC12-HSL. While the mechanisms by which GABA increases virulence of *P. aeruginosa* is still not well understood and the differences above were limited in significance, this study is one of the first to show that GABA can escalate virulence in a bacterium, when most studies in plant immunity show GABA decreases the virulence of a bacterium.

This effect was shown in another species of *Pseudomonas*, *P. fluorescens*, by the same group. *P. fluorescens*, a plant symbiont and rare human pathogen, showed a decrease in cytotoxicity in glial cells; however, very few physiological differences are

observed when *P. fluorescens* was exposed to GABA (Dagorn *et al.*, 2013a). Another significant change in *P. fluorescens* exposed to GABA was identified in LPS structure, specifically on lipid A. It is still unknown why GABA affects the structure of LPS, but the authors hypothesize that this difference can explain the difference in cytotoxicity observed in vitro (Dagorn *et al.*, 2013a). *P. fluorescens* has been shown to be a plant symbiont, but the authors did not examine the effect of GABA on this symbiosis.

The ability of GABA to enhance virulence of *Pseudomonas* spp. on eukaryotic cells but decreased virulence of bacterial plant pathogens presents a paradox. The effect of GABA on the animal pathogen *B. abortus* will be explored in chapter 4 and this paradox of the effect of GABA between animal- and plant-associated pathogens will be further discussed.

References

(2010) Introduction to Phytohormones. *The Plant Cell* **22**: tpc.110.tt0310.

Agriculture, P.D.o., (2018) Department of Agriculture Advises Consumers to Discard Unpasteurized Dairy from Lancaster County Farm, Quarantines Raw Cow's Milk Products. In., pp.

Akpinar, O., (2016) Historical perspective of brucellosis: a microbiological and epidemiological overview. *Infez Med* **24**: 77-86.

Al Dahouk, S., S. Kohler, A. Occhialini, M.P. Jimenez de Bagues, J.A. Hammerl, T. Eisenberg, G. Vergnaud, A. Cloeckaert, M.S. Zygmunt, A.M. Whatmore, F. Melzer, K.P. Drees, J.T. Foster, A.R. Wattam & H.C. Scholz, (2017) *Brucella* spp. of amphibians comprise genomically diverse motile strains competent for replication in macrophages and survival in mammalian hosts. *Sci Rep* **7**: 44420.

Bandara, A.B., A. Contreras, A. Contreras-Rodriguez, A.M. Martins, V. Dobrean, S. Poff-Reichow, P. Rajasekaran, N. Sriranganathan, G.G. Schurig & S.M. Boyle, (2007) *Brucella suis* urease encoded by *ure*1 but not *ure*2 is necessary for intestinal infection of BALB/c mice. *BMC Microbiol* **7**: 57.

Basrai, M.A., P. Hieter & J.D. Boeke, (1997) Small open reading frames: beautiful needles in the haystack. *Genome Res* **7**: 768-771.

Baum, G., S. Lev-Yadun, Y. Fridmann, T. Arazi, H. Katsnelson, M. Zik & H. Fromm, (1996) Calmodulin binding to glutamate decarboxylase is required for regulation of glutamate and GABA metabolism and normal development in plants. *EMBO J* **15**: 2988-2996.

Bhat, R., R. Axtell, A. Mitra, M. Miranda, C. Lock, R.W. Tsien & L. Steinman, (2010) Inhibitory role for GABA in autoimmune inflammation. *Proc Natl Acad Sci U S A* **107**: 2580-2585.

Bobrovskyy, M. & C.K. Vanderpool, (2014) The small RNA SgrS: roles in metabolism and pathogenesis of enteric bacteria. *Front Cell Infect Microbiol* **4**: 61.

Bown, A.W. & B.J. Shelp, (2016) Plant GABA: Not Just a Metabolite. *Trends Plant Sci* **21**: 811-813.

Brencic, A. & S.C. Winans, (2005) Detection of and response to signals involved in host-microbe interactions by plant-associated bacteria. *Microbiol Mol Biol Rev* **69**: 155-194.

Camilli, A. & B.L. Bassler, (2006) Bacterial small-molecule signaling pathways. *Science* **311**: 1113-1116.

Cangelosi, G.A., G. Martinetti, J.A. Leigh, C.C. Lee, C. Thienes & E.W. Nester, (1989) Role for [corrected] *Agrobacterium tumefaciens* ChvA protein in export of beta-1,2-glucan. *J Bacteriol* **171**: 1609-1615.

Caplan, A., L. Herrera-Estrella, D. Inze, E. Van Haute, M. Van Montagu, J. Schell & P. Zambryski, (1983) Introduction of genetic material into plant cells. *Science* **222**: 815-821.

Caswell, C.C., J.M. Gaines, P. Ciborowski, D. Smith, C.H. Borchers, C.M. Roux, K. Sayood, P.M. Dunman & R.M. Roop Ii, (2012) Identification of two small regulatory RNAs linked to virulence in *Brucella abortus* 2308. *Mol Microbiol* **85**: 345-360.

Caswell, C.C., A.G. Oglesby-Sherrouse & E.R. Murphy, (2014) Sibling rivalry: related bacterial small RNAs and their redundant and non-redundant roles. *Front Cell Infect Microbiol* **4**: 151.

CDC, (2017) People in Four States May Be Drinking Contaminated Raw Milk. In: CDC and partners urge families at risk to see their doctor for antibiotics. pp.

Celli, J., (2015) The changing nature of the *Brucella*-containing vacuole. *Cell Microbiol* **17**: 951-958.

Chevrot, R., R. Rosen, E. Haudecoeur, A. Cirou, B.J. Shelp, E. Ron & D. Faure, (2006) GABA controls the level of quorum-sensing signal in *Agrobacterium tumefaciens*. *Proc Natl Acad Sci U S A* **103**: 7460-7464.

Chilton, M.D., M.H. Drummond, D.J. Merio, D. Sciaky, A.L. Montoya, M.P. Gordon & E.W. Nester, (1977) Stable incorporation of plasmid DNA into higher plant cells: the molecular basis of crown gall tumorigenesis. *Cell* **11**: 263-271.

Cleene, J.D.L.a.M.D., (1976) The host range of crown gall. *The Botanical Review* **42**: 389-466.

Coelho, A.C. & J. Garcia Diez, (2015) Biological Risks and Laboratory-Acquired Infections: A Reality That Cannot be Ignored in Health Biotechnology. *Front Bioeng Biotechnol* **3**: 56.

Colwell, R.R., (1999) Alice C. Evans: breaking barriers. *Yale J Biol Med* **72**: 349-356.

Cossaboom, C.M., G.A. Kharod, J.S. Salzer, R.V. Tiller, L.P. Campbell, K. Wu, M.E. Negron, N. Ayala, N. Evert, J. Radowicz, J. Shuford & S. Stonecipher, (2018) Notes from the Field: *Brucella abortus* Vaccine Strain RB51 Infection and Exposures Associated with Raw Milk Consumption - Wise County, Texas, 2017. *MMWR Morb Mortal Wkly Rep* **67**: 286.

Dadar, M., Y. Shahali & A.M. Whatmore, (2018) Human brucellosis caused by raw dairy products: A review on the occurrence, major risk factors and prevention. *Int J Food Microbiol* **292**: 39-47.

Dagorn, A., A. Chapalain, L. Mijouin, M. Hillion, C. Duclairoir-Poc, S. Chevalier, L. Taupin, N. Orange & M.G. Feuilloley, (2013a) Effect of GABA, a bacterial metabolite, on *Pseudomonas fluorescens* surface properties and cytotoxicity. *Int J Mol Sci* **14**: 12186-12204.

Dagorn, A., M. Hillion, A. Chapalain, O. Lesouhaitier, C. Duclairoir Poc, J. Vieillard, S. Chevalier, L. Taupin, F. Le Derf & M.G. Feuilloley, (2013b) Gamma-aminobutyric acid acts as a specific virulence regulator in *Pseudomonas aeruginosa*. *Microbiology* **159**: 339-351.

Damiano, M.A., D. Bastianelli, S. Al Dahouk, S. Kohler, A. Cloeckaert, D. De Biase & A. Occhialini, (2015) Glutamate decarboxylase-dependent acid resistance in *Brucella* spp.: distribution and contribution to fitness under extremely acidic conditions. *Appl Environ Microbiol* **81**: 578-586.

De Biase, D. & E. Pennacchietti, (2012) Glutamate decarboxylase-dependent acid resistance in orally acquired bacteria: function, distribution and biomedical implications of the *gadBC* operon. *Mol Microbiol* **86**: 770-786.

de Figueiredo, P., T.A. Ficht, A. Rice-Ficht, C.A. Rossetti & L.G. Adams, (2015) Pathogenesis and immunobiology of brucellosis: review of *Brucella*-host interactions. *Am J Pathol* **185**: 1505-1517.

Dean, A.S., L. Crump, H. Greter, J. Hattendorf, E. Schelling & J. Zinsstag, (2012) Clinical manifestations of human brucellosis: a systematic review and meta-analysis. *PLoS Negl Trop Dis* **6**: e1929.

Deghelt, M., C. Mullier, J.F. Sternon, N. Francis, G. Laloux, D. Dotreppe, C. Van der Henst, C. Jacobs-Wagner, J.J. Letesson & X. De Bolle, (2014) G1-arrested newborn cells are the predominant infectious form of the pathogen *Brucella abortus*. *Nat Commun* **5**: 4366.

Detilleux, P.G., B.L. Deyoe & N.F. Cheville, (1990) Penetration and intracellular growth of *Brucella abortus* in nonphagocytic cells in vitro. *Infect Immun* **58**: 2320-2328.

Dorneles, E.M., N. Sriranganathan & A.P. Lage, (2015) Recent advances in *Brucella abortus* vaccines. *Vet Res* **46**: 76.

Ettema, T.J. & S.G. Andersson, (2009) The alpha-proteobacteria: the Darwin finches of the bacterial world. *Biol Lett* **5**: 429-432.

Ficht, T.A.a.A., L.G., (2009) Brucellosis. In: Vaccines for Biodefense and Emerging and Neglected Diseases. A.D.T.a.S. Barrett, L.R. (ed). Elsevier Ltd., pp. 807-829.

Fitzpatrick, D.A., C.J. Creevey & J.O. McInerney, (2006) Genome phylogenies indicate a meaningful alpha-proteobacterial phylogeny and support a grouping of the mitochondria with the *Rickettsiales*. *Mol Biol Evol* **23**: 74-85.

Fraley, R.T., S.G. Rogers, R.B. Horsch, D.A. Eichholtz, J.S. Flick, C.L. Fink, N.L. Hoffmann & P.R. Sanders, (1985) The SEV System: A New Disarmed Ti Plasmid Vector System for Plant Transformation. *Bio/Technology* **3**: 629-635.

Fretin, D., A. Fauconnier, S. Kohler, S. Halling, S. Leonard, C. Nijskens, J. Ferooz, P. Lestrate, R.M. Delrue, I. Danese, J. Vandenhaute, A. Tibor, X. DeBolle & J.J. Letesson, (2005) The sheathed flagellum of *Brucella melitensis* is involved in persistence in a murine model of infection. *Cell Microbiol* **7**: 687-698.

Friedman, R.C., S. Kalkhof, O. Doppelt-Azeroual, S.A. Mueller, M. Chovancova, M. von Bergen & B. Schwikowski, (2017) Common and phylogenetically widespread coding for peptides by bacterial small RNAs. *BMC Genomics* **18**: 553.

Godfroid, J., (2017) Brucellosis in livestock and wildlife: zoonotic diseases without pandemic potential in need of innovative one health approaches. *Arch Public Health* **75**: 34.

Gray, M.W., G. Burger & B.F. Lang, (1999) Mitochondrial evolution. *Science* **283**: 1476-1481.

Greco, E., O. El-Aguizy, M.F. Ali, S. Foti, V. Cunsolo, R. Saletti & E. Ciliberto, (2018) Proteomic Analyses on an Ancient Egyptian Cheese and Biomolecular Evidence of Brucellosis. *Anal Chem* **90**: 9673-9676.

Gupta, R.S. & A. Mok, (2007) Phylogenomics and signature proteins for the alpha proteobacteria and its main groups. *BMC Microbiol* **7**: 106.

Haudecoeur, E., S. Planamente, A. Cirou, M. Tannieres, B.J. Shelp, S. Morera & D. Faure, (2009) Proline antagonizes GABA-induced quenching of quorum-sensing in *Agrobacterium tumefaciens*. *Proc Natl Acad Sci U S A* **106**: 14587-14592.

He, Y., (2012) Analyses of *Brucella* pathogenesis, host immunity, and vaccine targets using systems biology and bioinformatics. *Front Cell Infect Microbiol* **2**: 2.

Hemm, M.R., B.J. Paul, J. Miranda-Rios, A. Zhang, N. Soltanzad & G. Storz, (2010) Small stress response proteins in *Escherichia coli*: proteins missed by Classical proteomic studies. *J Bacteriol* **192**: 46-58.

Hobbs, E.C., F. Fontaine, X. Yin & G. Storz, (2011) An expanding universe of small proteins. *Curr Opin Microbiol* **14**: 167-173.

Hobbs, E.C., X. Yin, B.J. Paul, J.L. Astarita & G. Storz, (2012) Conserved small protein associates with the multidrug efflux pump AcrB and differentially affects antibiotic resistance. *Proc Natl Acad Sci U S A* **109**: 16696-16701.

Hull, N.C. & B.A. Schumaker, (2018) Comparisons of brucellosis between human and veterinary medicine. *Infect Ecol Epidemiol* **8**: 1500846.

Hwang, H.-H., M. Yu & E.-M. Lai, (2017) *Agrobacterium-Mediated Plant Transformation: Biology and Applications*. SPIE.

Kado, C.I., (2014) Historical account on gaining insights on the mechanism of crown gall tumorigenesis induced by *Agrobacterium tumefaciens*. *Front Microbiol* **5**: 340.

Khan, M.Z. & M. Zahoor, (2018) An Overview of Brucellosis in Cattle and Humans, and its Serological and Molecular Diagnosis in Control Strategies. *Trop Med Infect Dis* **3**.

Kim, J.K., Y.S. Kim, H.M. Lee, H.S. Jin, C. Neupane, S. Kim, S.H. Lee, J.J. Min, M. Sasai, J.H. Jeong, S.K. Choe, J.M. Kim, M. Yamamoto, H.E. Choy, J.B. Park & E.K. Jo, (2018) GABAergic signaling linked to autophagy enhances host protection against intracellular bacterial infections. *Nat Commun* **9**: 4184.

Kohler, S., S. Michaux-Charachon, F. Porte, M. Ramuz & J.P. Liautard, (2003) What is the nature of the replicative niche of a stealthy bug named *Brucella*? *Trends Microbiol* **11**: 215-219.

Kosfeld, A. & K. Jahreis, (2012) Characterization of the Interaction Between the Small Regulatory Peptide SgrT and the EIICBGlc of the Glucose-Phosphotransferase System of *E. coli* K-12. *Metabolites* **2**: 756-774.

Lloyd, C.R., S. Park, J. Fei & C.K. Vanderpool, (2017) The Small Protein SgrT Controls Transport Activity of the Glucose-Specific Phosphotransferase System. *J Bacteriol* **199**.

Loper, J.E. & C.I. Kado, (1979) Host range conferred by the virulence-specifying plasmid of *Agrobacterium tumefaciens*. *J Bacteriol* **139**: 591-596.

Lu, D., G. Tang, D. Wang & L. Luo, (2013) The *Sinorhizobium meliloti* LysR family transcriptional factor LsrB is involved in regulation of glutathione biosynthesis. *Acta Biochim Biophys Sin (Shanghai)* **45**: 882-888.

Luo, L., S.Y. Yao, A. Becker, S. Ruberg, G.Q. Yu, J.B. Zhu & H.P. Cheng, (2005) Two new *Sinorhizobium meliloti* LysR-type transcriptional regulators required for nodulation. *J Bacteriol* **187**: 4562-4572.

Maddocks, S.E. & P.C. Oyston, (2008) Structure and function of the LysR-type transcriptional regulator (LTTR) family proteins. *Microbiology* **154**: 3609-3623.

Martijn, J., J. Vosseberg, L. Guy, P. Offre & T.J.G. Ettema, (2018) Deep mitochondrial origin outside the sampled alphaproteobacteria. *Nature* **557**: 101-105.

Matthysse, A.G., (1983) Role of bacterial cellulose fibrils in *Agrobacterium tumefaciens* infection. *J Bacteriol* **154**: 906-915.

McCraw, S.L., D.H. Park, R. Jones, M.A. Bentley, A. Rico, R.G. Ratcliffe, N.J. Kruger, A. Collmer & G.M. Preston, (2016) GABA (gamma-Aminobutyric Acid) Uptake Via the GABA Permease GabP Represses Virulence Gene Expression in *Pseudomonas syringae* pv. tomato DC3000. *Mol Plant Microbe Interact* **29**: 938-949.

Mick, V., G. Le Carrou, Y. Corde, Y. Game, M. Jay & B. Garin-Bastuji, (2014) *Brucella melitensis* in France: persistence in wildlife and probable spillover from Alpine ibex to domestic animals. *PloS one* **9**: e94168.

Miraglia, M.C., A.M. Rodriguez, P. Barrionuevo, J. Rodriguez, K.S. Kim, V.A. Dennis, M.V. Delpino & G.H. Giambartolomei, (2018) *Brucella abortus* Traverses Brain Microvascular Endothelial Cells Using Infected Monocytes as a Trojan Horse. *Front Cell Infect Microbiol* **8**: 200.

Moreno, E., (2014) Retrospective and prospective perspectives on zoonotic brucellosis. *Front Microbiol* **5**: 213.

Network, C.H.A., (2019) Third Case of Rifampin/Penicillin-Resistant Strain of RB51 *Brucella* from Consuming Raw Milk. In. Emergency.cdc.gov: CDC, pp.

Nonaka, S., T. Someya, S. Zhou, M. Takayama, K. Nakamura & H. Ezura, (2017) An *Agrobacterium tumefaciens* Strain with Gamma-Aminobutyric Acid Transaminase Activity Shows an Enhanced Genetic Transformation Ability in Plants. *Sci Rep* **7**: 42649.

Overloper, A., A. Kraus, R. Gurski, P.R. Wright, J. Georg, W.R. Hess & F. Narberhaus, (2014) Two separate modules of the conserved regulatory RNA AbcR1 address multiple target mRNAs in and outside of the translation initiation region. *RNA Biol* **11**: 624-640.

Palanivelu, R., L. Brass, A.F. Edlund & D. Preuss, (2003) Pollen tube growth and guidance is regulated by POP2, an *Arabidopsis* gene that controls GABA levels. *Cell* **114**: 47-59.

Pappas, G., P. Papadimitriou, N. Akritidis, L. Christou & E.V. Tsianos, (2006) The new global map of human brucellosis. *Lancet Infect Dis* **6**: 91-99.

Park, D.H., R. Mirabella, P.A. Bronstein, G.M. Preston, M.A. Haring, C.K. Lim, A. Collmer & R.C. Schuurink, (2010) Mutations in gamma-aminobutyric acid (GABA) transaminase genes in plants or *Pseudomonas syringae* reduce bacterial virulence. *Plant J* **64**: 318-330.

Pennacchietti, E., C. D'Alonzo, L. Freddi, A. Occhialini & D. De Biase, (2018) The Glutaminase-Dependent Acid Resistance System: Qualitative and Quantitative Assays and Analysis of Its Distribution in Enteric Bacteria. *Frontiers in microbiology* **9**: 2869.

Pizarro-Cerda, J., S. Meresse, R.G. Parton, G. van der Goot, A. Sola-Landa, I. Lopez-Goni, E. Moreno & J.P. Gorvel, (1998) *Brucella abortus* transits through the autophagic pathway and replicates in the endoplasmic reticulum of nonprofessional phagocytes. *Infect Immun* **66**: 5711-5724.

Planamente, S., A. Vigouroux, S. Mondy, M. Nicaise, D. Faure & S. Morera, (2010) A conserved mechanism of GABA binding and antagonism is revealed by structure-function analysis of the periplasmic binding protein Atu2422 in *Agrobacterium tumefaciens*. *J Biol Chem* **285**: 30294-30303.

Porte, F., A. Naroeni, S. Ouahrani-Bettache & J.P. Liautard, (2003) Role of the *Brucella suis* lipopolysaccharide O antigen in phagosomal genesis and in inhibition of phagosome-lysosome fusion in murine macrophages. *Infection and immunity* **71**: 1481-1490.

Pulawska, J., (2010) Crown Gall of Stone Fruits and Nuts, Economic Significance and Diversity of its Causal Agents: Tumorigenic *Agrobacterium* spp. *Journal of Plant Pathology* **92**.

Raina, M., A. King, C. Bianco & C.K. Vanderpool, (2018) Dual-Function RNAs. *Microbiol Spectr* **6**.

Raina, M. & G. Storz, (2017) SgrT, a Small Protein That Packs a Sweet Punch. *J Bacteriol* **199**.

Ramputh, A.I. & A.W. Bown, (1996) Rapid [gamma]-Aminobutyric Acid Synthesis and the Inhibition of the Growth and Development of Oblique-Banded Leaf-Roller Larvae. *Plant Physiol* **111**: 1349-1352.

Rhyan, J.C., P. Nol, C. Quance, A. Gertonson, J. Belfrage, L. Harris, K. Straka & S. Robbe-Austerman, (2013) Transmission of brucellosis from elk to cattle and bison, Greater Yellowstone area, U.S.A., 2002-2012. *Emerging infectious diseases* **19**: 1992-1995.

Rice, J.B. & C.K. Vanderpool, (2011) The small RNA SgrS controls sugar-phosphate accumulation by regulating multiple PTS genes. *Nucleic Acids Res* **39**: 3806-3819.

Roop, R.M., 2nd, J.M. Gaines, E.S. Anderson, C.C. Caswell & D.W. Martin, (2009) Survival of the fittest: how *Brucella* strains adapt to their intracellular niche in the host. *Med Microbiol Immunol* **198**: 221-238.

Salzberg, S., (2016) The Problem With The Raw Food Movement. In. forbes.com: Forbes, pp.

Sangari, F.J., A. Seoane, M.C. Rodriguez, J. Aguero & J.M. Garcia Lobo, (2007) Characterization of the urease operon of *Brucella abortus* and assessment of its role in virulence of the bacterium. *Infect Immun* **75**: 774-780.

Service, U.S.D.o.A.A.a.P.H.I., (2018) National Brucellosis Eradication Program. In., pp.

Sheehan, L.M., J.A. Budnick, C. Blanchard, P.M. Dunman & C.C. Caswell, (2015) A LysR-family transcriptional regulator required for virulence in *Brucella abortus* is highly conserved among the alpha-proteobacteria. *Mol Microbiol* **98**: 318-328.

Sheehan, L.M. & C.C. Caswell, (2017) A 6-Nucleotide Regulatory Motif within the AbcR Small RNAs of *Brucella abortus* Mediates Host-Pathogen Interactions. *MBio* **8**.

Sheehan, L.M. & C.C. Caswell, (2018) An account of evolutionary specialization: the AbcR small RNAs in the *Rhizobiales*. *Mol Microbiol* **107**: 24-33.

Shelp, B.J., A.W. Bown & M.D. McLean, (1999) Metabolism and functions of gamma-aminobutyric acid. *Trends Plant Sci* **4**: 446-452.

Smith, E.F. & C.O. Townsend, (1907) A Plant-Tumor of Bacterial Origin. *Science* **25**: 671-673.

Stachel, S.E., E.W. Nester & P.C. Zambryski, (1986) A plant cell factor induces *Agrobacterium tumefaciens* vir gene expression. *Proc Natl Acad Sci U S A* **83**: 379-383.

Starr, T., R. Child, T.D. Wehrly, B. Hansen, S. Hwang, C. Lopez-Otin, H.W. Virgin & J. Celli, (2012) Selective subversion of autophagy complexes facilitates completion of the *Brucella* intracellular cycle. *Cell Host Microbe* **11**: 33-45.

Starr, T., T.W. Ng, T.D. Wehrly, L.A. Knodler & J. Celli, (2008) *Brucella* intracellular replication requires trafficking through the late endosomal/lysosomal compartment. *Traffic* **9**: 678-694.

Storz, G., Y.I. Wolf & K.S. Ramamurthi, (2014) Small proteins can no longer be ignored. *Annu Rev Biochem* **83**: 753-777.

Suarez-Esquivel, M., N. Ruiz-Villalobos, C. Jimenez-Rojas, E. Barquero-Calvo, C. Chacon-Diaz, E. Viquez-Ruiz, N. Rojas-Campos, K.S. Baker, G. Oviedo-Sanchez, E. Amuy, E. Chaves-Olarte, N.R. Thomson, E. Moreno & C. Guzman-Verri, (2017) *Brucella neotomae* Infection in Humans, Costa Rica. *Emerg Infect Dis* **23**: 997-1000.

Subramoni, S., N. Nathoo, E. Klimov & Z.C. Yuan, (2014) *Agrobacterium tumefaciens* responses to plant-derived signaling molecules. *Front Plant Sci* **5**: 322.

Sun, Y.H., M.F. de Jong, A.B. den Hartigh, C.M. Roux, H.G. Rolan & R.M. Tsolis, (2012) The small protein CydX is required for function of cytochrome bd oxidase in *Brucella abortus*. *Front Cell Infect Microbiol* **2**: 47.

Taminiau, B., M. Daykin, S. Swift, M.L. Boschiroli, A. Tibor, P. Lestrate, X. De Bolle, D. O'Callaghan, P. Williams & J.J. Letesson, (2002) Identification of a quorum-sensing signal molecule in the facultative intracellular pathogen *Brucella melitensis*. *Infection and immunity* **70**: 3004-3011.

Tang, G., Q. Li, S. Xing, N. Li, Z. Tang, L. Yu, J. Yan, X. Li & L. Luo, (2018) The LsrB protein is required for *Agrobacterium tumefaciens* interaction with host plants. *Molecular plant-microbe interactions : MPMI*.

Tang, G., Y. Wang & L. Luo, (2014) Transcriptional regulator LsrB of *Sinorhizobium meliloti* positively regulates the expression of genes involved in lipopolysaccharide biosynthesis. *Applied and environmental microbiology* **80**: 5265-5273.

Thomashow, M.F., C.G. Panagopoulos, M.P. Gordon & E.W. Nester, (1980) Host range of *Agrobacterium tumefaciens* is determined by the Ti plasmid. *Nature* **283**: 794-796.

Thrash, J.C., A. Boyd, M.J. Huggett, J. Grote, P. Carini, R.J. Yoder, B. Robbertse, J.W. Spatafora, M.S. Rappe & S.J. Giovannoni, (2011) Phylogenomic evidence for a common ancestor of mitochondria and the SAR11 clade. *Scientific reports* **1**: 13.

Tian, M., Z. Lian, Y. Bao, S. Bao, Y. Yin, P. Li, C. Ding, S. Wang, T. Li, J. Qi, X. Wang & S. Yu, (2018) Identification of a novel, small, conserved hypothetical protein involved in *Brucella abortus* virulence by modifying the expression of multiple genes. *Transbound Emerg Dis*.

Tuon, F.F., R.B. Gondolfo & N. Cerchiari, (2017) Human-to-human transmission of *Brucella* - a systematic review. *Trop Med Int Health* **22**: 539-546.

Valentine, L., (2003) *Agrobacterium tumefaciens* and the plant: the David and Goliath of modern genetics. *Plant Physiol* **133**: 948-955.

Vanderpool, C.K. & S. Gottesman, (2004) Involvement of a novel transcriptional activator and small RNA in post-transcriptional regulation of the glucose phosphoenolpyruvate phosphotransferase system. *Mol Microbiol* **54**: 1076-1089.

Vassallo, D.J., (1992) The corps disease: brucellosis and its historical association with the Royal Army Medical Corps. *J R Army Med Corps* **138**: 140-150.

von Bargen, K., J.P. Gorvel & S.P. Salcedo, (2012) Internal affairs: investigating the *Brucella* intracellular lifestyle. *FEMS Microbiol Rev* **36**: 533-562.

Wadler, C.S. & C.K. Vanderpool, (2007) A dual function for a bacterial small RNA: SgrS performs base pairing-dependent regulation and encodes a functional polypeptide. *Proc Natl Acad Sci U S A* **104**: 20454-20459.

Wang, C., H.B. Zhang, L.H. Wang & L.H. Zhang, (2006) Succinic semialdehyde couples stress response to quorum-sensing signal decay in *Agrobacterium tumefaciens*. *Mol Microbiol* **62**: 45-56.

Wang, Z. & M. Wu, (2015) An integrated phylogenomic approach toward pinpointing the origin of mitochondria. *Scientific reports* **5**: 7949.

Waters, L.S. & G. Storz, (2009) Regulatory RNAs in bacteria. *Cell* **136**: 615-628.

Whatmore, A.M., C.E. Dawson, P. Groussaud, M.S. Koylass, A.C. King, S.J. Shankster, A.H. Sohn, W.S. Probert & W.L. McDonald, (2008) Marine mammal *Brucella* genotype associated with zoonotic infection. *Emerg Infect Dis* **14**: 517-518.

White, P.R. & A.C. Braun, (1942) A Cancerous Neoplasm of Plants. Autonomous Bacteria-Free Crown-Gall Tissue. *Cancer Research* **2**: 597-617.

Wilms, I., B. Voss, W.R. Hess, L.I. Leichert & F. Narberhaus, (2011) Small RNA-mediated control of the *Agrobacterium tumefaciens* GABA binding protein. *Mol Microbiol* **80**: 492-506.

Wise, R.I., (1980) Brucellosis in the United States. Past, present, and future. *JAMA* **244**: 2318-2322.

Wyatt, H.V., (2005) How Themistocles Zammit found Malta Fever (brucellosis) to be transmitted by the milk of goats. *J R Soc Med* **98**: 451-454.

Xin, X.F. & S.Y. He, (2013) *Pseudomonas syringae* pv. tomato DC3000: a model pathogen for probing disease susceptibility and hormone signaling in plants. *Annu Rev Phytopathol* **51**: 473-498.

Yang, X., T.J. Tschaplinski, G.B. Hurst, S. Jawdy, P.E. Abraham, P.K. Lankford, R.M. Adams, M.B. Shah, R.L. Hettich, E. Lindquist, U.C. Kalluri, L.E. Gunter, C. Pennacchio & G.A. Tuskan, (2011) Discovery and annotation of small proteins using genomics, proteomics, and computational approaches. *Genome Res* **21**: 634-641.

Young, D.A., (1995) Florence Nightingale's fever. *BMJ* **311**: 1697-1700.

Zaenen, I., N. Van Larebeke, M. Van Montagu & J. Schell, (1974) Supercoiled circular DNA in crown-gall inducing *Agrobacterium* strains. *J Mol Biol* **86**: 109-127.

Zupan, J., T.R. Muth, O. Draper & P. Zambryski, (2000) The transfer of DNA from *agrobacterium tumefaciens* into plants: a feast of fundamental insights. *Plant J* **23**: 11-28.

Chapter 2: Characterization of three small proteins in *Brucella abortus* linked to fucose utilization

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Abstract

Elucidating the function of proteins less 50 amino acids in length is no small task. Nevertheless, small proteins can play vital roles in the lifestyle of bacteria and influence the virulence of pathogens; thus, the investigation of the small proteome is warranted. Recently our group identified the *Brucella abortus* protein VtlR as a transcriptional activator of four genes, one of which is the well-studied small regulatory RNA, AbcR2, while the other three genes encode hypothetical small proteins, two of which are highly conserved among the Order *Rhizobiales*. This study provides evidence that all three genes encode authentic small proteins and all three are highly expressed under oxidative stress, low pH, and stationary phase growth conditions. Fractionation of the cells revealed that the proteins are localized to the membranes of *B. abortus*. We demonstrate that the small proteins under the transcriptional control of VtlR are not accountable for attenuation observed with the *B. abortus vtlR* deletion strain. However, there is an association between VtlR-regulated genes and growth inhibition in the presence of the sugar L-fucose. Subsequent transcriptomic analyses revealed that *B. abortus* initiates the transcription of a locus encoding a putative sugar transport and utilization system when the bacteria are cultured in the presence of L-fucose. Altogether, our observations characterize the role of the VtlR-controlled small proteins BAB1_0914, BAB2_0512, and BAB2_0574 in the biology of *B. abortus*, particularly in the capacity of the bacteria to utilize L-fucose.

Importance

Despite being one of the most common zoonoses worldwide, there is currently no human vaccine to combat brucellosis. Therefore, a better understanding of the pathogenesis and biology of *Brucella* spp., the causative agent of brucellosis, is essential for the discovery of novel therapeutics against these highly infectious bacteria. In this study, we further characterize the virulence-associated transcriptional regulator VtlR in *Brucella abortus*. Our findings not only shed light on our current understanding of a virulence related genetic system in *Brucella*, but also increase our knowledge of small proteins in the field of bacteriology.

Introduction

"Small protein" is an unrestricting term encompassing any protein that is less than 50 amino acids in length without post-translational processing (Storz *et al.*, 2014). Generally considered too small to contribute to the physiology and biology of bacteria, small proteins are often left unannotated; and without optimized protocols to investigate small proteins, the study of small proteins has been neglected (Ramamurthi & Storz, 2014). This is not completely unwarranted due to the large number of open reading frames ranging from 2-99 codons in prokaryotic and eukaryotic genomes. It was calculated that the number of open reading frames between 2 and 99 codons exceeded 250,000 in *Saccharomyces cerevisiae* (Basrai *et al.*, 1997). Several studies have been published exploring the ability to identify small proteins in both eukaryotes and bacteria (Basrai *et al.*, 1997, Friedman *et al.*, 2017). This presents the problem of distinguishing between genes that encode functional small proteins and those that are meaningless. However, in the last decade, several small proteins have been well characterized in bacteria, and small proteins have been characterized for their various roles in sporulation, cell division, oxidative stress response, and as regulators of other proteins (Cutting *et al.*, 1997, Sun *et al.*, 2012, Bisson-Filho *et al.*, 2015, Lippa & Goulian, 2009).

To date, only a handful of studies have described the role of small proteins found in *Brucella* spp., which are Gram-negative intracellular bacterial pathogens that are members of the *Alphaproteobacteria* Class of bacteria. Each species preferentially infects a specific host; however, several *Brucella* strains adapted the ability to infect other hosts, including humans (Atluri *et al.*, 2011). Infection leads to spontaneous abortions and infertility in cows and swine, while causing a debilitating relapsing fever in humans (de

Figueiredo *et al.*, 2015). Upon infection, *Brucella* spp. traffic through human macrophages and dendritic cells to form an intracellular replicative niche (von Bargen *et al.*, 2012). Regarding small proteins in *Brucella*, one of the most robust studies was performed when Sun et al. characterized whether 30 putative small proteins in *Brucella abortus* were important for the colonization of J774 macrophage-like cells or infection of a mouse model. Their findings indicated that four of the identified small proteins were important for either the infection of J774 macrophage-like cells or mice (Sun *et al.*, 2012). The authors went on to characterize one of these small proteins, CydX, as a contributor to the function of cytochrome bd oxidase, but did not elucidate the mechanistic roles of the other small proteins. While microbiologists have historically overlooked small proteins, this study is a prime example of the potentially large value they bring to microbial physiology.

Recently, our group described the role of VtlR, a LysR-Type Transcriptional Regulator (LTTR), in the virulence of *B. abortus* 2308 (Sheehan *et al.*, 2015). We showed that VtlR is required for the ability of *B. abortus* to 1) survive and replicate in naïve peritoneal macrophages, and 2) colonize the spleens of BALB/c mice. Microarray and northern blot analyses revealed that VtlR transcriptionally activates four genes; *abcR2*, *bab1_0914 (bab_rs20300)*, *bab2_0512 (bab_rs28790)* and *bab2_0574 (bab_rs29075)* (Sheehan *et al.*, 2015). The small regulatory RNA (sRNA) AbcR2 has a sibling sRNA, AbcR1, that has redundant regulatory functions to that of AbcR2, and a deletion of one *abcR* gene does not cause a decrease in the ability of *B. abortus* to colonize the spleens of C57BL/6 mice (Caswell *et al.*, 2012a). Since VtlR only activates transcription of *abcR2*, and not *abcR1*, we hypothesized that a lack of expression of

bab1_0914, *bab2_0512* and/or *bab2_0574* in a Δ*vtlR* strain of *B. abortus* was accountable for the attenuation of the *B. abortus* Δ*vtlR* strain in macrophages and mice. The data in the present study does not support this hypothesis since isogenic deletions or deletion of all three genes encoding small proteins does not lead to attenuation of *B. abortus* in a BALB/c mouse model of infection. Despite this, we were able to further characterize the localization and the expression profiles of the three small proteins. Moreover, we also observed an interesting phenotype involving the ability of *B. abortus* to utilize L-fucose during growth and sensitivity to this sugar when *vtlR* is deleted from the *Brucella* chromosome. Altogether, this study describes three novel small proteins in *B. abortus*, and furthermore characterizes the link between these small proteins and the utilization of L-fucose by *Brucella*.

Results

BAB1_0914 and BAB2_0512 are well conserved among the Order *Rhizobiales* and are highly similar in amino acid sequence.

bab1_0914 and *bab2_0512* encode small hypothetical proteins of unknown function, and these putative proteins are predicted to be 48 amino acids in length (Figure 2.1). *bab1_0914* is flanked by *bab1_0915*, which encodes a hypothetical protein, and by the methyltransferase-encoding gene *bab1_0913*. *bab2_0512* is flanked by *bab2_0513* that encodes the glycine cleavage system protein T (GcvT), and *bab2_0511* that encodes an oxidoreductase. Of note, the amino acid sequences of BAB1_0914 and BAB2_0512 are highly similar (Figure 2.1D). In fact, BAB1_0914 and BAB2_0512 share over 75% amino acid identity and over 85% similarity. Another gene of interest whose expression is linked to VtlR in *B. abortus* is *bab2_0574*, which encodes a small hypothetical protein of 45 amino acids in length (Figure 2.1C). *bab2_0574* is located on chromosome II and is surrounded by *bab2_0573* that encodes a transporter and *bab2_0575* that encodes a hypothetical protein. While BAB2_0574 is well conserved across *Brucella* strains, *bab1_0914* and *bab2_0512* are well conserved throughout many members of the Order *Rhizobiales* in the Class *Alphaproteobacteria*. Moreover, it appears that *bab1_0914* and *bab2_0512* are homologous to a single gene found in other bacteria in the *Rhizobiales*. These include *sMc02051* in *Sinorhizobium meliloti* 1021, *atu1667* in *Agrobacterium tumefaciens* C58, and *rleg2_1502* in *Rhizobium leguminosarum* bv *trifolii* WSM2304.

Utilizing BAB1_0914 as the template amino acid sequence and the online tool DNA Subway, a PHYLIP ML phylogenetic tree was constructed showing the homologs of BAB1_0914 throughout the *Rhizobiales* (Figure 2.1E). This analysis illustrated that

genes encoding BAB1_0914-type small proteins are highly abundant in bacteria of the *Rhizobiales*, an Order of bacteria that are extremely divergent in terms of their environmental niches. Related to this analysis, it should be noted that a homolog of BAB2_0512 is not listed for *B. melitensis*, because the gene encoding this protein is not annotated in the genome; however, an unannotated region of DNA with 100% nucleotide identity to *bab2_0512* can be found in the *B. melitensis* genome downstream of *bmeII0557*.

***bab1_0914*, *bab2_0512*, and *bab2_0574* encode small proteins and are expressed under oxidative and acidic stress.**

The first question we sought to answer was whether or not *bab1_0914*, *bab2_0512*, and *bab2_0574* encode authentic peptides. For this, a 3xFLAG tag was incorporated onto the C-terminus of BAB1_0914, BAB2_0512, and BAB2_0574 at each chromosomal locus, and we demonstrated the production of each protein (Figure 2.2A). According to NCBI gene, BAB1_0914 and BAB2_0512 are both predicted to be 48 amino acids in length and BAB2_0574 is predicted to be 45 amino acids in length. With the addition of the 22 amino acid 3xFLAG tag, each protein is predicted to be 8-10 kDa. This is consistent with the bands observed in the western blot analyses depicted in Figure 2A.

To begin to understand if *bab1_0914*, *bab2_0512*, and *bab2_0574* are transcribed constitutively or under specific conditions, northern blot analysis was conducted on RNA isolated from cultures of *B. abortus* 2308 grown under a variety of conditions. *B. abortus* 2308 was grown to mid-log phase in brucella broth, washed with PBS, and then used to inoculate either brucella broth, Gerhardt's Minimal Media (GMM), E medium (low nutrients and low pH), or brucella broth with the addition of hydrogen peroxide (5 μM),

citric acid to lower pH to 4.5, or deoxycholate (0.5% final concentration). A culture of *B. abortus* 2308 was also grown to stationary phase in brucella broth. Cultures were incubated for 30 minutes and then RNA was extracted. Northern blot analysis was performed to determine expression of AbcR1, AbcR2, BAB1_0914, BAB2_0512, BAB2_0574, and 5S rRNA as a control (Figure 2.2B). This experiment revealed that AbcR2 was expressed at higher levels in stationary phase of growth and in the presence of H_2O_2, low pH, and in E medium compared to the other conditions. BAB1_0914, BAB2_0512, and BAB2_0574 showed similar expression patterns to that of AbcR2 with the exception of E medium. BAB1_0914 and BAB2_0512 were not expressed at a detectable level in the presence of E medium. It is unknown why *bab2_0512* and *bab2_0574* produce two bands in the northern blot. It is possible that one of the bands represents a processed or truncated form of the transcript; however, it should be noted that a deletion of *bab2_0512* or *bab2_0574* abolished the presence of both bands in a northern blot, indicated that the two RNA bands are encoded by a single gene (data not shown).

The experimental design described above was repeated with the *B. abortus* strains harboring 3xFLAG versions of BAB1_0914, BAB2_0512, and BAB2_0574 with the intent of assessing protein production under the tested conditions. Using western blot analysis, we observed similar expression levels of protein to that of RNA in the BAB1_0914-3xFLAG tagged strain, with higher expression in stationary phase cells and those exposed to lower pH and oxidative stress (Figure 2.2C). Alternatively, the BAB2_0512 protein exhibited robust production in both exponential and stationary phases of growth, as well as in response to hydrogen peroxide stress, growth in E

medium, and in the presence of deoxycholate (Figure 2.2D). This observed discrepancy between RNA and protein levels of BAB2_0512 is interesting, and it is possible that a post-transcriptional regulatory mechanism exists to coordinate the levels of BAB2_0512 protein under certain environmental conditions. The increased production of BAB2_0574 was observed in cells grown to stationary phase and when the bacteria were exposed to hydrogen peroxide, and BAB2_0574 production was slightly induced when the bacteria were cultured in nutrient-limiting GMM and in medium with increased acidity (i.e. pH 4.5) (Figure 2.2E). Altogether, these experiments demonstrate that *bab1_0914*, *bab2_0512*, and *bab2_0574* encode authentic proteins in *B. abortus* 2308, and moreover, that production of these small proteins is driven by conditions that are biologically relevant to the brucellae.

Localization of the BAB1_0914, BAB2_0512, and BAB2_0574 in the *B. abortus* cell. Fractionation of *B. abortus* was developed from Thein et al., and western blot analyses were applied to localize the three small proteins to the membrane fractions of *B. abortus* (Figure 2.3) (Thein *et al.*, 2010). Control western blot analyses to support the successful fractionation method performed included anti-SodC antibody for the periplasmic fraction, anti-GroEL antibody for the cytoplasmic fraction, anti-Omp89 antibody for the membrane fraction. Anti-FLAG antibody was used to detect each 3xFLAG tagged small protein.

The fractionated samples contained little contamination between the cytoplasmic, periplasmic, and membrane fractions in all three samples. The membrane fraction isolated from BAB1_0914-3xFLAG was contaminated with cytoplasmic proteins as indicated by the presence of GroEL in the membrane fraction, and the cytoplasmic

fraction from BAB2_0512-3xFLAG was contaminated with periplasmic proteins as indicated by SodC in the cytoplasmic fraction. Despite this, no other fractions other than the membrane fraction contained the protein Omp89 and thus, the appearance of FLAG tagged protein in the membrane fraction alone reveals that BAB1_0914, BAB2_0512, and BAB2_0574 are localized to the membrane alone (Figure 2.3).

These data are supported due to the large number of charged and hydrophobic residues in the amino acid sequences of BAB1_0914, BAB2_0512, and BAB2_0574 (Figure 2.1), and protein structure homology-modeling via the free online server SWISS-MODEL revealed the predicted structures for BAB1_0914, BAB2_0512, and BAB2_0574 all contain alpha-helices (Figure 2.4) (Benkert *et al.*, 2011, Bertoni *et al.*, 2017, Biasini *et al.*, 2014, Bienert *et al.*, 2017, Guex *et al.*, 2009). It should be noted that homology modeling provided BAB1_0914 and BAB2_0512 with the same predicted structure due to the highly similar amino acid sequences of the two small proteins.

Phenotypic analysis of the VtlR regulon.

Biolog Phenotype Microarray Plates for Microbial Cells were utilized to identify phenotypic differences between wild-type *B. abortus* 2308 and a deletion of the entire VtlR regulon (i.e., a strain with deletions of *abcR2, bab1_0914, bab2_0512,* and *bab2_0574*) to tease out any potential functions for any of the small proteins and/or AbcR2. For this, the parental *B. abortus* 2308 and the *B. abortus* 2308::Δ*abcR2*-Δ*bab1_0914*-Δ*bab2_0512*-Δ*bab2_0574* deletion strain (i.e., Δquad) strains were used to inoculate 20 Biolog Phenotype Microarray plates and grown statically at 37^{o}C for 84 hours. Metabolic activity, measured by cell respiration due to the reduction of a tetrazolium dye (clear to purple), was measured via O.D. 590 nm every 12 to 24 hours

(Data not shown). When compared to *B. abortus* 2308, the quadruple deletion strain contained two major differences in growth. The *B. abortus* Δquad strain was unable to utilize L-fucose as the sole carbon source for growth in minimal media in plate 1 well B4 and showed minimal growth over the 84-hour incubation time (Figure 2.5A); while the parental strain was able to utilize L-fucose as a carbon source and grew to an O.D. of over 0.8. However, Δquad was able to grow in minimal media pH 4.5 with the addition of hydroxylysine in plate 10 well D8 while the growth of the parental strain was inhibited (Figure 2.5B). The experiment was repeated by inoculating plate 1 and plate 10 of the Biolog system with the Δ*bab1_0914*, Δ*bab2_0512*, Δ*bab2_0574*, and Δ*abcR2* strains, as well as a double deletion of *bab1_0914* and *bab2_0512* strain and a triple deletion of *bab1_0914*, *bab2_0512*, and *bab2_0574* strain (Δtriple). With regards to plate 10, the quadruple deletion strain was able to grow in the presence of pH4.5+hydroxylysine. Conversely, the double and triple deletion strain had intermediate growth and *B. abortus* 2308 and the isogenic deletions of *bab1_0914* and *bab2_0512* exhibited little to no growth in the presence of pH4.5+Hydroxylysine (Figure 2.5B). In plate 1, only *B. abortus* 2308 and the isogenic deletion of *bab2_0574* were able to utilize fucose as the sole carbon source in minimal media (Figure 2.5A). We sought to further exam these phenotypes outside of the Biolog system.

Effect of L-fucose on *B. abortus* growth.

While we were unsuccessful in reproducing the growth characteristics of *B. abortus* in the presence of hydroxylysine that we observed in the Biolog plates, we were able to independently verify the ability of *B. abortus* to utilize fucose during growth outside of the Biolog system. First, we tested the effect of supplementing minimal medium with 24

mM L-fucose on *B. abortus* growth. GMM +/- 24 mM L-fucose was inoculated with ~$5x10^4$ CFU/ml and incubated for 96 hours. Samples were collected every 24 hours and serial diluted to calculate CFU brucellae/ml. The data revealed that after 96 hours of growth, *B. abortus* 2308 grew to a larger cell density in the presence of 24 mM L-fucose (Figure 2.6A). The following experiment tested the ability of VtlR regulon deletion strains to utilize L-fucose when grown in GMM. The data revealed that after 96 hours of growth the *B. abortus* Δquad strain grew to a lower cell density than that of the parental strain *B. abortus* 2308 (Figure 2.6B). *B. abortus* 2308::Δ*bab2_0574* showed no significant difference compared to *B. abortus* 2308. Surprisingly, the growth of *B. abortus* 2308::Δ*bab1_0914* was not significantly decreased, but *B. abortus* 2308::Δ*bab2_0512* cell density was significantly lower than *B. abortus* 2308 after 96 hours of incubation. As a proof of principle, we tested the effect of L-fucose on *B. abortus* 2308::Δ*vtlR*. Not only was cell density significantly less in *B. abortus* 2308::Δ*vtlR* cultures, but growth was inhibited by the presence of L-fucose (Figure 2.6C). Indeed, the *vtlR* deletion strain exhibits significantly decreased bacterial numbers compared to the parental strain 2308 at all time points during the growth experiment (data not shown), indicating that the *vtlR* deletion strain was unable to utilize fucose. Rhamnose and fucose are degraded similarly in *E. coli,* and both lead to the secretion of the byproduct propanediol (Baldoma & Aguilar, 1988). Thus, we tested the sensitivity of the Δ*vtlR* strain to these compounds, as well as glucose to test whether there is a general sensitivity to sugars (Figure 2.7). Our data demonstrate that Δ*vtlR* growth was not inhibited by rhamnose, propanediol, or glucose when incubated in GMM, suggesting that the phenotype observed is reserved solely for L-fucose.

Identification of genes induced in the presence of L-fucose.

Due to our observations that *B. abortus* grows to a higher density when exposed to L-fucose (Figure 2.6A), we hypothesized that *B. abortus* imports L-fucose and expression of a fucose import system and genes related to fucose import and utilization would fluctuate in the presence of L-fucose . Therefore, we set out to define the transcriptional response of *B. abortus* to L-fucose in Order to identify potential L-fucose transport and/or utilization systems, and RNA-sequencing (RNAseq) analysis was employed to detect differences in gene expression in cultures of *B. abortus* growth in the presence or absence of L-fucose. RNA was isolated from *B. abortus* 2308 incubated in GMM with and without the addition of 100 μM L-fucose, and RNAseq analysis revealed very few genes that displayed significantly different levels of expression between the conditions tested. Indeed, only twelve genes exhibited >4-fold (i.e., $Log_2 \geq 2$) increased expression in cultures with the addition of 100 μM L-fucose compared to cultures without the addition of L-fucose (Table 2.1). All 12 genes belong to a single genetic locus on chromosome I of *B. abortus* 2308, and the genes in this locus encode a putative IclR-family transcriptional regulator (BAB1_0237), putative ABC-type sugar transport system (BAB1_0238- 0241), and proteins potentially involved in sugar metabolism (BAB1_0242-0248).

To test whether genes in this locus are involved in fucose metabolism or virulence, an isogenic deletion of *bab1_0238*, the putative periplasmic binding protein in the identified putative fucose transporter (Table 2.1), was constructed and the deletion strain was evaluated for the capacity to grow in minimal medium with the addition of L-fucose and for the ability to survive and replicate within macrophages. A deletion of

bab1_0238 resulted in no difference in bacterial growth in the presence of L-fucose or difference in the ability to survive and replicate in macrophages when compared to the parental strain *B. abortus* 2308 (Figure 2.8).

***bab1_0914*, *bab2_0512* or *bab2_0574* do not contribute to the ability of the brucellae to colonize BALB/c mice.**

It was previously shown that *bab1_0914*, *bab2_0512* and *bab2_0574* do not contribute to the ability of *B. abortus* to survive and replicate in a macrophage model of infection (Sheehan *et al.*, 2015). However, a lack of attenuation by a mutant strain of *B. abortus* in a macrophage model does not always correlate with a difference in the ability for the strain to infect a mouse model. Therefore, multiple strains of *B. abortus* 2308 were constructed with different combinations of mutations in the genes associated with the VtlR regulon to determine which, if any, genes in the described VtlR regulon are responsible for the decrease in spleen colonization previously observed in the *vtlR* mutant in the mouse model. Isogenic mutants of each small hypothetical protein were constructed, a strain containing a mutation of all three hypothetical small proteins (Δ*bab1_0914*, Δ*bab2_0512*, Δ*bab2_0574*) and a quadruple mutant (Δ*bab1_0914*, Δ*bab2_0512*, Δ*bab2_0574*, Δ*abcR2*) were also constructed. Female BALB/c mice were infected intraperitoneally with approximately $5x10^4$ CFU/mL of either *B. abortus* 2308 or the mutant strains. After 8 weeks of infection, spleens were removed, homogenized and serial diluted to determine CFU/spleen, and it was determined that there is no difference in the ability of *B. abortus* 2308 and any of the deletion strains tested to colonize the spleens of BALB/c mice (Figure 2.9).

Discussion

In the present study, we characterize three hypothetical small protein-encoding genes activated by VtlR in *B. abortus* 2308, and importantly, none of these hypothetical small proteins nor their homologous proteins found in other bacteria have been characterized previously. Our previous study describing VtlR in *B. abortus* 2308 revealed that isogenic mutations of the small hypothetical proteins, as well as a quadruple mutant of the entire VtlR regulon, survive and replicate in naïve peritoneal macrophages at a similar degree to that of parental strain *B. abortus* 2308. In the present study, we sought to further characterize the role that the small hypothetical proteins activated by VtlR contribute to *B. abortus* biology and/or virulence *in vivo*. We demonstrated that similar to the previously performed infection of naïve macrophages, *B. abortus* strains containing deletions of the VtlR regulon do not significantly differ in their ability to colonize the spleens of BALB/c mice compared to *B. abortus* 2308 and do not account for the observed attenuation of the Δ*vtlR* strain *in vivo* (Figure 2.9). Despite this, we further characterized the three small proteins and proved their translation, defined their expression under biologically relevant conditions, localized them within *B. abortus*, as well as proposed a functional role for *bab1_0914* and *bab2_0512* in the utilization of L-fucose by *B. abortus*.

Our virulence data leads us to believe that VtlR has a separate *in vivo* regulon from our previously published *in vitro* regulon. The original VtlR regulon was characterized via microarray analysis, and this transcriptional analysis was performed on RNA from brucellae cultured in nutrient rich broth. In that *in vitro* analysis, all three genes encoding the small hypothetical proteins showed decreased expression in a Δ*vtlR*

strain. We formed the hypothesis that these small hypothetical proteins constituted the VtlR regulon, and therefore, deletions of these small hypothetical proteins would have similar phenotypes *in vivo* to an isogenic deletion of *vtlR*. Conversely, deletion of individual or combinations of the genes in the observed VtlR regulon showed no difference in the ability to successfully colonize and survive in naïve peritoneal macrophages or a BALB/c mouse model compared to the parental strain (Figure 2.9) (Sheehan *et al.*, 2015). This was the first indication that VtlR may have an alternative transcriptional regulon *in vivo*. The second indication of multiple VtlR regulons was observed by measuring the transcriptional expression of BAB1_0914, BAB2_0512, and BAB2_0574 under different biological conditions. Transcription of all three genes changed under the different conditions tested. *bab1_0914*, *bab2_0512*, and *bab2_0574* were highly expressed during stationary phase of growth, under oxidative stress and acidic conditions. There are multiple hypotheses to account for the change in expression of *bab1_0914*, *bab2_0512*, and *bab2_0574* in different culture conditions. One hypothesis is that regulators other than VtlR could regulate expression of *bab1_0914*, *bab2_0512*, and *bab2_0574* in *B. abortus.* Another hypothesis is that the activity of VtlR changes under different growth conditions. Thus, under changing environmental conditions, such as those encountered during intramacrophagic trafficking, the expression of VtlR transcriptional targets may also change. To date, we have not empirically tested this hypothesis, and future experiments are underway to identify and characterize the *in vivo* gene targets of VtlR transcriptional regulation.

Our results also further our understanding of the AbcR small RNAs in *B. abortus*, as it was previously unknown whether AbcR1 and AbcR2 were differentially expressed

in response to various growth and stress conditions. Our data show that under the conditions tested, AbcR1 is consistently produced while AbcR2 is activated in response to specific conditions, including stationary growth, oxidative stress, acidic growth medium, and E medium (Figure 2.2). AbcR1 and AbcR2 have been shown to be redundant regulators of target mRNAs in *B. abortus* (Sheehan *et al.*, 2015), yet our expression data indicate that AbcR1 is constitutively expressed under the conditions tested. This does not seem beneficial to the cell, because if AbcR1 is always present, then all of the AbcR target mRNAs would be constitutively regulated as well. Thus, AbcR1 is most likely not constitutively expressed within *Brucella*, and our group is currently working to identify a transcriptional regulator of AbcR1 in *B. abortus*. Moreover, while all of the AbcR target mRNAs have not been individually studied in *Brucella*, homologs of AbcR1 and AbcR2 target mRNAs are necessary for the pathogenesis or symbiosis of other closely-related bacteria (Sheehan *et al.*, 2015, Becker *et al.*, 2014), and ongoing work in our laboratory is continuing to define the links between the control of *abcR* expression, AbcR-mediated regulation of mRNA levels, and *Brucella* virulence.

Up to this point, we have discussed how our findings have changed our understanding of the activity of the transcriptional regulator VtlR and new discoveries of AbcR sRNA expression in *Brucella*, but perhaps the most intriguing data observed from this study is the potential interaction between L-fucose and *Brucella*. It has been shown that many strains of *B. melitensis* and *B. abortus* can utilize L-fucose, but in general, very little is known about L-fucose metabolism in *Brucella* spp. (Al Dahouk *et al.*, 2010). The ability of a bacterium to sense and potentially utilize L-fucose has recently been documented as an important virulence determinant, as is has been demonstrated that

some enteric bacteria can use L-fucose to aid in their ability to persist in the gut. For example, *Campylobacter jejuni* can transport L-fucose, and a strain containing a mutation in this transport system is less able to successfully colonize the host (Stahl *et al.*, 2011). Similar to *in vitro* growth analyses in *C. jejuni*, we demonstrated that *B. abortus* 2308 can grow to a higher density in minimal media supplemented with L-fucose (Figure 2.6). Enterohemorrhagic *E. coli* (EHEC) can both metabolize host-derived L-fucose and sense L-fucose using a two component regulatory system, which regulates virulence associated genes (Keeney & Finlay, 2013, Pacheco *et al.*, 2012). Additionally, *Klebsiella pneumoniae* incorporates fucose into its capsule during infection of a mouse model (Wu *et al.*, 2008). While these studies highlight the ability for different bacteria to use fucose to their benefit, little information is available about bacterial sensitivity to fucose, as we observed in our experiments with *B. abortus* mutants (Figure 2.5 and 2.6). Regarding fucose availability, *Brucella* infections commonly occur via the oral route, and thus, it is likely that *Brucella* spp. would encounter L-fucose in the gut during infection.

Our data indicate that *B. abortus* strains containing mutations in *vtlR*, *bab1_0914* and *bab2_0512* exhibit increased growth inhibition to the presence of L-fucose in the growth medium, but what accounts for this L-fucose toxicity? While fucose metabolism has not been studied in *Brucella* spp., it has been shown in *E. coli* and *C. phytofermentans* that fucose and rhamnose are digested similarly to produce lactaldehyde, which is a precursor of lactic acid and 1,2-propanediol (Petit *et al.*, 2013, Baldoma & Aguilar, 1988). Our data demonstrate that the *vtlR* deletion strain is not sensitive to the presence of rhamnose or 1,2-propanediol during growth (Figure 2.7), and thus, it is possible that an accumulation of lactaldehyde in the Δ*vtlR* strain leads to the

toxicity observed when the strain in grown in the presence of L-fucose. However, this hypothesis is based on the assumption that the metabolism of fucose in *B. abortus* is similar to fucose metabolism in *E. coli* and *C. phytofermentans*. As such, a more detailed analysis of fucose metabolism in *Brucella* is needed to elucidate the mechanism of L-fucose-mediated toxicity observed for the *B. abortus* Δ*vtlR* strain. While at least one small protein, SgrT, has been shown to be involved in sugar transport in *E. coli*, there are few examples of the role of small proteins with sugar utilization in general (Lloyd *et al.*, 2017, Raina & Storz, 2017). The presented study warrants further research to exam the role of small proteins in fucose utilization and metabolism in other organisms.

Regarding the response of *B. abortus* to L-fucose, RNA sequencing analysis revealed a putative fucose-activated sugar transport and utilization system (Table 2.1), and we hypothesize that this system is responsible for the transport and utilization of fucose by *B. abortus*. Of the genes exhibiting elevated expression in response to L-fucose, ten genes (i.e., *bab1_0238*-*bab1_0248*) encode a putative sugar ABC-transport system and proteins potentially involved in the metabolism of sugars (e.g., oxidoreductases, isomerases, and dehydratases). Our data revealed that an isogenic deletion of *bab1_0238* from the *B. abortus* genome did not result in any significant differences in the capability to grow in minimal medium with the addition of L-fucose or the ability to survive and replicate in a macrophage model of infection when compared to the parental strain *B. abortus* 2308 (Figure 2.8). Nonetheless, it is possible that the presence of orphan periplasmic binding proteins encoded in the *Brucella* genome may function in place of BAB1_0238, and this may account for the lack of an observable fucose-linked phenotype in the *bab1_0238* deletion strain. Moreover, it is possible that

another ABC transport systems can transport L-fucose in the absence of the BAB1_0238-0248 system (Thomas, 2010, Durmort & Brown, 2015). Overall, additional studies are needed to fully characterize the function of the putative fucose transport and utilization system, as well as the role of fucose in the biology and virulence of *B. abortus*.

Two additional genes, *bab1_0236* and *bab1_0237*, are divergently oriented from the putative fucose ABC-transporter and utilization genes, and *bab1_0237* encodes a putative transcriptional regulatory protein from the IclR family, while *bab1_0236* encodes a putative amidohydrolase. A highly similar genomic locus in *Sinorhizobium meliloti* 1021 is induced in cultures by the addition of L- and D-fucose (Mauchline *et al.*, 2006). With regards to *Brucella*, Lamontagne et al. showed that within 24 hours of infection of murine RAW264.7 macrophages with *B. abortus*, the expression of *bab1_0238* and *bab1_0246* were both significantly decreased, along with several other sugar transport systems (Lamontagne *et al.*, 2009). This led the authors to conclude that sugars imported by these systems may be in low abundance intracellularly (Lamontagne *et al.*, 2009). Indeed, we hypothesize that fucose may play a more important role in the gut of an infected host rather than during intracellular trafficking. It is important to note that while many studies have focused on the interactions between fucose and microbes in the gut, fucose can be found throughout the mammalian body, including the reproductive tract and the brain (Staudacher *et al.*, 1999, Ma *et al.*, 2006, Domino *et al.*, 2001, Mountford *et al.*, 2015). Thus, the role of fucose in the virulence of *Brucella* spp. could be important during different stages of *Brucella* pathogenesis, different routes of infection, or the infection of different hosts.

To summarize, while many small proteins do not have enzymatic functions, several have been shown to act as facilitators of enzymatic function for larger proteins (Storz *et al.*, 2014). Because of this, observable phenotypes in strains with mutated or deleted small proteins can be subtle or nonexistent. Deletion of *bab1_0914* and *bab2_0512* in *B. abortus* revealed a minor sensitivity to the sugar L-fucose, which led us to discover a robust phenotype in a deletion of the transcriptional regulator VtlR. As previously stated, it is hypothesized that VtlR may be operating in response to an environmental stimulus, which could include the sugar L-fucose during infection (Sheehan *et al.*, 2015). Overall, this study serves as an example of the benefits of studying small proteins, which are continually overlooked as insignificant but may play major roles in bacterial physiology and virulence.

Materials and Methods

Bacterial strains and growth conditions.

Brucella abortus 2308 and derivative strains were routinely grown on Schaedler blood agar (SBA), composed of Schaedler agar (Acumedia, Burton, MI) containing 5% defibrinated bovine blood (Quad Five, Ryegate, MT), or in brucella broth (BD, Sparks, MD). For cloning, *Escherichia coli* strain DH5α was grown on tryptic soy agar (MP, Solon, OH) or in Luria-Bertani (LB) broth. When appropriate, growth media were supplemented with kanamycin (45 μg/ml) or L-fucose (24 mM). All work with live *Brucella* strains was performed in a BSL3 facility.

Construction of *Brucella abortus* mutants and 3xFLAG tag strains by complementation.

Strains containing non-polar, unmarked isogenic deletions of *bab1_0914*, *bab2_0512* and *bab2_0574* as well as a quadruple deletion strain of *abcR2*, *bab1_0914*, *bab2_0512*, and *bab2_0574* in *Brucella abortus* 2308 were previously constructed and utilized in the publication by Sheehan et al. and are utilized in this publication (Sheehan *et al.*, 2015). A double deletion strain of *bab1_0914* and *bab2_0512*; triple deletion of *bab1_0914*, *bab2_0512*, and *bab2_0574*, and an isogenic deletion strain of *bab1_0238* were also constructed using the strategy previously stated (Sheehan *et al.*, 2015). Briefly, a 1 kb fragment upstream of the gene of interest plus the addition of the first codon of the gene of interest was amplified using Up Forward and Reverse primers and a 1 kb fragment downstream of the gene of interest plus the addition of the last codon of the gene of interest was amplified using Down Forward and Reverse primers and genomic *B. abortus* 2308 DNA. The upstream fragment was digested with *BamHI* and the downstream

fragment was digested with *PstI*. Both fragments were phosphorylated using polynucleotide kinase in the presence of ATP. The upstream and downstream fragments were then ligated to each other and then into a *BamHI/PstI* digest pNPTS138 vector (M.R.K. Alley, unpublished) using T4 DNA ligase (Monserate Biotechnology Group, San Diego, CA). The primers utilized for the construction of the deletion constructs were used in our previous study and can be found in Table 2.1. The deletion constructs were introduced into *B. abortus* 2308 and merodiploid transformants were selected on SBA+kanamycin. Merodiploid transformants were then incubated at 37^{o}C for 7 hours and then plated onto SBA+10% sucrose. Colonies that were sucrose resistant and kanamycin sensitive were screen via PCR for a loss of the genes of interest. The double and triple mutant strains were constructed by sequential mutagenesis of *B. abortus* with the deletion constructs to obtain the correct combination of genetic deletions. The double mutant of *bab1_0914* and *bab2_0512* was named JB003 and the triple mutant of *bab1_0914*, *bab2_0512*, and *bab2_0574* was named JB004.

Addition of a C-terminus 3xFLAG tag (5'-GACTACAAAGACCATGACGGTGATTATAAAGATCATGACATCGACTACAAGG ATGACGATGACAAG-3') to the small hypothetical protein nucleotide sequence was carried out by reconstruction of the deleted loci on the *B. abortus* chromosome. This process is similar to the one described above, however, new primers were constructed to replace Up-Rev and Down-For, each containing half of the 3xFLAG tag. The amplified upstream and down stream regions encompassing the genes of interest and the addition of the 3xFLAG Tag were digested with *BamHI* and *PstI* respectively. The fragments were phosphorylated and ligated together and then ligated into the pNPTS138 plasmid and

transformation and selection for the reconstructed tagged strains was carried out as described above.

RNA expression and protein production under different growth conditions.

For expression experiments under different growth conditions, *B. abortus* 2308, *B. abortus* 2308::*bab1_0914*-3xFLAG, or *B. abortus* 2308::*bab2_0512*-3xFLAG were used to inoculate brucella broth at a concentration of 10^9 CFU/ml or 10^6 CFU/ml for stationary phase and exponential phase grown cells respectively and grown for approximately 30 hours to the appropriate O.D. Exponential Phase cells at an O.D. of 0.15 were collected, washed with PBS and used to inoculate the appropriate media at a final concentration of 10^9 CFU/ml. Brucella broth, brucella broth pH 4.5, brucella broth supplemented with 0.5% deoxycholate, brucella broth supplemented with 5 μM H_2O_2, GMM, and E medium were utilized. After 30 minutes of incubation at 37°C with shaking, cultures were diluted 1:1 with ethanol:acetone and RNA or protein was extracted as previously described (Caswell *et al.*, 2012b).

Fractionation of *Brucella abortus* 2308.

Fractionation of *B. abortus* 2308 was performed as previously described by Thein et al with revisions optimized for *B. abortus* 2308 (Thein *et al.*, 2010). 100 mL of brucella broth (BD) were inoculated with appropriate strain of *B. abortus* and grown to late exponential phase at 37°C. 100 mL of 1:1 ethanol:acetone was added to the culture and death of *Brucella* was established for 10 days post addition of ethanol:acetone prior to the removal of the samples from the BSL3 facility. Cells were pelleted at 10,000 x g for 10 minutes and supernatant was discarded. The pellet was resuspended in a 10 mL solution 1 (0.2 M Tris-HCl pH 8, 1 M sucrose, 1 mM EDTA, lysozyme (1mg/mL)). The suspension

was vortexed and incubated at room temperature for 5 minutes. 40 mL of dH_2O was added to the suspension and placed on ice for 10 minutes. The suspension was centrifuged at 200,000 x *g* for 45 minutes at 4°C. The supernantant was collected as the periplasmic fraction. The pellet was resuspended in 7.5 mL of ice-cold solution 2 (10 mM Tris-HCl pH 7.5, 5 mM EDTA, 0.2 mM DTT supplemented with 50 μL DNase (1 mg/mL)). Cells were ruptured via 2 passes through a French Press at 10^6 Pa. Unbroken cells were spun down by centrifugation at ~2000 x *g* for 10 minutes. The pellet was discarded. The supernatant was centrifuged at 300000 x *g* for 3 hours at 4°C. The supernatant was collected as the cytoplasmic fraction. Pellet containing the crude membranes was separated by sucrose gradient. The pellet was resuspended in 1mL solution (10 mM Tris pH7.5, 15% sucrose, 5 mM EDTA, 0.2 mM DTT). Samples were stored at -20°C until analyzed via western blotting.

Western blot analyses.

Protein samples were prepared with the addition of 4X Laemmli Buffer and boiled for 5 minutes prior to being loaded into a 10 to 20% polyacrylamide gel. BAB1_0914-3xFLAG, BAB2_0512-3xFLAG, and BAB2_0574-3xFLAG were detected using mouse monoclonal anti-FLAG at a 1:1,000 dilution (Thermo Fisher Scientific). SodC was detected using rabbit polyclonal anti-SodC at a dilution of 1:1,000. Omp89 was detected with the addition of serum directly isolated from rabbit serum. GroEL was detected using mouse monoclonal anti-GroEL at a 1:1,000 dilution (Origene). HRP-conjugated anti-mouse or anti-rabbit was used as secondary antibody when appropriate and protein detection was carried out via femtoLUCENT Luminol Solution (G Biosciences).

Growth in Biolog Phenotype Microarray plates.

Biolog Phenotype Microarray plates (Biolog, Inc., Hayward, CA.) were utilized to determine phenotypic differences between different *B. abortus* strains. Strains were grown on SBA plates to produce a lawn of bacteria. Bacteria was collected and suspended in IF-0a GN/GP Base (Biolog). The protocol "PM Procedures for GN Fastidious Bacteria" provided by Biolog were followed and Biolog Phenotype Microarray plates 1-20 were inoculated at a final concentration of 10^9 CFU/ml. Plates were grown statically at 37°C for 84 hours and O.D. 590 nm was measured every 12 to 24 hours.

RNA sequencing analysis of L-fucose treated cultures.

Brucella broth was inoculated with *B. abortus* 2308 and incubated at 37°C with shaking for ~24 hours until the cultured obtained an O.D. 600 nm of 0.15. Cells were then washed with PBS and cells were used to inoculate either GMM or GMM with the addition of 100 μM L-fucose at a concentration of 10^9 CFU/mL. Cultures were incubated for 20 minutes at 37°C with shaking. Following incubation, an equal volume of 1:1 ethanol:acetone was added to each culture and cultures were frozen at -80°C until RNA isolation. This was performed in triplicate for each condition. RNA was isolated from each culture and DNase treated prior to submission for RNAseq analysis.

(i) Stranded RNA Library Construction for Prokaryotic RNA-Seq

1 μg of total RNA with RIN ≥ 8.0 was depleted of rRNA using Illumina's Ribo-Zero rRNA Removal Kit (Gram-Positive and Gram-Negative Bacteria) (P/N MRZB12424, Illumina, CA). The depleted RNA is fragmented and converted to first strand cDNA using reverse transcriptase and random primers using using Illumina's TruSeq Stranded mRNA HT Sample Prep Kit (Illumina, RS-122-2103). This is followed by second strand

synthesis using polymerase I and RNAse H, and dNTPs that contain dUTP instead of dTTP. The cDNA fragments then go through end repair, addition of a single 'A' base, and then ligation of adapters and indexed individually. The products are then purified and the second strand digested with N-Glycosylase, thus resulting in stranded template. The template molecules with the adapters are enriched by 10 cycles of PCR to create the final cDNA library. The library generated is validated using Agilent 2100 Bioanalyzer and quantitated using Quant-iT dsDNA HS Kit (Invitrogen) and qPCR. 16 individually indexed cDNA libraries were pooled and sequenced on Illumina NextSeq.

(ii) Illumina NextSeq Sequencing

The libraries are clustered and sequenced using, NextSeq 500/550 High Output kit V2 (150 cycles) (P/N FC-404-2002) to 2 x 75 cycles to generate paired end reads. The Illumina NextSeq Control Software v2.1.0.32 with Real Time Analysis RTA v2.4.11.0 was used to provide the management and execution of the NextSeq 500 and to generate BCL files. The BCL files were converted to FASTQ files and demultiplexed using bcl2fastq Conversion Software v2.20. The FASTQ files are provided to the investigator for further analysis.

(iii) RNA-Seq data processing and analysis

The *Brucella abortus* (strain 2308) gene and genome sequences, as well as corresponding annotations from NCBI (https://www.ncbi.nlm.nih.gov/) were used as the reference. Raw reads were quality-controlled and filtered with FastqMcf (Aronesty, 2013), resulting in an average of 1,971 Mbp (1,730 to 2,305 Mbp) nucleotides. The remaining reads were mapped to the gene reference using BWA (Li & Durbin, 2009) with default parameters. Differential expression of genes was calculated using the edgeR (Robinson *et al.*, 2010)

package in R software (http://www.r-project.org/), with Benjamini–Hochberg adjusted P-values of 0.05 considered to be significant. The GenBank accession number for the RNAseq data is SRP141183.

Virulence of *Brucella* strains in experimentally infected mice and macrophages.

The infection of primary, peritoneal murine macrophages was describe previously by Sheehan et al (Sheehan *et al.*, 2015). Macrophages were harvested from mice and seeded into 96-well plates in Dulbecco's modified Eagle's medium with 5% fetal bovine serum. The following day, macrophages were infected with opsonized brucellae at a multiplicity of infection of 100:1. After 2 h, the infected macrophages were treated with gentamicin (50 $\mu g\,ml^{-1}$) for 1 h. The macrophages were then lysed with 0.1% deoxycholate in PBS, and serial dilutions were plated on Schaedler blood agar (SBA). For the 24 and 48 h time points, macrophages were washed with PBS following gentamicin treatment and fresh cell culture medium containing gentamicin (20 $\mu g\,ml^{-1}$) was added to the monolayer. At the indicated time points, macrophages were lysed and serial dilutions were plated on SBA in triplicates.

The infection of mice by *Brucella* strains was performed as described previously by Sheehan et al (Sheehan *et al.*, 2015). Female BALB/c mice (5 per *Brucella* strain per experiment) were infected intraperitoneally with ~10^5 CFU of each *Brucella* strain in sterile PBS. The mice were sacrificed 8 weeks post-infection, and serial dilutions of spleen homogenates were plated on SBA to determine CFU/spleen of each strain.

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References

Al Dahouk, S., H.C. Scholz, H. Tomaso, P. Bahn, C. Gollner, W. Karges, B. Appel, A. Hensel, H. Neubauer & K. Nockler, (2010) Differential phenotyping of *Brucella* species using a newly developed semi-automated metabolic system. *BMC Microbiol* **10**: 269.

Aronesty, E., (2013) Comparison of Sequencing Utility Programs. *The Open Bioinformatics Journal* **7**: 1-8.

Atluri, V.L., M.N. Xavier, M.F. de Jong, A.B. den Hartigh & R.M. Tsolis, (2011) Interactions of the human pathogenic *Brucella* species with their hosts. *Annu Rev Microbiol* **65**: 523-541.

Baldoma, L. & J. Aguilar, (1988) Metabolism of L-fucose and L-rhamnose in *Escherichia coli*: aerobic-anaerobic regulation of L-lactaldehyde dissimilation. *J Bacteriol* **170**: 416-421.

Basrai, M.A., P. Hieter & J.D. Boeke, (1997) Small open reading frames: beautiful needles in the haystack. *Genome Res* **7**: 768-771.

Becker, A., A. Overlöper, J.-P. Schlüter & J. Reinkensmeier, (2014) Riboregulation in plant-associated α-proteobacteria. *RNA biology* **11**: 550-562.

Benkert, P., M. Biasini & T. Schwede, (2011) Toward the estimation of the absolute quality of individual protein structure models. *Bioinformatics* **27**: 343-350.

Bertoni, M., F. Kiefer, M. Biasini, L. Bordoli & T. Schwede, (2017) Modeling protein quaternary structure of homo- and hetero-oligomers beyond binary interactions by homology. *Sci Rep* **7**: 10480.

Biasini, M., S. Bienert, A. Waterhouse, K. Arnold, G. Studer, T. Schmidt, F. Kiefer, T. Gallo Cassarino, M. Bertoni, L. Bordoli & T. Schwede, (2014) SWISS-MODEL: modelling protein tertiary and quaternary structure using evolutionary information. *Nucleic Acids Res* **42**: W252-258.

Bienert, S., A. Waterhouse, T.A. de Beer, G. Tauriello, G. Studer, L. Bordoli & T. Schwede, (2017) The SWISS-MODEL Repository-new features and functionality. *Nucleic Acids Res* **45**: D313-D319.

Bisson-Filho, A.W., K.F. Discola, P. Castellen, V. Blasios, A. Martins, M.L. Sforca, W. Garcia, A.C. Zeri, H.P. Erickson, A. Dessen & F.J. Gueiros-Filho, (2015) FtsZ filament capping by MciZ, a developmental regulator of bacterial division. *Proc Natl Acad Sci U S A* **112**: E2130-2138.

Caswell, C.C., J.M. Gaines, P. Ciborowski, D. Smith, C.H. Borchers, C.M. Roux, K. Sayood, P.M. Dunman & R.M. Roop Ii, (2012a) Identification of two small regulatory RNAs linked to virulence in *Brucella abortus* 2308. *Molecular microbiology* **85**: 345-360.

Caswell, C.C., J.M. Gaines & R.M. Roop, 2nd, (2012b) 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* **194**: 3-14.

Cutting, S., M. Anderson, E. Lysenko, A. Page, T. Tomoyasu, K. Tatematsu, T. Tatsuta, L. Kroos & T. Ogura, (1997) SpoVM, a small protein essential to development in *Bacillus subtilis*, interacts with the ATP-dependent protease FtsH. *J Bacteriol* **179**: 5534-5542.

de Figueiredo, P., T.A. Ficht, A. Rice-Ficht, C.A. Rossetti & L.G. Adams, (2015) Pathogenesis and immunobiology of brucellosis: review of *Brucella*-host interactions. *Am J Pathol* **185**: 1505-1517.

Domino, S.E., L. Zhang, P.J. Gillespie, T.L. Saunders & J.B. Lowe, (2001) Deficiency of reproductive tract alpha(1,2)fucosylated glycans and normal fertility in mice with targeted deletions of the FUT1 or FUT2 alpha(1,2)fucosyltransferase locus. *Mol Cell Biol* **21**: 8336-8345.

Durmort, C. & J.S. Brown, (2015) Chapter 10 - *Streptococcus pneumoniae* Lipoproteins and ABC Transporters. In: *Streptococcus Pneumoniae.* Amsterdam: Academic Press, pp. 181-206.

Friedman, R.C., S. Kalkhof, O. Doppelt-Azeroual, S.A. Mueller, M. Chovancova, M. von Bergen & B. Schwikowski, (2017) Common and phylogenetically widespread coding for peptides by bacterial small RNAs. *BMC Genomics* **18**: 553.

Guex, N., M.C. Peitsch & T. Schwede, (2009) Automated comparative protein structure modeling with SWISS-MODEL and Swiss-PdbViewer: a historical perspective. *Electrophoresis* **30 Suppl 1**: S162-173.

Keeney, K.M. & B.B. Finlay, (2013) Microbiology: EHEC downregulates virulence in response to intestinal fucose. *Curr Biol* **23**: R108-110.

Lamontagne, J., A. Forest, E. Marazzo, F. Denis, H. Butler, J.F. Michaud, L. Boucher, I. Pedro, A. Villeneuve, D. Sitnikov, K. Trudel, N. Nassif, D. Boudjelti, F. Tomaki, E. Chaves-Olarte, C. Guzman-Verri, S. Brunet, A. Cote-Martin, J. Hunter, E. Moreno & E. Paramithiotis, (2009) Intracellular adaptation of *Brucella abortus*. *J Proteome Res* **8**: 1594-1609.

Li, H. & R. Durbin, (2009) Fast and accurate short read alignment with Burrows-Wheeler transform. *Bioinformatics* **25**: 1754-1760.

Lippa, A.M. & M. Goulian, (2009) Feedback inhibition in the PhoQ/PhoP signaling system by a membrane peptide. *PLoS Genet* **5**: e1000788.

Lloyd, C.R., S. Park, J. Fei & C.K. Vanderpool, (2017) The Small Protein SgrT Controls Transport Activity of the Glucose-Specific Phosphotransferase System. *J Bacteriol* **199**.

Ma, B., J.L. Simala-Grant & D.E. Taylor, (2006) Fucosylation in prokaryotes and eukaryotes. *Glycobiology* **16**: 158R-184R.

Mauchline, T.H., J.E. Fowler, A.K. East, A.L. Sartor, R. Zaheer, A.H. Hosie, P.S. Poole & T.M. Finan, (2006) Mapping the *Sinorhizobium meliloti* 1021 solute-binding protein-dependent transportome. *Proceedings of the National Academy of Sciences of the United States of America* **103**: 17933-17938.

Mountford, C., S. Quadrelli, A. Lin & S. Ramadan, (2015) Six fucose-alpha(1-2) sugars and alpha-fucose assigned in the human brain using *in vivo* two-dimensional MRS. *NMR Biomed* **28**: 291-296.

Pacheco, A.R., M.M. Curtis, J.M. Ritchie, D. Munera, M.K. Waldor, C.G. Moreira & V. Sperandio, (2012) Fucose sensing regulates bacterial intestinal colonization. *Nature* **492**: 113-117.

Petit, E., W.G. LaTouf, M.V. Coppi, T.A. Warnick, D. Currie, I. Romashko, S. Deshpande, K. Haas, J.G. Alvelo-Maurosa, C. Wardman, D.J. Schnell, S.B. Leschine & J.L. Blanchard, (2013) Involvement of a bacterial microcompartment

in the metabolism of fucose and rhamnose by *Clostridium phytofermentans*. *PloS one* **8**: e54337.

Raina, M. & G. Storz, (2017) SgrT, a Small Protein That Packs a Sweet Punch. *J Bacteriol* **199**.

Ramamurthi, K.S. & G. Storz, (2014) The small protein floodgates are opening; now the functional analysis begins. *BMC Biol* **12**: 96.

Robinson, M.D., D.J. McCarthy & G.K. Smyth, (2010) edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. *Bioinformatics* **26**: 139-140.

Sheehan, L.M., J.A. Budnick, C. Blanchard, P.M. Dunman & C.C. Caswell, (2015) A LysR-family transcriptional regulator required for virulence in *Brucella abortus* is highly conserved among the alpha-proteobacteria. *Molecular microbiology*.

Stahl, M., L.M. Friis, H. Nothaft, X. Liu, J. Li, C.M. Szymanski & A. Stintzi, (2011) L-fucose utilization provides *Campylobacter jejuni* with a competitive advantage. *Proc Natl Acad Sci U S A* **108**: 7194-7199.

Staudacher, E., F. Altmann, I.B. Wilson & L. Marz, (1999) Fucose in N-glycans: from plant to man. *Biochim Biophys Acta* **1473**: 216-236.

Storz, G., Y.I. Wolf & K.S. Ramamurthi, (2014) Small proteins can no longer be ignored. *Annu Rev Biochem* **83**: 753-777.

Sun, Y.H., M.F. de Jong, A.B. den Hartigh, C.M. Roux, H.G. Rolan & R.M. Tsolis, (2012) The small protein CydX is required for function of cytochrome bd oxidase in *Brucella abortus*. *Front Cell Infect Microbiol* **2**: 47.

Thein, M., G. Sauer, N. Paramasivam, I. Grin & D. Linke, (2010) Efficient subfractionation of Gram-negative bacteria for proteomics studies. *J Proteome Res* **9**: 6135-6147.

Thomas, G.H., (2010) Homes for the orphans: utilization of multiple substrate-binding proteins by ABC transporters. *Molecular microbiology* **75**: 6-9.

von Bargen, K., J.P. Gorvel & S.P. Salcedo, (2012) Internal affairs: investigating the *Brucella* intracellular lifestyle. *FEMS Microbiol Rev* **36**: 533-562.

Wu, J.H., A.M. Wu, C.G. Tsai, X.Y. Chang, S.F. Tsai & T.S. Wu, (2008) Contribution of fucose-containing capsules in *Klebsiella pneumoniae* to bacterial virulence in mice. *Exp Biol Med (Maywood)* **233**: 64-70.

Tables

Table 2.1: Differential gene expression of *B. abortus* 2308 in GMM + 100 μM L-fucose vs. GMM.

BAB Designation	BAB_RS Designation	Gene Description	Log_2 Fold Change [GMM + 100 μM L-fucose vs. GMM]
BAB1_0238	BAB_RS17035	sugar ABC transporter substrate-binding protein	4.4
BAB1_0239	BAB_RS17040	sugar ABC transporter permease	4.3
BAB1_0241	BAB_RS17050	sugar ABC transporter ATPase	4.2
BAB1_0240	BAB_RS17045	maltose ABC transporter permease	4.1
BAB1_0242	BAB_RS17055	mandelate racemase	4.1
BAB1_0244	BAB_RS17065	oxidoreductase	4.0
BAB1_0246	BAB_RS17075	oxidoreductase	4.0
BAB1_0247	BAB_RS17080	2-hydroxyhepta-2,4-diene-1,7-dioate isomerase	3.9
BAB1_0248	BAB_RS17085	fuconate dehydratase	3.8
BAB1_0243	BAB_RS17060	L-rhamnose mutarotase	3.7
BAB1_0237	BAB_RS17030	IclR family transcriptional regulator	3.2
BAB1_0236	BAB_RS17025	amidohydrolase	2.2

RNAseq analysis was performed using total cellular RNA from *Brucella* strains grown in minimal media to late exponential phase, and those genes whose expression was shown to be more than 4-fold altered in minimal media with 100 μM L-fucose compared to minimal media without 100 μM L-fucose are shown in the list.

Table 2.2: Oligonucleotide primers used in this study.

Primer name	Sequence (5'→3')
1_0914-Up-For	TAGGATCCCCAGTATCAGCCCTATGCCAATC
1_0914-Dn-Rev	TACTGCAGAGCGTTTCCGGTCCGCGCTC
1_0914-Up-3X-FLAG-Rev	**ATCTTTATAATCACCGTCATGGTCTTTGTAGTC**GCGCGC TTTGGCCTGACG
1_0914-Dn-3X-FLAG-For	**CATGACATCGACTACAAGGATGACGATGACAAG**TAATC ACAAGTTTCTCCG
2_0512-Up-For	TAGGATCCCTCGATGTCGGCATCGGCATTG
2_0512-Dn-Rev	TACTGCAGGGACGTGCGCAATGTCGTGAT
2_0512-Up-3X-FLAG-Rev	**ATCTTTATAATCACCGTCATGGTCTTTGTAGTC**GCGGGC AGCGGCCTTGCG
2_0512-Dn-3X-FLAG-For	**CATGACATCGACTACAAGGATGACGATGACAAG**TGATA GCGCCGCCTCCAA
2_0574-Dn-Rev	TACTGCAGTCCAACGCAAAACCGCTACG
2_0574-Up-For	TAGGATCCGATCATCTGGCTATATTGCGGTG
2_0574-Up-3X-FLAG-Rev	**ATCTTTATAATCACCGTCATGGTCTTTGTAGTC**TCGACC GAAGACGGCGCT
2_0574-Dn-3X-FLAG-For	**CATGACATCGACTACAAGGATGACGATGACAAG**TAACA GTTATGGCGCGGC
1_0238-Up-For	ATGGATCCCACGCCTACATCCGCGCCCAC
1_0238-Up-Rev	TTTGAAATCCTGCATTCGTCTCC
1_0238-Dn-For	GCCGGCGACCTTTGACC
1_0238-Dn-Rev	ATCTGCAGACTACCACTCCGCTCCGATCTGC

*Underlined sequences depict a restriction endonuclease recognition site.
Bold sequence depicts 3X-FLAG sequence

Table 2.3: Plasmids used in this study.

Plasmid name	Description	Reference
pNPTS138	Cloning vector; contains *sacB* gene; Kan^R	(M.R.K. Alley, unpublished)
pJB006	Intact *bab2_0512* gene plus 3X-FLAG Tag at C-terminus and 1 kb of each flanking region in pNPTS138	This Study
pJB007	Intact *bab2_0574* gene plus 3X-FLAG Tag at C-terminus and 1 kb of each flanking region in pNPTS138	This Study
pJB008	Intact *bab1_0914* gene plus 3X-FLAG Tag at C-terminus and 1 kb of each flanking region in pNPTS138	This Study
pΔ*bab1_0238*	In-frame deletion of *bab1_0238* plus 1 kb of each flanking region in pNPTS138	This Study

Figures/Figure Legends

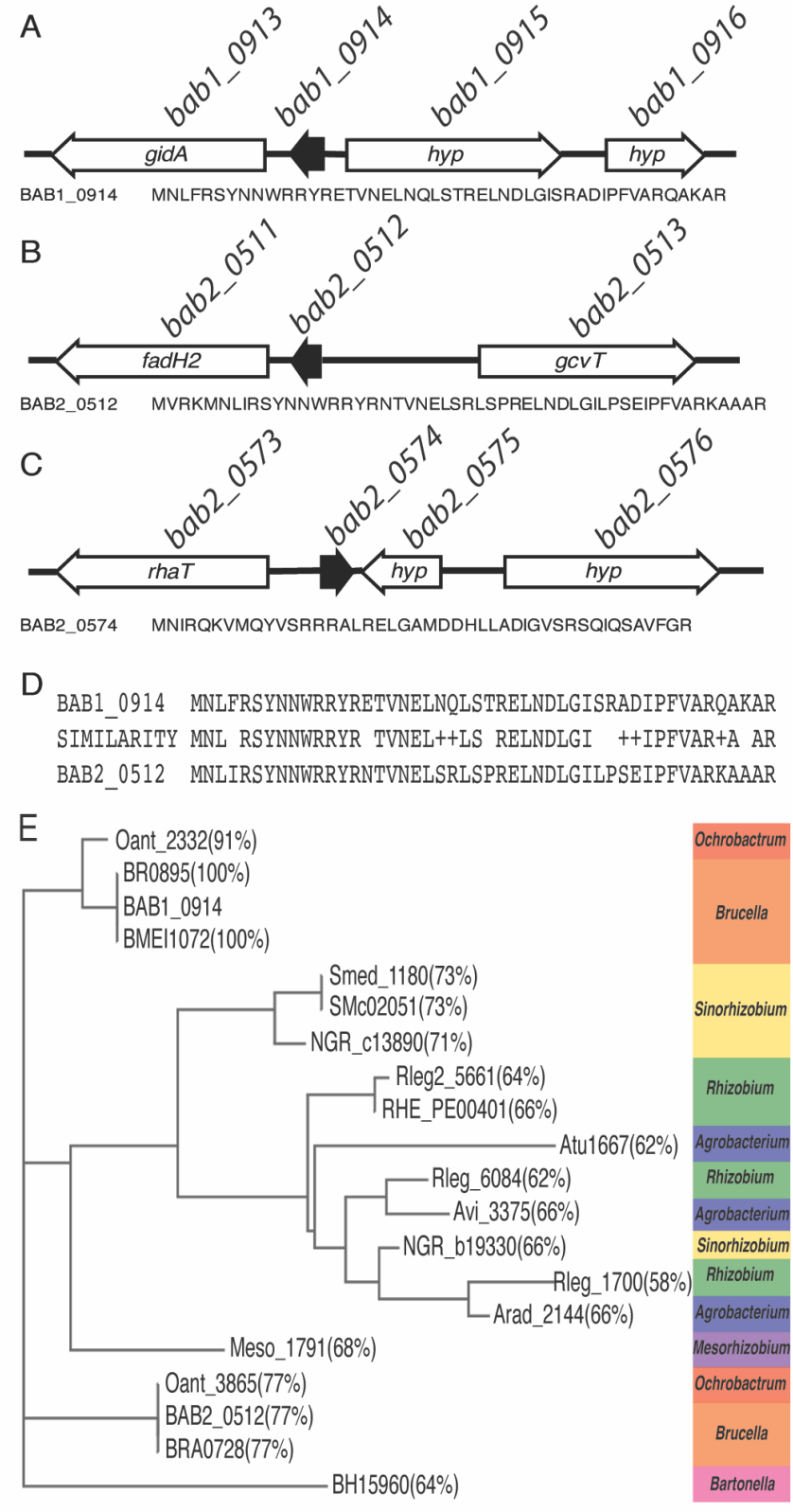


Figure 2.1: Organization of *bab1_0914*, *bab2_0512*, and *bab2_0574* in *Brucella abortus* 2308 and amino acid sequence similarity among BAB1_0914 and BAB2_0512.

Genetic contexts and encoding amino acid sequences of *bab1_0914 (bab_rs20300)* (A), *bab2_0512 (bab_rs28790)* (B), and *bab2_0574 (bab_rs29075)* (C). (D) Amino acid sequence alignment of BAB1_0914 and BAB2_0512 created by NCBI Align Sequences Protein Blast. Identical amino acids are depicted by abbreviated amino acid symbol and similar amino acids are depicted by +. (E) PHYLIP ML phylogenetic tree of BAB1_0914 and BAB2_0512 homologs created using "Determine Sequence Relationships" tool https://dnasubway.cyverse.org/. Percentages represent amino acid identity to BAB1_0914.

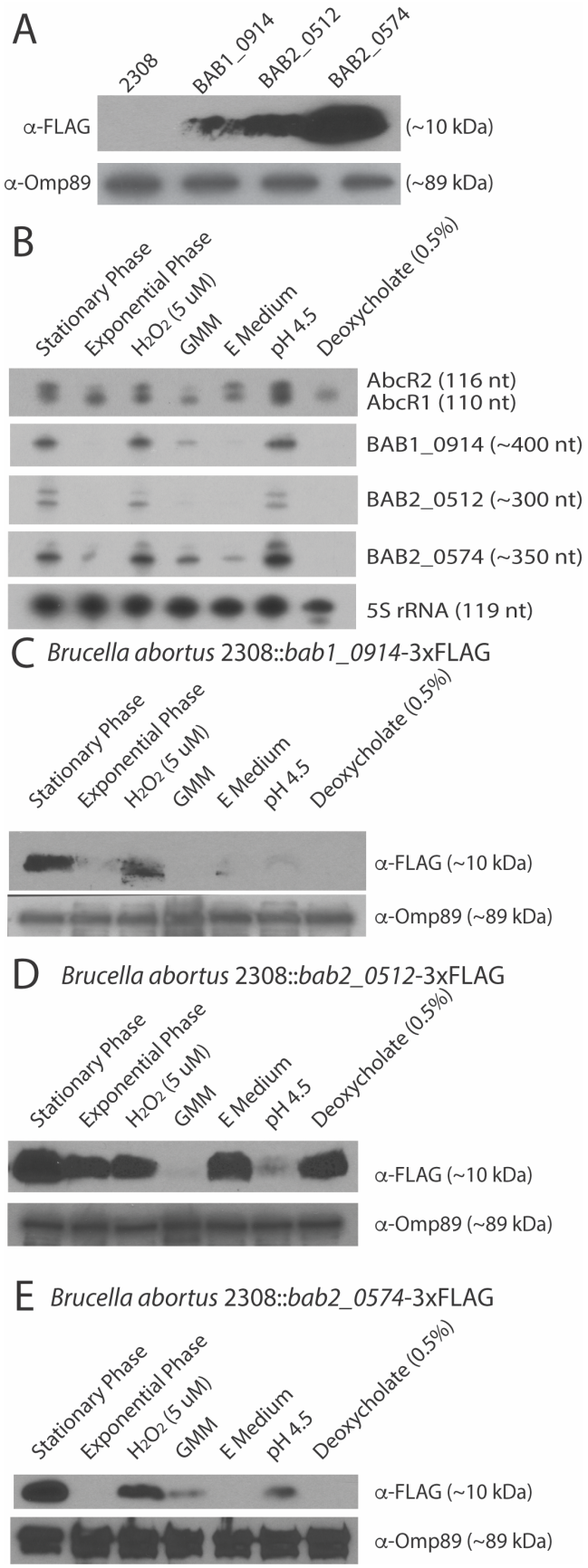

A
2308
BAB1_0914
BAB2_0512
BAB2_0574
α-FLAG
(~10 kDa)
α-Omp89
(~89 kDa)
B
Stationary Phase
Exponential Phase
H2O2 (5 uM)
GMM
E Medium
pH 4.5
Deoxycholate (0.5%)
AbcR2 (116 nt)
AbcR1 (110 nt)
BAB1_0914 (~400 nt)
BAB2_0512 (~300 nt)
BAB2_0574 (~350 nt)
5S rRNA (119 nt)
C Brucella abortus 2308::bab1_0914-3xFLAG
Stationary Phase
Exponential Phase
H2O2 (5 uM)
GMM
E Medium
pH 4.5
Deoxycholate (0.5%)
α-FLAG (~10 kDa)
α-Omp89 (~89 kDa)
D Brucella abortus 2308::bab2_0512-3xFLAG
Stationary Phase
Exponential Phase
H2O2 (5 uM)
GMM
E Medium
pH 4.5
Deoxycholate (0.5%)
α-FLAG (~10 kDa)
α-Omp89 (~89 kDa)
E Brucella abortus 2308::bab2_0574-3xFLAG
Stationary Phase
Exponential Phase
H2O2 (5 uM)
GMM
E Medium
pH 4.5
Deoxycholate (0.5%)
α-FLAG (~10 kDa)
α-Omp89 (~89 kDa)

Figure 2.2: *bab1_0914*, *bab2_0512*, and *bab2_0574* are activated during stationary phase of growth, low pH, and in the presence of H_2O_2.
A. Western blot analyses for protein expression of *B. abortus* 2308, *B. abortus* 2308::*bab1_0914*-3xFLAG, *B. abortus* 2308::*bab2_0512*-3xFLAG, and *B. abortus* 2308::*bab2_0574*-3xFLAG. Probes include anti-FLAG and anti-Omp89.
B. Northern blot analyses for RNA expression of AbcR1, AbcR2, BAB1_0914, BAB2_0512, and BAB2_0574 during stationary phase and exponential phase of growth in brucella broth, GMM and E medium, and in the presence of H_2O_2 (5 μM), pH4.5, and deoxycholate (0.5%) in brucella broth.
C.D.E. Western blot analyses for protein expression of BAB1_0914-3xFLAG, BAB2_0512-3xFLAG, BAB2_0574-3xFLAG respectively during stationary phase and exponential phase of growth in brucella broth, GMM and E medium, and in the presence of H_2O_2 (5 μM), pH4.5, and deoxycholate (0.5%) in brucella broth. Probes include anti-FLAG and anti-Omp89.

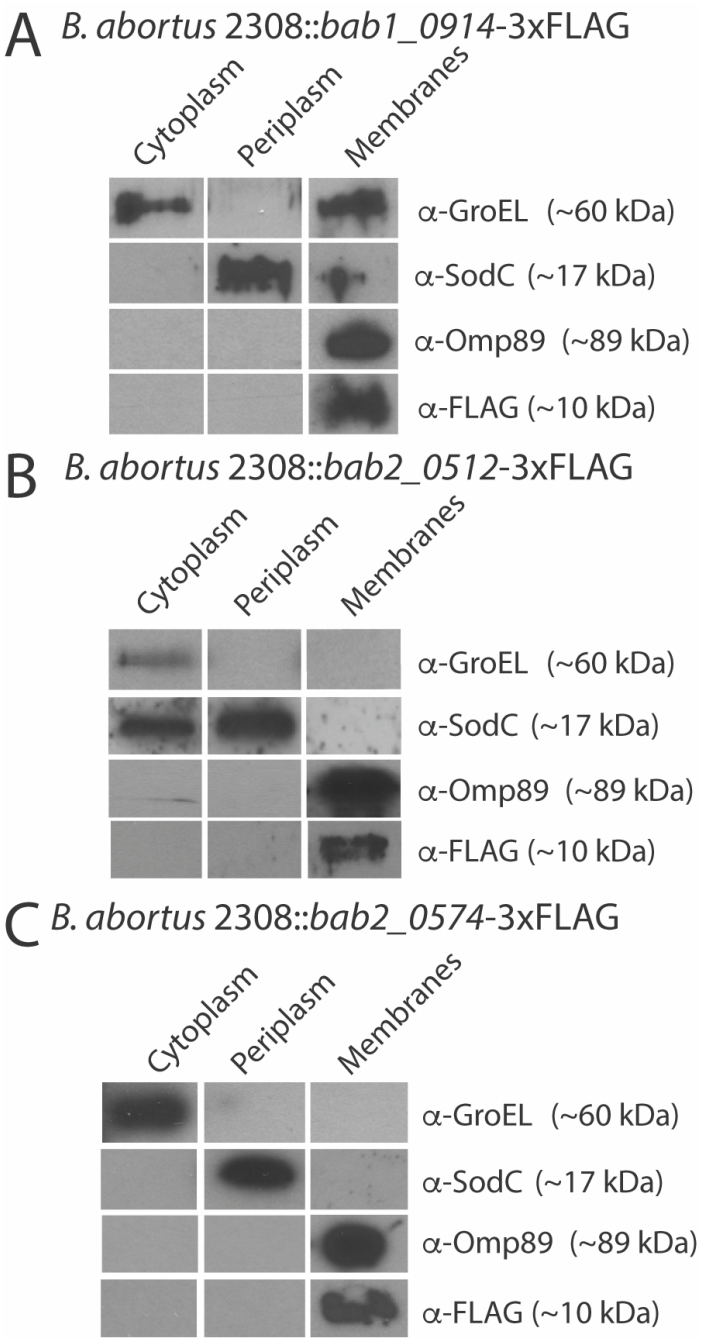


Figure 2.3: Localization of BAB1_0914, BAB2_0512, and BAB2_0574.
Western blot analysis of *Brucella* strains grown to stationary phase followed by fractionation methods described in methods for *B. abortus* 2308::*bab1_0914*-3xFLAG (A), *B. abortus* 2308::*bab2_0512*-3xFLAG (B), and *B. abortus* 2308::*bab2_0574*-3xFLAG (C). Antibodies utilized include α-FLAG, α-Omp89, α-SodC, and α-GroEL.

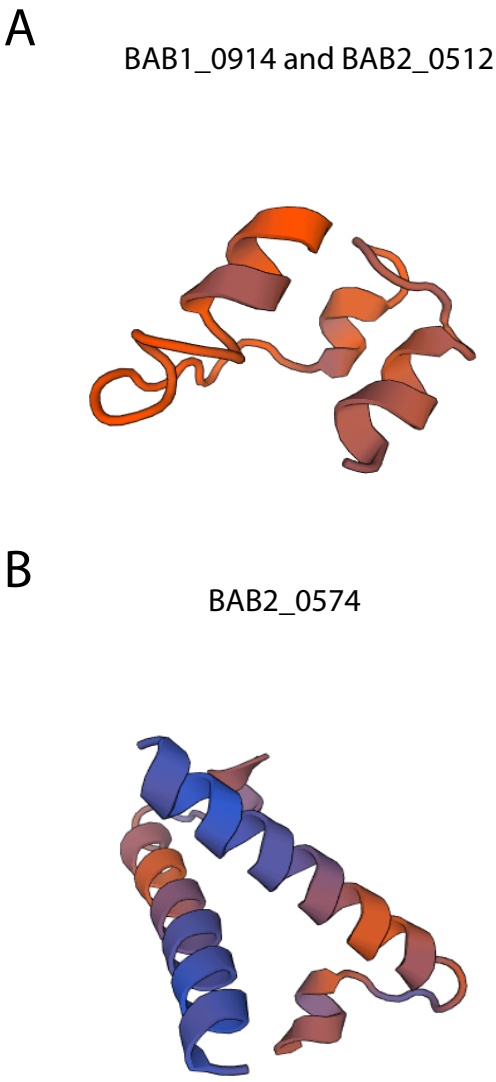
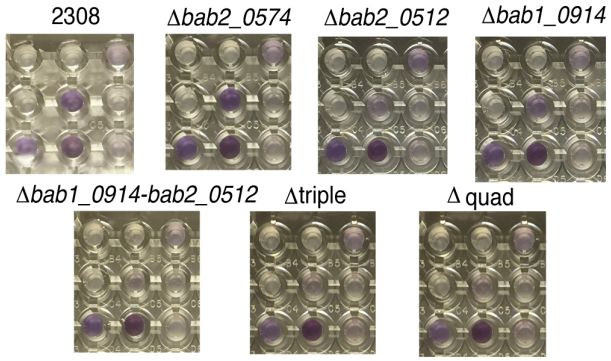
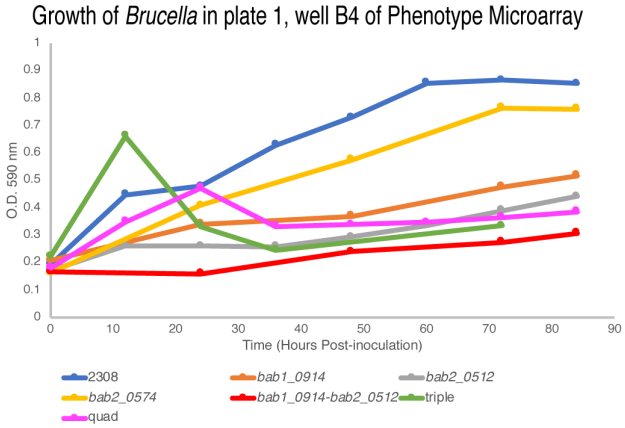


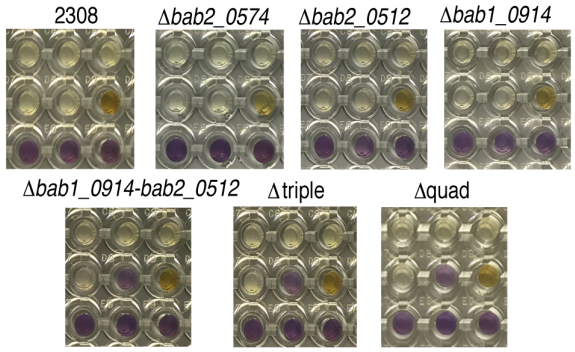
Figure 2.4: Predicted protein structures of BAB1_0914, BAB2_0512, and BAB2_0574.
Protein structure homology-modelling as depicted by the free online server SWISS-MODEL for BAB1_0914 and BAB2_0512 (A) and BAB2_0574 (B).

A Growth of *Brucella* strains in Biolog Phenotype Microarray Plate 1 (L-Fucose center well)





B Growth of *Brucella* strains in Biolog Phenotype Microarray Plate 10 (pH4.5+Hydroxylysine center well)



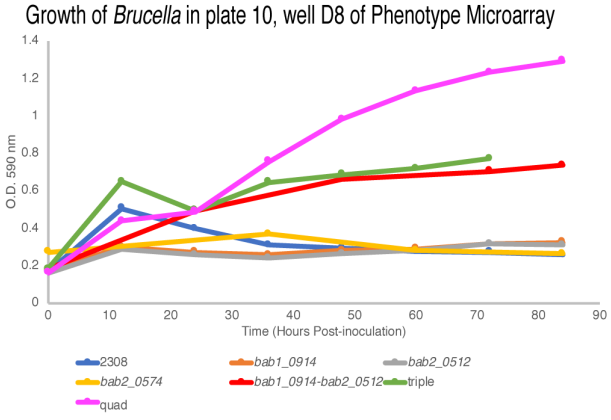


Figure 2.5: Characterization of *B. abortus* 2308 and VtlR regulon mutants using Biolog Phenotype Microarray plates.
A. Growth of *Brucella* strains in plate 1, well B4, in minimal medium+24 mM L-fucose, over 84 hours of growth. Pictures of plate 1 well B4 (center well) after 84 hours of growth. O.D. 590 nm of plate 1 well B4 over 84 hours of incubation.
B. Growth of *Brucella* strains in plate 10 well D8, minimal medium pH4.5+hydroxylysine, over 84 hours of growth. Pictures of plate 10 well D8 (center well) after 84 hours of growth. O.D. 590 nm of plate 10 well D8 over 84 hours of incubation.

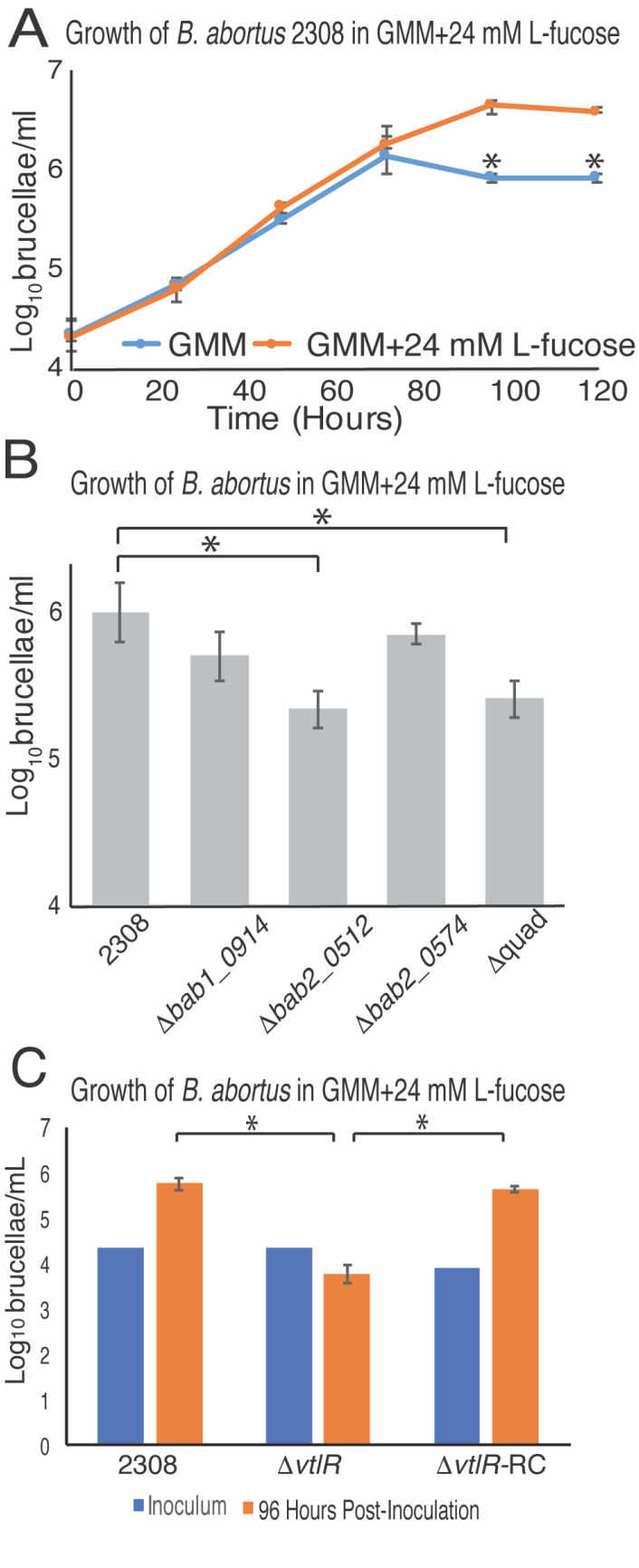

A
Growth of B. abortus 2308 in GMM+24 mM L-fucose
Log10 brucellae/ml
Time (Hours)
GMM
GMM+24 mM L-fucose
B
Growth of B. abortus in GMM+24 mM L-fucose
Log10 brucellae/ml
2308
Δbab1_0914
Δbab2_0512
Δbab2_0574
Δquad
C
Growth of B. abortus in GMM+24 mM L-fucose
Log10 brucellae/mL
2308
ΔvtlR
ΔvtlR-RC
Inoculum
96 Hours Post-Inoculation

Figure 2.6: L-fucose contribution to *B. abortus* growth and growth inhibition in VtlR regulon mutants.

Cultures of Gerhardt's minimal medium (GMM) supplemented with and without the addition of 24 mM L-fucose were inoculated with *Brucella* strains at an initial concentration of $5x10^4$ CFU/ml and incubated at 37^oC.

A. *B. abortus* 2308 grows to a higher cell density in GMM supplemented with 24 mM L-fucose than GMM without 24 mM L-fucose.

B. In the presence of L-fucose, *B. abortus* 2308::Δ*bab2_0512* and *B. abortus* 2308::Δ*abcR2*-Δ*bab1_0914*-Δ*bab2_0512*-Δ*bab2_0574* (Δ*quad*) growth is significantly lower than *B. abortus* 2308.

C. *B. abortus* 2308::Δ*vtlR* is sensitive to the presence of 24 mM L-fucose in GMM. Statistical significance (*) was determined using one-way analysis of variance (ANOVA, $P<0.05$).

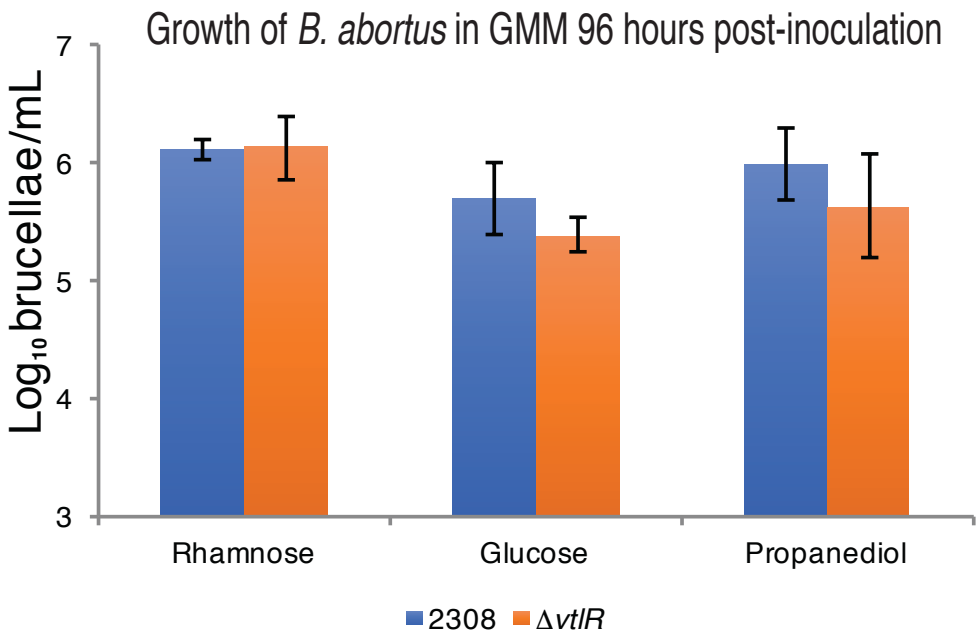


Figure 2.7: *B. abortus* 2308::Δ*vtlR* sensitivity to L-rhamnose, D-glucose, and propanediol.
B. abortus 2308::Δ*vtlR* growth 96 hours post-inoculation in GMM in the presence of 24 mM L-rhamnose, D-glucose, or propanediol.

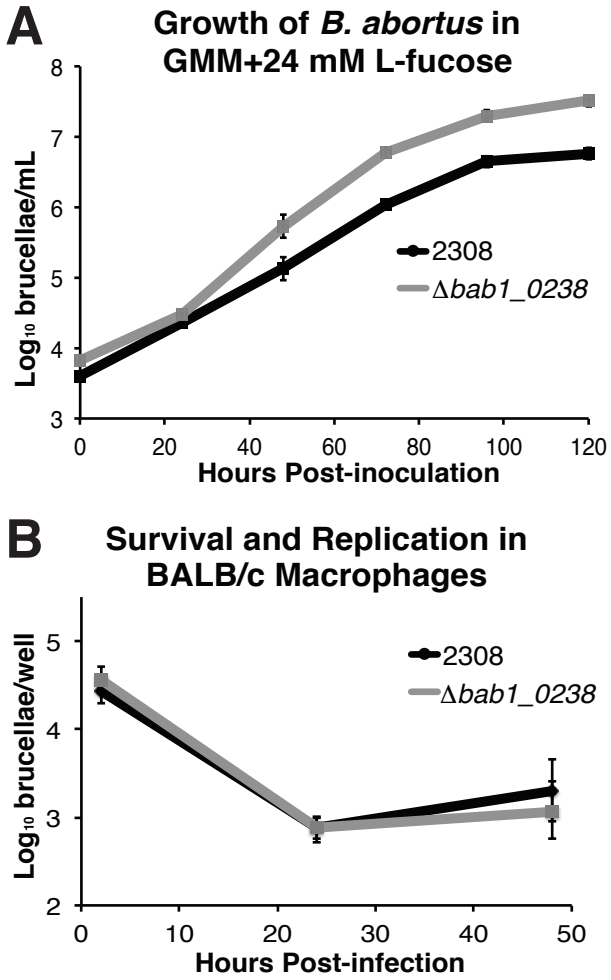


Figure 2.8: Growth kinetics and virulence of *B. abortus* 2308 and *B. abortus* 2308::Δ*bab1_0238*.

A. Growth of *B. abortus* strains in Gerhardt's minimal medium (GMM) + 24 mM L-fucose. Cultures of (GMM) supplemented with and without the addition of 24 mM L-fucose were inoculated with *Brucella* strains at an initial concentration of ~5x10^3 CFU/ml and incubated at 37^{o}C. Samples were collected and serial diluted to calculate Log10 brucellae/mL every 24 hours.

B. Macrophage survival and replication experiments. Primary peritoneal macrophages from BALB/c mice were infected with *B. abortus* 2308 or the isogenic *bab1_0238* deletion strain (Δ*bab1_0238*). Macrophages were lysed 2, 24, and 48 hours post-infection, and the number of intracellular brucellae was determined by serial dilatation and plating on agar medium.

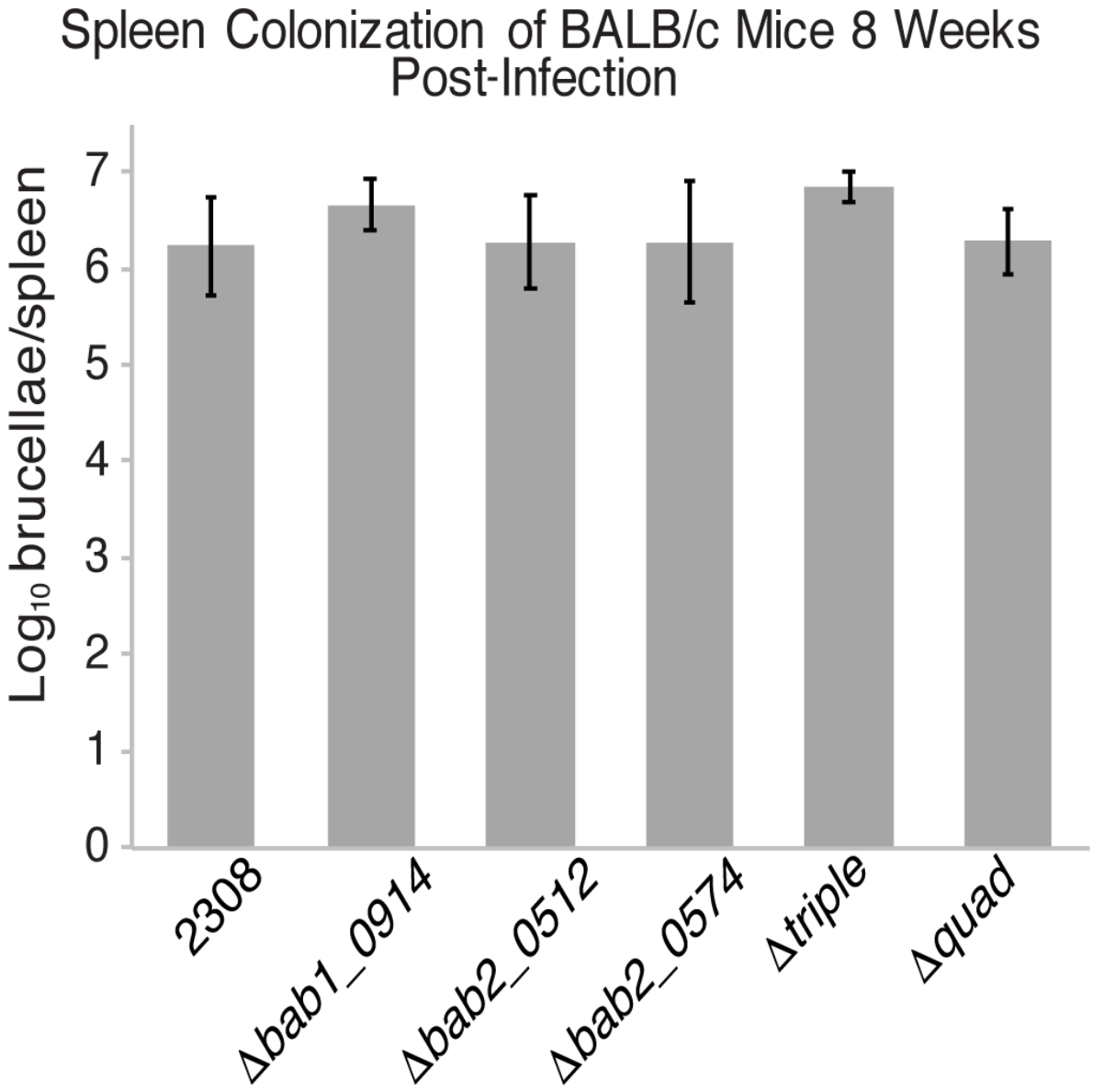


Figure 2.9: *bab1_0914*, *bab2_0512*, and *bab2_0574* do not contribute to the ability of *B. abortus* 2308 to colonize the spleens of BALB/c mice.
BALB/c mice were infected intraperitoneally with 10^5 CFU *B. abortus* 2308, *B. abortus* 2308::Δ*bab1_0914, B. abortus* 2308::Δ*bab2_0512*, *B. abortus* 2308::Δ*bab2_0574*; *B. abortus* 2308::Δ*bab1_0914*-Δ*bab2_0512*-Δ*bab2_0574* (Δtriple); or *B. abortus* 2308::Δ*abcR2*-Δ*bab1_0914*-Δ*bab2_0512*-Δ*bab2_0574* (Δquad). Mice were sacrificed at 8 weeks post-infection, and the number of brucellae colonizing the spleens was determined. The data is presented as the average brucellae +/- the standard deviation from the 5 mice colonized with a specific *Brucella* strain.

Chapter 3: Activation of small regulatory RNAs by VtlR during transcriptional regulation of myriad genes in *A. tumefaciens* str. C58.

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*J.A.B. and L.M.S. contributed equally to this work.

Abstract

LysR-type transcriptional regulators (LTTRs) are the most common type of transcriptional regulators in prokaryotes and function by altering gene expression in response to environmental stimuli. In the Class *Alphaproteobacteria*, a conserved LTTR named VtlR is critical to the establishment of host-microbe interactions. In the mammalian pathogen *Brucella abortus*, VtlR is required for full virulence in a mouse model of infection, and VtlR activates the expression of *abcR2*, which encodes a small regulatory RNA (sRNA). In the plant symbiont *Sinorhizobium meliloti*, the ortholog of VtlR, named LsrB, is involved in the symbiosis of the bacterium with alfalfa. *Agrobacterium tumefaciens* is a close relative of both *B. abortus* and *S. meliloti*, and this bacterium is the causative agent of crown gall disease in plants. In the present study, we demonstrate that VtlR is involved in the ability of *A. tumefaciens* to grow appropriately in artificial medium, and an *A. tumefaciens vtlR* deletion strain is defective in biofilm formation and tumorigenesis of potato discs. RNA-sequencing analyses revealed that more than 250 genes are dysregulated in the Δ*vtlR* strain, and importantly, VtlR directly controls the expression of three sRNAs in *A. tumefaciens*. Taken together, these data support a model in which VtlR indirectly regulates hundreds of genes via management of sRNA pathways in *A. tumefaciens*, and moreover, while the VtlR/LsrB protein is present and structurally conserved in many members of the *Alphaproteobacteria*, the VtlR/LsrB regulatory circuitry has diverged in Order to accommodate the unique environmental niche of each organism.

Importance

The Class *Alphaproteobacteria* encompasses a highly diverse group of bacteria containing host-associated symbionts and pathogens, as well as free-living organisms. Questions often rise about the conservation of gene function between phylogenetically related organisms, and the present study sheds light on the commonalities and differences of an important regulatory pathway in three related *Alphaproteobacteria*: *Agrobacterium tumefaciens*, *Brucella abortus*, and *Sinorhizobium meliloti*. This work sought to characterize the regulatory mechanism of a highly conserved transcriptional regulator, called VtlR/LsrB, in these members of the *Alphaproteobacteria*, while highly similar in amino acid sequence, the VtlR/LsrB regulators have considerably different regulatory activities in each bacterium. Altogether, while some assumptions can be made across organisms, genes and proteins deemed "highly similar" may in fact serve very dissimilar functions to the physiology and biology of related bacteria.

Introduction

LysR-type transcriptional regulators (LTTRs) are well represented in the three domains of life, and encompass the most common type of transcriptional regulator in prokaryotes (Maddocks & Oyston, 2008). First documented in 1988, this Class of regulators can act as both activators and repressors of gene expression (Henikoff *et al.*, 1988). LTTRs are composed of two domains: a well-conserved N-terminal DNA-binding domain and a variable C-terminal substrate-binding domain.

The LTTR N-terminal domain is most commonly found as a helix-turn-helix, and regulated targets have a 'Classic' LTTR-binding box sequence of TTA-$N_{7/8}$-TAA (Maddocks & Oyston, 2008). The C-terminal domain is more variable among LTTRs. This domain can bind to a specific substrate and alter the activity of the protein. Some examples of substrate-sensing LTTRs include AphB from *Vibrio cholerae* (Kovacikova *et al.*, 2010, Liu *et al.*, 2011, Liu *et al.*, 2016, Privett *et al.*, 2017, Taylor *et al.*, 2012), BenM and CatM from *Acinetobacter baylyi* (Craven *et al.*, 2009, Ezezika *et al.*, 2006, Ruangprasert *et al.*, 2010), CbbR from *Rhodobacter sphaeroides* (Dangel *et al.*, 2005, Smith & Tabita, 2002, Tichi & Tabita, 2002), and OccR from *Agrobacterium tumefaciens* (Akakura & Winans, 2002, Wang *et al.*, 1992). Alternatively, LTTRs can also undergo conformational changes without binding to a substrate. This is the case with OxyR, a redox-sensing LTTR responsible for activating genes important for responding to reactive oxygen species (Kullik *et al.*, 1995, Zheng *et al.*, 1998). Overall, LTTRs play important regulatory roles in bacteria, allowing organisms to sense environmental cues and, in turn, swiftly alter gene expression through transcriptional activation and/or repression.

In the Class *Alphaproteobacteria*, one highly conserved LTTR has been linked to efficient and effective host-bacterium interactions. First identified in the plant symbiont *Sinorhizobium meliloti*, the LTTR named LsrB (for **L**ysR-type **s**ymbiosis **r**egulator) is critical for the symbiosis of the bacterium and its host alfalfa (*Medicago sativa*) (Luo *et al.*, 2005). Regarding regulatory roles, *S. meliloti* LsrB is involved in the regulation of genes required for synthesizing glutathione and lipopolysaccharide (Lu *et al.*, 2013, Tang *et al.*, 2014). Recently, *S. meliloti* LsrB was shown to have a similar sensing mechanism as OxyR, where the formation of intermolecular disulfide bonds in LsrB is involved in adaptation to oxidative stress, regulation of gene expression, proper alfalfa nodulation, and effective nitrogen fixation (Tang *et al.*, 2017).

An orthologous LTTR has also been characterized in the mammalian pathogen *Brucella abortus* (Sheehan *et al.*, 2015a). In *B. abortus*, this LTTR, named VtlR (for **v**irulence-associated **t**ranscriptional **L**ysR-family **r**egulator), was shown to be a critical component in the ability of the bacterium to cause infection in both macrophages and mice. Microarray analysis revealed that *B. abortus* VtlR activates the expression of three genes encoding for small proteins. In addition, VtlR also positively regulates *abcR2*, encoding a sibling small regulatory RNA (sRNA) of the AbcR family. The AbcR sRNAs have been well documented to be involved in nutrient acquisition in the *Rhizobiales* (Becker *et al.*, 2014, Caswell *et al.*, 2012, Overloper *et al.*, 2014, Sheehan & Caswell, 2017, Torres-Quesada *et al.*, 2013, Torres-Quesada *et al.*, 2014, Wilms *et al.*, 2011, Sheehan *et al.*, 2015a).

sRNAs are key regulatory components in bacteria and allow for rapid modification of gene expression, most commonly through post-transcriptional activation

or repression of target mRNAs (Storz *et al.*, 2011). Two sRNAs found throughout the Order *Rhizobiales* are the sibling AbcR sRNAs, AbcR1 and AbcR2 (Sheehan & Caswell, 2018). The AbcR sRNAs regulate target mRNAs encoding ABC-type transport systems, many of which are responsible for transporting nutrients in specific environmental conditions (Becker *et al.*, 2014, Caswell *et al.*, 2012, Overloper *et al.*, 2014, Sheehan & Caswell, 2017, Torres-Quesada *et al.*, 2013, Torres-Quesada *et al.*, 2014, Wilms *et al.*, 2011). Several of these mRNA targets encode transport systems found in *A. tumefaciens*, *S. meliloti* and *B. abortus*, and moreover, have been shown to be regulated by one or both of the AbcR sRNAs. However, it was unknown if the conservation of the AbcR system includes the transcriptional regulation of the sRNAs by VtlR/LsrB.

The present study aimed to characterize the LTTR VtlR in the plant pathogen *Agrobacterium tumefaciens*. Recently, it was reported that the VtlR ortholog in *A. tumefaciens* is required for efficient host-bacterium interactions, as well as other important processes, such as exopolysaccharide production, biofilm formation, and resistance to oxidative stress (Tang *et al.*, 2018). The present study confirms that VtlR is important for interactions between *A. tumefaciens* and plants, but this work also demonstrates that the significant global gene dysregulation observed in the *A. tumefaciens* Δ*vtlR* strain results predominantly from the direct activation of three small transcripts, all of which are authentic or purported small regulatory RNAs. Interestingly, we also demonstrate that LsrB was not involved in the expression of the AbcR sRNAs in *S. meliloti*, indicating that the VtlR/LsrB regulatory pathway has diverged significantly across members of the *Alphaproteobacteria*. Overall, the regulatory activity of VtlR

differs dramatically from one bacterium to another, suggesting VtlR has evolved to fulfill the regulatory and environmental requirements of each particular bacterium.

Results

Expression of the sRNA AbcR1 is dependent on VtlR, and deletion of *vtlR* in *A. tumefaciens* results in a significant lag in growth *in vitro.*

The *vtlR* gene, designated as *atu2186,* is located on the circular chromosome of *A. tumefaciens* strain C58 (Figure 3.1A). Directly upstream of *vtlR* is *trxB* (*atu2185*), a gene encoding a thioredoxin reductase; and downstream of *vtlR* are the genes encoding the AbcR sRNAs, *abcR1* and *abcR2*. It is interesting to note that the genetic organization of *trxB* and *vtlR* is well conserved in the Class *Alphaproteobacteria*.

In *B. abortus*, VtlR has been shown to positively regulate the tandemly encoded sRNA *abcR2* on chromosome I; however the expression of *abcR1* on chromosome II of *B. abortus* is not regulated by VtlR (Sheehan *et al.*, 2015a). Dissimilar from *B. abortus*, *abcR1* and *abcR2* are encoded directly downstream of *vtlR/lsrB* on the same chromosome in *A. tumefaciens* str. C58 (Figure 3.1A) and *S. meliloti 1021*. It was unknown, however, if either of these sRNAs were regulated by the homolog of VtlR/LsrB (Atu2186 and SMc01225) in *A. tumefaciens* or *S. meliloti* respectively. To test whether VtlR regulated *abcR1* and/or *abcR2* in *A. tumefaciens*, northern blot analyses were performed to measure expression of AbcR1 and AbcR2 in wild-type *A. tumefaciens*, the isogenic deletion strains of *abcR1* (Δ*abcR1*) and *abcR2* (Δ*abcR2*), a double deletion strain of *abcR1 and abcR2* (Δ*abcR1/2*), and an isogenic deletion strain of *vtlR* (Δ*vtlR*) (Figure 3.1B). Bands representing the AbcR1 and AbcR2 transcripts were clearly visible when the *A. tumefaciens* culture was grown to an O.D. of 4.0 (Figure 3.1B). Importantly, the AbcR1 and AbcR2 transcripts were not present in the isogenic deletion strains, confirming that these strains represent the desired deletions of the indicated genes. Furthermore, the

northern blot analyses also demonstrated that the expression level of AbcR2 was unaffected in Δ*vtlR*, but AbcR1 production was abolished in Δ*vtlR*. These data indicated that VtlR positively influenced the expression of AbcR1, but not AbcR2, in *A. tumefaciens*. A similar strategy was employed to measure the expression of AbcR1 and AbcR2 in *S. meliloti 1021* and *S. meliloti 1021*::Δ*lsrB*. Northern blot analysis revealed no change in the expression of the AbcRs in the absence of *lsrB*; indicating that *lsrB* does not regulate the expression of AbcR1 or AbcR2 in *S. meliloti* (Figure 3.2).

In the plant symbiont *S. meliloti*, a deletion of *lsrB* resulted in a significant growth defect (Luo *et al.*, 2005, Tang *et al.*, 2014); however, a deletion of *vtlR* in *B. abortus* resulted in no significant differences in growth when grown in nutrient rich or nutrient limiting media (Sheehan *et al.*, 2015a). *A. tumefaciens* str. C58, Δ*vtlR*, Δ*abcR1*, Δ*abcR2*, and Δ*abcR1/2* were grown in a nutrient-rich medium, and the number of colony-forming units (CFU) were measured every 12 hours to examine growth over time in the deletion backgrounds to assess the necessity of *vtlR* for growth of *A. tumefaciens* (Figure 3.1C). *A. tumefaciens* Δ*vtlR* displayed no difference in growth kinetics compared to the parental strain; however, similar to *S. meliloti*, *A. tumefaciens* Δ*vtlR* exhibited a lag in growth in liquid medium and small colony phenotype on agar medium when compared to the parental strain C58 (Figure 3.1C and 3.1D). Isogenic deletions of *abcR1* and *abcR2*, and a double deletion of *abcR1* and *abcR2,* resulted in no differences in *A. tumefaciens* growth when compared to the parental strain (Figure 3.1C). The lag in growth of *A. tumefaciens* Δ*vtlR* was rescued by in-trans complementation with the plasmid pSRK-Km harboring an IPTG-inducible wild-type *vtlR* gene (Figure 3.1D and 3.9) (Khan *et al.*, 2008). As a control, *A. tumefaciens* Δ*vtlR* harboring an empty pSRK-Km plasmid showed no

difference in growth compared to *A. tumefaciens* Δ*vtlR* (Figure 3.1D). Altogether, these data revealed that VtlR positively regulates the sRNA AbcR1, and that VtlR is critical to the growth of *A. tumefaciens* in artificial medium.

VtlR is critical to the virulence and biofilm formation of *A. tumefaciens.*

VtlR was previously shown to be necessary for the symbiosis and pathogenesis of *S. meliloti* and *B. abortus* (Luo *et al.*, 2005, Sheehan *et al.*, 2015a). Moreover, recent work has demonstrated a role for VtlR in the pathogenesis of *A. tumefaciens* for attachment of the bacteria to the roots of *Arabidopsis* plants, as well as for the efficient transformation of tobacco leaves (Tang *et al.*, 2018). Since attachment and transformation are crucial virulence factors for the pathogenesis of *A. tumefaciens*, we hypothesized that *A. tumefaciens* Δ*vtlR* would also exhibit reduced tumorigenesis compared to the wild-type strain C58. To test this hypothesis, *A. tumefaciens* Δ*vtlR* was assessed for its ability to form tumors in experimentally infected potatoes (Figure 3.3A). Compared to *A. tumefaciens* C58, the *vtlR* deletion strain caused, on average, the formation of fewer tumors; however, given the significant inter-experiment variability, this reduction in tumors was not statistically significant. Furthermore, no other strains tested (i.e., Δ*abcR1*, Δ*abcR2*, or Δ*abcR1/2*) were attenuated compared to the wild-type strain (data not shown).

To further examine the function of VtlR in *A. tumefaciens*, biofilm production by Δ*vtlR* was assessed (Figure 3.3B). *A. tumefaciens* Δ*abcR1*, Δ*abcR2*, and Δ*abcR1/2* displayed no differences in biofilm production when compared to the parental strain C58 (data not shown). However, the *A. tumefaciens* Δ*vtlR* produced significantly less biofilm than the parental strain (Figure 3.3B). This phenotype was genetically complemented

with the introduction of the pSRK-Km plasmid harboring the *A. tumefaciens vtlR* gene. Taken together, these data demonstrated that VtlR is important for the ability of *A. tumefaciens* to efficiently produce biofilms, and this is consistent with previous work produced by Tang et al. linking VtlR to *A. tumefaciens* biofilm formation (Tang *et al.*, 2018).

The small RNA AbcR1 primarily regulates ABC-type transport systems.

To better understand the role of VtlR in *A. tumefaciens* pathogenesis, we sought next to define the transcriptional regulons of VtlR, AbcR1, and AbcR2. RNA-sequencing (RNA-seq) was carried out to compare RNA levels in Δ*vtlR*, Δ*abcR1*, and Δ*abcR2* to the parental strain C58 when cultured to late exponential phase in nutrient rich broth. Although AbcR1 was dispensable for *A. tumefaciens* virulence, the regulon of AbcR1 is robust with almost 100 genes differentially expressed ≥3-fold in Δ*abcR1* when compared to the wild-type strain C58 (Table 3.1). In comparison, only 8 genes were differentially expressed in Δ*abcR2*, and interestingly, half of the AbcR2-regulated genes encode hypothetical proteins (Table 3.2). The significant difference in terms of the number of transcripts showing altered levels between AbcR1 over AbcR2 is in line with previous studies showing that AbcR1 exhibits more regulatory activity that AbcR2 in *Agrobacterium* (Overloper *et al.*, 2014, Wilms *et al.*, 2011). Overall, the majority of genes differentially expressed in the *abcR1* deletion strain encode components of ABC-type transporters and membrane proteins (56%), followed by hypothetical proteins (22%), and genes involved in enzymatic processes (18%) (Figure 3.4A). Regulation of these targets is confirmed via previously published proteomic analysis of target gene

expression differences between the parental strain and an *abcR1* deletion strain (Overloper *et al.*, 2014).

The *A. tumefaciens* VtlR transcriptional regulon is comprised of over 250 genes, including the AvhB type IV secretion system and a conjugal transfer system.

RNA-seq analysis identified over 250 dysregulated genes in *A. tumefaciens* Δ*vtlR* when grown in LB broth (i.e., nutrient rich medium). Genes differentially expressed ≥3-fold in Δ*vtlR* are depicted in Table 3.3, and qRT-PCR was utilized to validate differential expression of several of these targets in Δ*vtlR*. An outline of the functional Classification of the proteins encoded by the differentially expressed genes is shown in Figure 3.4B. Of these dysregulated genes, 33% are predicted to encode membrane proteins and transport systems, 33% encode hypothetical proteins, and approximately 19% encode protein involved in metabolism, signaling, and enzymatic processes (Figure 3.4B).

A comparison of the VtlR and AbcR1 regulons shed light on the dependent and independent regulatory functions each possesses (Figure 3.4C). For example, the VtlR regulon was found to be significantly larger than the AbcR1 regulon, with 254 dysregulated genes in Δ*vtlR* compared to 95 dysregulated genes in Δ*abcR1*. Approximately half of the genes differentially expressed ≥3-fold in *abcR1* were also differentially expressed in Δ*vtlR*. Altogether, these data revealed >210 genes that could potentially be regulated by VtlR in an AbcR1-independent manner.

***A. tumefaciens* VtlR directly activates the expression of *abcR1* and *atu1667*, a small hypothetical protein.**

To further characterize the regulatory mechanism of *A. tumefaciens* VtlR, electrophoretic mobility shift assays (EMSAs) were employed to test for the ability of recombinantly

purified VtlR (rVtlR) to interact directly with the promoter regions of putative regulatory targets. As expected, VtlR bound to the promoter region on *abcR1* in a concentration-dependent manner (Figure 3.5A). Moreover, the addition of unlabeled *abcR1* promoter region DNA competitively inhibited binding between rVtlR and the radiolabeled *abcR1* promoter, while excess unlabeled *abcR2* promoter DNA did not affect the formation of the rVtlR-P_{abcR1} binding complex. Overall, these data demonstrated that VtlR binds directly to the *abcR1* promoter region to activate *abcR1* expression in *A. tumefaciens*.

In *A. tumefaciens*, the gene *atu1667* encodes for a small hypothetical protein that is orthologous to BAB1_0914 and BAB2_0512 in *B. abortus*. *bab1_0914* and *bab2_0512* are directly transcriptionally activated by VtlR in *B. abortus*, and thus, it was hypothesized that VtlR also directly activates the expression of *atu1667* in *A. tumefaciens* (Sheehan *et al.*, 2015a). To test this hypothesis, northern blot analysis was used to assess Atu1667 RNA levels in the *A. tumefaciens vtlR* deletion strain (Figure 3.5B). Northern blot analysis and RNAseq analysis (>8-fold differentially expressed in Δ*vtlR*) showed that Atu1667 RNA levels were significantly decreased in *A. tumefaciens* Δ*vtlR* compared to the parental strain, suggesting that VtlR activates expression of *atu1667*. To assess potential binding between VtlR and the *atu1667* promoter region, EMSAs were performed with a radiolabeled promoter region of *atu1667* and rVtlR (Figure 3.5C). As a result of these experiments, rVtlR bound directly and specifically to the promoter of *atu1667*, indicating that VtlR is a direct transcriptional activator of *atu1667* in *A. tumefaciens*.

A bioinformatic approach was employed to align the upstream regions of *abcR1* and *atu1667* and determine sequence similarities between the two promoters to identify a

binding motif. Alignment of the two promoters led to the identification of a putative VtlR-binding sequence composed of 15 DNA base pairs (Figure 3.5D).

Identification of a VtlR-binding consensus sequence and discovery of a novel VtlR-regulated sRNA.

Bioinformatic analyses of this putative VtlR consensus sequence (Figure 3.5D) was utilized to search for other potential VtlR binding sites in the *A. tumefaciens* genome via the use of the online service Virtual Footprint (Munch *et al.*, 2005). Surprisingly, only one area displayed a match to the *abcR1/atu1667* VtlR-binding consensus sequence using this approach, and the identified area corresponded to an intergenic region flanked by *atu4669* and *atu4670* (Figure 3.6A and 3.6B). Subsequently, an EMSA was performed with rVtlR and DNA that encompasses the *atu4669* and *atu4670* intergenic region (Figure 3.6C). rVtlR bound directly and specifically to this intergenic region DNA.

Importantly, the promoter regions of *atu4669* and *atu4670* are within this intergenic region of the chromosome; in contrast, this intergenic region is located between the 3' ends of both *atu4669* and *atu4670* (Figure 3.6B). Therefore, if VtlR is binding to a promoter region of a gene it regulates, then there may be a previously unannotated gene transcribed antisense to either *atu4669* or *atu4670*. To determine if an unannotated gene in encoded in this region, northern blot analysis was carried out with RNA isolated from C58 and Δ*vtlR*. Northern blot analysis revealed the presence of a ~300 nucleotide transcript antisense to *atu4670* (Figure 3.6D). This small RNA was first identified as "L4" by Wilms et al. during a screen for previously unidentified sRNAs in the *Agrobacterium tumefaciens* genome (Wilms *et al.*, 2012).

The expression of the "L4" transcript was abolished in Δ*vtlR*. The evidence of direct binding of rVtlR to this DNA region and the decreased expression of the newly identified transcript in the *vtlR* deletion strain suggested that this new small RNA is under the direct transcriptional control of VtlR in *A. tumefaciens*. Thus, we have named this small RNA VrsA, for VtlR regulated small RNA.

The role of VtlR in sRNA regulation.

Mentioned above, Wilms et al utilized a differential RNA sequencing (dRNA-seq) strategy to identify novel sRNAs on all four of the *A. tumefaciens* replicons. They identified 228 new sRNAs in the *A. tumefaciens* genome via dRNA-seq and confirmed the existence of 22 of these sRNAs via northern blot analysis (Wilms *et al.*, 2012). The Δ*vtlR* RNAseq data was examined for differentially expressed sRNAs identified in the Wilms et al. dataset. Of the 228 sRNAs identified by Wilms et al., 24 sRNAs were predicted to be differentially expressed in *A. tumefaciens* Δ*vtlR* (Table 3.5). Under the conditions tested, RNA was isolated from *A. tumefaciens* grown in LB broth to late exponential phase, but only 7 of these sRNAs could be visualized via northern blot analyses and 2 were differentially expressed in *A. tumefaciens* Δ*vtlR* with restored expression in the complemented strain Δ*vtlR*::pSRK-Km-*vtlR*; one of which was the aforementioned VrsA (Figure 3.7). The second dysregulated sRNA in Δ*vtlR* is located within the intergenic region between *atu0985* and *atu0986* and is approximately 225 nucleotides in length; however, the proposed VtlR binding box is not found upstream of this new sRNA.

VrsA does not contribute to the growth of *A. tumefaciens* in LB broth.

A. tumefaciens Δ*abcR1* did not exhibit similar phenotypes observed in Δ*vtlR* and RNAseq analysis showed that only a portion (~15%) of the genes dysregulated in Δ*vtlR* are potentially regulated in an AbcR1-dependent manner. With the discovery that VtlR also regulated the expression of *vrsA*, it is plausible that a large portion (>210 genes) are VrsA-dependently regulated genes in Δ*vtlR*. To examine this hypothesis, an unmarked in-frame deletion of *atu4670*, the cis-encoded gene to *vrsA*, was constructed and utilized to perform phenotypic and RNAseq analyses.

A growth curve was performed similarly to what was performed for Figure 3.1C. LB broth was inoculated with either $5x10^3$ CFU/mL *A. tumefaciens* str. C58 or *A. tumefaciens* Δ*vtlR* and grown at 28°C with shaking for 72 hours. Log_{10}CFU/mL was determined for each culture every 12 hours. *A. tumefaciens* Δ*vrsA* growth was not significantly different when compared to the parental strain *A. tumefaciens* C58 (Figure 3.8). Further phenotypic and transcriptomic analyses are currently being conducted in our lab.

Heterologous complementation of *A. tumefaciens* Δ*vtlR* with *S. meliloti lsrB* or *B. abortus vtlR.*

VtlR/LsrB proteins are highly conserved in many members of the *Rhizobiales*, and thus, it was hypothesized that the *A. tumefaciens vtlR* deletion strain could be functionally complemented with a heterologous allele. The *A. tumefaciens* VtlR and *S. meliloti* LsrB, proteins share over 88% identity in amino acid sequence, whereas *A. tumefaciens* VtlR and *B. abortus* VtlR share 68% amino acid sequence identity. In *S. meliloti*, deletion of *lsrB* results in a severe growth defect in rich medium (Luo *et al.*, 2005). In contrast, there was no difference in the growth kinetics of *B. abortus* when *vtlR* was deleted (Sheehan *et*

al., 2015a). To test our hypothesis, *vtlR* from *A. tumefaciens*, *vtlR* from *B. abortus,* and *lsrB* from *S. meliloti* were individually cloned into the expression vector pSRK-Km to complement the *A. tumefaciens vtlR* deletion strain, and the strains were assessed for growth *in vitro*. When cultured on AT agar, wild-type colony size was restored with complementation of all three *vtlR* genes (Figure 3.1D and 3.9A). Thus, heterologous complementation of *vtlR* from *A. tumefaciens*, *vtlR* from *B. abortus,* and *lsrB* from *S. meliloti* was able to restore the small colony phenotype of *A. tumefaciens* Δ*vtlR*.

To assess the ability of VtlR orthologs to complement the *A. tumefaciens vtlR* growth defect in liquid medium, growth curves over time were conducted in rich medium supplemented with 100 μM IPTG and 45 μg/mL kanamycin (Figure 3.9B). As a control, the growth defect displayed by *A. tumefaciens* Δ*vtlR* was not complemented by the presence of an empty pSRK-Km plasmid. *A. tumefaciens* Δ*vtlR* harboring the *lsrB* allele from *S. meliloti* or *vtlR* from *A. tumefaciens* restored wild-type growth kinetics (Figure 3.9B). However, *vtlR* from *B. abortus* only partially restored growth of the *A. tumefaciens* Δ*vtlR*, revealing a potential divergence in function of *B. abortus* VtlR from other *Rhizobiales*.

Discussion

In the present study, we have characterized a conserved LTTR named VtlR in the plant pathogen *A. tumefaciens* by defining its role in biological processes (i.e., tumorigenesis and biofilm formation) and genetic regulation (Figure 3.1-3.9; Table 3.3). Furthermore, this study revealed similarities and differences amongst three VtlR/LsrB orthologs from *A. tumefaciens*, *S. meliloti*, and *B. abortus* (Figure S3). This work also further characterizes the regulatory capabilities of the AbcR sRNAs in *Agrobacterium* pathogenesis (Figure 3.1, 3.4; Tables 3.1 and 3.2).

The requirement of *vtlR* in *A. tumefaciens* pathogenesis resembles what was previously reported in two other *Rhizobiales*: *B. abortus* and *S. meliloti* (Luo *et al.*, 2005, Sheehan *et al.*, 2015a). Moreover, our data supported the observation that VtlR is important for host-bacterium interactions of *A. tumefaciens* with plants (Tang *et al.*, 2018). However, the dispensability of *abcR1* and *abcR2* in *A. tumefaciens* for efficient host-bacterium interactions and for proper growth of the bacteria differs from *S. meliloti* and *B. abortus*. In *S. meliloti*, deletion of *abcR1* or *abcR2* does not affect symbiosis, but a deletion of *abcR1* causes an acute growth defect in nutrient-rich medium (Torres-Quesada *et al.*, 2013). In *B. abortus*, AbcR1 and AbcR2 are functionally redundant, and despite displaying no differences in bacterial growth in nutrient-rich or nutrient-limiting conditions, a *B. abortus* deletion strain of both *abcR1* and *abcR2* is less able to colonize and survive in macrophages and experimentally infected mice (Caswell *et al.*, 2012). Conversely, AbcR1 and AbcR2 in *A. tumefaciens* are not functionally redundant. In *A. tumefaciens,* AbcR1 contains two RNA-binding motifs, named M1 (for motif 1) and M2 (for motif 2), and these motifs are utilized by AbcR1 to interact with target mRNAs

(Overloper *et al.*, 2014). The reason AbcR2 lacks robust regulatory functionality may have to do with the absence of M1 from its nucleotide sequence (Sheehan & Caswell, 2018). Altogether, data from this study supports previous work, where RNA-seq analysis found a deletion of *abcR1*, not *abcR2*, to lead to significant gene dysregulation (Figure 3.4; Tables 3.1 and 3.2) (Overloper *et al.*, 2014).

Although deletion of *abcR1* did not result in any phenotypic differences with regards to growth *in vitro*, virulence, or biofilm formation, we sought to further analyze the AbcR1 regulon (Figure 3.4; Table 3.1). RNA-seq analyses uncovered 95 differentially expressed genes (≥3-fold) in the *abcR1* deletion strain compared to the parental strain C58. Previously, Overlöper and colleagues utilized proteomic and bioinformatics analyses to characterize 16 targets of AbcR1, the majority of which are components of ABC-type transport systems (e.g., *chvE*, *malE*, *atu2422*, *atu4678*, and *atu1879*) (Overloper *et al.*, 2014). Indeed, our transcriptomic data largely resembles the proteomic data from that study (Table 3.1). However, aside from the ABC-type transport systems, RNA-seq revealed additional AbcR1 targets, such as genes encoding transcriptional regulators, chemoreceptors, and a variety of enzymes. Remarkably, several genes previously shown to be necessary for *A. tumefaciens* virulence, including *chvE* and *attC*, are dysregulated in Δ*abcR1* (Table 3.6) (Kemner *et al.*, 1997, Matthysse *et al.*, 2008). Yet, a deletion of *abcR1* did not affect the ability of *A. tumefaciens* to form tumors in experimentally infected potatoes (Data not shown). A possible explanation for this result may reside in the expression of these virulence-associated targets in Δ*abcR1*. While these target mRNAs exhibited decreased, but not completely abrogated, levels in Δ*abcR1*, it is possible that even the low level of expression of virulence-associated targets in Δ*abcR1* is

sufficient to sustain infection, thus resulting in a lack of attenuation. Further investigation is needed to fully define the direct regulation and sRNA-mRNA interactions between AbcR1 and the newly identified targets in *A. tumefaciens*.

In *Agrobacterium*, VtlR is the transcriptional activator of *abcR1*, and directly binds to the promoter region of *abcR1* to exert its regulatory function (Fig 3.1, 3.5). Following identification of gene dysregulation in both *A. tumefaciens* Δ*abcR1* and Δ*vtlR*, we sought to compare the transcriptomic profiles of the two deletion strains (Figure 3.4). Importantly, several genetic systems differentially expressed in Δ*vtlR* showed no difference in *abcR1*, suggesting these systems could be key for *Agrobacterium* pathogenesis. Of note, genes necessary for a type IV secretion system (*avhB*), a conjugation system (*tra*), polysaccharide biosynthesis (*exo*), and 10 transcriptional regulators are all differentially expressed in Δ*vtlR* and show no difference in expression in Δ*abcR1*. However, none of these genes have been shown to contribute to or are associated with the virulence of *A. tumefaciens* (Chen *et al.*, 2002). With regards to biofilm production, one gene, *divK*, may contribute to differences in biofilm production observed in Δ*vtlR* (Figure 3.3). A deletion of *divK* has been reported to result in decreased biofilm production in *Agrobacterium* (Kim *et al.*, 2013). In *A. tumefaciens* Δ*vtlR*, *divK* is over-expressed, suggesting the possibility that *divK* dysregulation could alter biofilm formation. While no other genes from the Δ*vtlR* RNA-seq analysis have been linked directly to biofilm formation, several genes have been speculated to be necessary for biofilm production (e.g., *glcF* and *gguB*) (Heindl *et al.*, 2015).

The difference in size of the VtlR regulons in *Brucella* and *Agrobacterium* is striking. The *B. abortus* VtlR regulon is comprised of 10 genes, and the *A. tumefaciens*

VtlR regulon is comprised of >200 genes (Table 3.3) (Sheehan *et al.*, 2015a). The complete regulon of LsrB in *S. meliloti* is currently unknown, although several genes involved in LPS synthesis, glutathione synthesis, and oxidative stress are regulated by LsrB (Lu *et al.*, 2013, Tang *et al.*, 2014). Since *S. meliloti* LsrB was shown to directly bind to the promoter region of the *lrp3-lpsCDE* operon, it was hypothesized that the homologous *lrp3-lpsCDE* system in *A. tumefaciens* may be similarly regulated (Tang *et al.*, 2014). However, EMSAs performed showed no binding of *A. tumefaciens* rVtlR to the promoter region of *lrp3-lpsCDE* (Figure 3.10). Furthermore, the VtlR/LsrB systems have diverged in their regulation of the *abcR* sRNAs between organisms, where *S. meliloti* LsrB did not regulate the *abcR* sRNAs under the conditions tested (Figure 3.2). Regarding glutathione production, Tang et al. demonstrated that the genes *gshA* and *gshB* are regulated by OxyR (Tang *et al.*, 2014). OxyR, a LTTR responsible for aiding the cell in responding to oxidative stress, was identified to be transcriptionally activated by LsrB in *S. meliloti* (Tang *et al.*, 2014). Similar to *S. meliloti*, *A. tumefaciens* OxyR is necessary for protection against oxidative stress and important for host-microbe interactions (Eiamphungporn *et al.*, 2003, Nakjarung *et al.*, 2003). In contrast to *S. meliloti*, *oxyR* did not appear to be regulated by VtlR in *A. tumefaciens*, as no difference in *oxyR* expression in the *A. tumefaciens vtlR* deletion strain was found (Table 3.3). Overall, these findings suggested a divergence of function between *S. meliloti* LsrB and *A. tumefaciens* VtlR.

To further clarify the functional homology between VtlR orthologs, heterologous complementation of *A. tumefaciens* Δ*vtlR* with *lsrB* from *S. meliloti* or *vtlR* from *B. abortus* was assessed via assays to measure growth *in vitro*, biofilm formation, and tumor formation on potato discs (Figure 3.9). While all *vtlR/lsrB* alleles complimented the small

colony phenotype observed in the *A. tumefaciens vtlR* deletion strain; *lsrB* from *S. meliloti*, but not *vtlR* from *B. abortus,* could complement the growth defect of *A. tumefaciens* Δ*vtlR* in liquid medium. Without knowing the complete regulon of *S. meliloti* LsrB, it is difficult to speculate as to why *S. meliloti* LsrB and *A. tumefaciens* VtlR appear to be more functionally similar to each other than to *B. abortus* VtlR. Since *Sinorhizobium* and *Agrobacterium* are both soil-dwelling bacteria, these two *Rhizobiales* share a similar environmental niche compared to the facultative intracellular mammalian pathogen *B. abortus*, which may partially explain successful heterologous complementation by *lsrB* from *S. meliloti*.

As mentioned in the introduction, the N-terminal domain LysR-type transcriptional regulators (LTTRs) most commonly include a helix-turn-helix domain, and regulated targets have a 'Classic' LTTR-binding box sequence of TTA-$N_{7/8}$-TAA, in an A/T rich region (Maddocks & Oyston, 2008). Thus, the proposed VtlR binding box confirmed in *B. abortus* and hypothesized in *A. tumefaciens* of GCAT-N_3-TG-N_3-T is divergent from typical LTTRs and may be unique to this regulator among the *Rhizobiales*.

Of particular interest, a bioinformatics approach led to the identification of a novel sRNA regulated by VtlR in *A. tumefaciens* by searching the *A. tumefaciens* genome for this novel LTTR binding motif (Munch *et al.*, 2005). Initially, EMSA experiments were performed with rVtlR and the promoter regions of genes that exhibited significantly differential expression in Δ*vtlR* (Table 3.3), including promoters from the following genes: *atu0036*, *atu0055*, *atu0157*, *atu0323*, *atu0463*, *atu2708*, *atu3939*, *atu4669*, *atu5116*, *atu5119*, *atu5121*, *atu5161*, *atu5118*, *atu0484*, *atu0828*, *atu1296*, *atu2187*,

atu2350, *atu2384*, *atu3252*, *atu4782*, *atrA*, *atrB*, *avhB1*, and *chvE*. Surprisingly, we determined that the *A. tumefaciens* rVtlR protein did not interact with any of these promoter regions under the conditions tested, aside from *abcR1* (data not shown).

Identification of a putative VtlR binding box, Figure 3.5D, and genome-wide search of this sequence in *A. tumefaciens* identified a region upstream of a novel sRNA previously described as "L4" but has been renamed VrsA (VtlR-regulated sRNA) (Wilms *et al.*, 2012). Our data showed that VrsA was directly activated by VtlR, but a deletion of *vrsA* did not result in a lag in growth similar to that observed in Δ*vtlR* (Figure 3.8). Analyses of biofilm formation, tumorigenesis, and transcriptomics of Δ*vrsA* are currently being conducted in the laboratory to understand if similar phenotypes are observed between *A. tumefaciens* Δ*vrsA* and *A. tumefaciens* Δ*vtlR*. Additionally, *atu1667* encodes a putative hypothetical protein that has not yet been characterized in *Agrobacterium* and Atu1667 could participate in gene regulation of the VtlR regulon as an sRNA or small protein.

A recent study described the necessity of VtlR in *A. tumefaciens* host-bacterium interactions (Tang *et al.*, 2018). There are several similarities observed between our work and the study by Tang *et al*., but the studies also contain differences with regards to experimental approach and results observed. One major difference is the *Agrobacterium* strain utilized. The work presented here employed *A. tumefaciens* str. C58, which is a naturally occurring strain of *A. tumefaciens*, whereas Tang *et al.* utilized a strain named *A. tumefaciens* C58C1 (Tang *et al.*, 2018). *A. tumefaciens* C58C1 is a modified *A. tumefaciens* strain in which the original virulence plasmid pTiC58 is replaced by the *A. rhizogenes* virulence plasmid. While seemingly minor, this is an important distinction

considering the virulence plasmid pTiC58 in *A. tumefaciens* contains the *virB* operon, which is necessary for virulence, while the *A. rhizogenes* virulence plasmid is lacking the *virB* operon (Berger & Christie, 1994). While the C58C1 strain is still able to form host-microbe interactions and cause disease in its plant host, this absence may affect the function of VtlR in *A. tumefaciens*.

While there are many similarities in gene expression when comparing the present RNA-seq dataset to the previously published Δ*vtlR* dataset, there are some significant differences (Table 3) (Tang *et al.*, 2018). It should be noted that between the studies, *A. tumefaciens* strains were grown in different media. In the presented study, RNA was isolated from *A. tumefaciens* grown in LB broth, whereas the previous study isolated RNA from bacteria grown in TY broth (Tang *et al.*, 2018). The largest difference between the VtlR regulons from these two studies is the differential gene expression observed with regards to AbcR1. Our data and data previously presented by Overlöper and colleagues show differences in expression and regulation of *chvE*, *atu2422*, *atu4678*, and *atu1879* in Δ*abcR1* and Δ*vtlR (*Table 3.6 and 3) (Overloper *et al.*, 2014). None of these genes appear to be differentially regulated in the VtlR regulon described previously (Tang *et al.*, 2018). Altogether, these differences may be explained by the variation in experimental design, particularly in regards to the conditions under which the bacteria were cultured before RNA isolation.

In conclusion, the present work has demonstrated that VtlR was necessary for efficient *in vitro* growth and tumorigenesis of the plant pathogen *A. tumefaciens*. The *A. tumefaciens* VtlR protein bound directly to a conserved binding box in three promoter regions, but VtlR was linked to the regulation of over 250 genes. Taken together, we

propose a model of regulation by VtlR in *A. tumefaciens* in which VtlR plays a central role in the activation of sRNAs that, in turn, control the expression of a wide variety of mRNA targets (Figure 3.11). In this model, VtlR activates the expression of AbcR1, the newly described sRNA, VrsA, and the putative sRNA Atu1667, and these sRNAs are the major regulatory elements responsible for propagating the VtlR genetic circuit. Overall, this study demonstrates the functional importance of VtlR in *A. tumefaciens*, and provides insight into the evolutionary similarities and differences that exist in the VtlR/LsrB systems of members of the *Rhizobiales*.

Materials and Methods

Bacterial strains and growth conditions.

Agrobacterium tumefaciens str. C58 and derivative strains were routinely grown on Luria-Bertani (LB) broth (Fisher Bioreagents, Fair Lawn, NJ) and cultures were routinely grown in LB broth. For cloning, *Escherichia coli* strain DH5α was grown on tryptic soy agar (BD) or in Luria-Bertani (LB) broth. When appropriate, growth media were supplemented with kanamycin (45 μg/ml for *E. coli* and 300 μg/ml for *A. tumefaciens*), sucrose (5%), or IPTG (100 μM).

Construction and complementation of *A. tumefaciens* deletion strains.

(i) Mutagenesis of *A. tumefaciens vtlR, abcR1, abcR2, and vrsA*

The *vtlR* gene (*atu2186*) was mutated utilizing an unmarked gene excision strategy previously described (Sheehan & Caswell, 2017, Sheehan *et al.*, 2015a). An approximately 1-kb fragment of the upstream region of *atu2186* was amplified via PCR with *A. tumefaciens* genomic DNA, primers *atu2186*-Up-For and *atu2186*-Up-Rev, and *Taq* polymerase (Monserate Biotechnology Group). Similarly, an approximately 1-kb fragment of the downstream region of *atu2186* was amplified with primers *atu2186*-Dn-For and *atu2186*-Dn-Rev. All oligonucleotides are listed in Table 3.6. These fragments were then digested with the appropriate restriction enzymes, and subsequently phosphorylated with polynucleotide kinase (Monserate Biotechnology Group). Fragments were then combined in a single ligation with digested pNPTS138 and T4 DNA ligase (Monserate Biotechnology Group) (Spratt *et al.*, 1986). The resulting plasmid was introduced into *A. tumefaciens* C58 by electroporation. Following electroporation of this plasmid into C58 primary integration of this non-replicating plasmid was confirmed

using primer pairs USP003/*atu2186*-con-For and USP003/*atu2186*-con-Rev. Counter-selection on 5% sucrose resulted in excision of the integrated plasmid. Deletion of the *atu2186* (*vtlR*) locus was confirmed using primer pair *atu2186*-con-For/*atu2186*-con-Rev. All plasmid constructs are listed in Table 3.7. This method was utilized to construct the *abcR1*, *abcR2*, and *abcR1/2* deletion constructs and strains.

(ii) *In-trans* complementation of *A. tumefaciens* str. C58::Δ*vtlR* and *A. tumefaciens* str. C58::Δ*vrsA*

Complementation of the *A. tumefaciens vtlR* deletion strain was done using IPTG-inducible overexpression plasmid pSRK-Km (Khan *et al.*, 2008). Briefly, the *A. tumefaciens vtlR* gene was amplified via PCR with *A. tumefaciens* genomic DNA, primers *atu2186*-comp-For and *atu2186*-comp-Rev and *Taq* polymerase. All oligonucleotides are listed in Table 3.6. The fragment was digested with the appropriate restriction enzymes, and subsequently ligated into digested pSRK-Km. The resulting plasmid was then introduced into the *A. tumefaciens vtlR* strain by electroporation. The strain harboring the complementation plasmid was selected for on AT-agar plates supplemented with kanamycin (45 μg ml^{-1}). All plasmid constructs are listed in Table 3.7.

(ii) *In-trans* heterologous complementation of *A. tumefaciens vtlR*

Heterologous complementation was carried out as described above. For complementation with *lsrB* from *Sinorhizobium meliloti*, the *lsrB* gene (*SMc01226*) was amplified via PCR with *S. meliloti* 1021 genomic DNA, primers *lsrB*-comp-For and *lsrB*-comp-Rev and *Taq* polymerase. All oligonucleotides are listed in Table 3.6. The fragment was digested with the appropriate restriction enzymes, and ligated into digested pSRK-Km. The resulting

plasmid was then introduced into the *A. tumefaciens vtlR* deletion strain by electroporation. All plasmid constructs are listed in Table 3.7.

For complementation with *vtlR* from *Brucella abortus*, the *vtlR* gene (*bab1_1517*) was amplified via PCR with *B. abortus* 2308 genomic DNA, primers *vtlR*-comp-For and *vtlR*-comp-Rev and *Taq* polymerase. All oligonucleotides are listed in Table 3.6. The fragment was digested with the appropriate restriction enzymes, and ligated into digested pSRK-Km. The resulting plasmid was then introduced into the *A. tumefaciens vtlR* strain by electroporation. All plasmid constructs are listed in Table 3.7.

Potato Tumor Assay.

To test the virulence of the constructs, tumor formation on disks of red potato was measured. Organic, red potatoes were scrubbed to remove dirt and debris, sterilized in dilute bleach for twenty minutes, and finally sterilized with UV light for no less than twenty minutes. Potato disks were created by coring the potatoes and cutting the cores into .5 centimeter wide slices. The slices were placed onto an agar plate with no added nutrients, and each plate had five technical replicates. The disks were inoculated with the indicated strains, which were grown overnight in ATGN containing 100 μg/ml kanamycin. Half of the inoculants contained 100 μM isopropyl β-D-1-thiogalactopyranoside to induce expression of each *vtlR* homologue. The overnight cultures were diluted to an optical density (600 nm) of 0.06 prior to inoculation, then ten microliters of the strain was placed on each potato disk. The plates were sealed with parafilm and left undisturbed at room temperature for four weeks. Tumor formation was counted at day 14 and 21.

Static Biofilm Assay.

To test the ability of a strain to form a biofilm, a static biofilm assay was performed. Overnight cultures of each strain were grown in either LB or ATGN with 100 μg/ml kanamycin. Half of the overnight cutlures also contained 100 μM isopropyl β-D-1-thiogalactopyranoside to induce expression of each *vtlR* homologue. The following morning each culture was subcultured to an optical density (600 nm) of 0.1, and once the cultures reach exponential growth, they were diluted to an optical density (600 nm) of 0.05. Three milliliters of each culture was placed in a nine-well polystyrene plate. Previously, polyvinyl chloride coverslips had been placed in each well, and then the plates were UV sterilized for no less than twenty minutes. After inoculation, the plates were incubated at room temperature for 48 hours. The coverslips were rinsed to remove excess or weakly attached organisms, then stained with 0.1% crystal violet. After staining, the excess crystal violet was rinsed off and the adherent crystal violet was re-solubilized in 33% acetic acid. Three hundred microliters of re-solubilized crystal violet and culture were loaded into a ninety-six well plate. The absorbance of the crystal violet solution was measured at 600 nm (A_{600nm}), and the optical density of the culture at 600 nm was measured (OD_{600nm}). For data presentation biofilm formation was normalized to growth using the formula A_{600nm}/OD_{600nm} and expressed relative to biofilm formation by the wild-type background.

Northern blot analysis.

A. tumefaciens RNA was isolated from cultures using the methodology previously described (Sheehan & Caswell, 2017, Sheehan *et al.*, 2015a). Ten micrograms of RNA were separated on a denaturing 10% polyacrylamide gel with 7 M urea and 1 × TBE (89 mM Tris base, 89 mM boric acid, 2 mM EDTA). To determine size, a low molecular

weight DNA ladder (New England Biolabs) was labelled with [γ-^{32}P]ATP (PerkinElmer) and polynucleotide kinase (Monserate Biotechnology Group). Following electrophoresis, the ladder and RNA samples were transferred to a Amersham Hybond^{TM}-N^{+} membrane (GE Heathcare) by electroblotting in 1 × TBE buffer. Samples were then UV cross-linked to the membrane, and membranes were then pre-hybridized in ULTRAhyb®-Oligo Buffer (Ambion) for 1 hour at 45°C in a rotating hybridization oven. Oligonucleotide probes were end-labelled with [γ-^{32}P]ATP and polynucleotide kinase. All oligonucleotides are listed in Table 3.6. Radiolabeled probes were incubated with pre-hybridized membranes at 45°C in a rotating hybridization oven overnight. The following day, membranes were washed four times with 2 × SSC (300 mM sodium chloride and 30 mM sodium citrate), 1 × SSC, 0.5 × SSC, and 0.25 × SSC at 45°C in a rotating hybridization oven for 30 minutes each. Each SSC washing solution contained 0.1% sodium dodecyl sulphate (SDS). Membranes were exposed to X-ray film and visualized by autoradiography.

Protein purification.

Recombinant *A. tumefaciens* VtlR (rVtlR) was constructed utilizing the *Strep*-tag II system (IBA), and subsequently cloned and expressed in *E. coli* BL21 cells. The coding region of *atu2186* was amplified via PCR using *A. tumefaciens* C58 genomic DNA as a template, primers rAtu2186-For and rAtu2186-Rev and *Taq* polymerase (Monserate Biotechnology Group). All oligonucleotides are listed in Table 3.6. The DNA was then digested with *Bsa*I and ligated into pASK-IBA7, which encodes an amino-terminal Strep-tag II on the protein of interest. Following sequencing of the plasmid, the *E. coli* BL21 strain harboring rVtlR-pIBA7 was grown to an OD_{600} nm of 0.7 before recombinant gene expression was induced by 200 µg ml^{-1} anhydrotetracycline (AHT). Following 3 hours of

constant shaking at 37°C, the cells were collected by centrifugation (4,200 × *g* for 10 minutes at 4°C) and lysed with CelLytic B (Sigma) in the presence of the protease inhibitor phenylmethanesulfonylfluoride (PMSF). The supernatant from the lysed cells was cleared by centrifugation (14,000 × *g* for 10 minutes at 4°C) and passed through a Strep-Tactin Sepharose affinity column. The column was then washed two times with Buffer W (100 mM Tris-HCl, 300 mM NaCl, pH 8.0) and the rVtlR was eluted with 2.5 mM desthiobiotin in Buffer W. The degree of purity of the rVtlR was high as judged by visualization of a single band on SDS-PAGE (data not shown).

Electrophoretic mobility shift assays (EMSAs).

EMSAs with rVtlR were carried out as previously described (Sheehan *et al.*, 2015a). All EMSAs were done in a final volume of 20 µL reaction mixture that included a binding buffer composed of 10 mM Tris-HCl (pH 7.4), 50 mM KCl, 1 mM dithiothreitol, 6% glycerol, 50 µg/ml bovine serum albumin and 50 µg ml^{-1} salmon sperm DNA. DNA fragments of the *abcR1*, *abcR2*, *atu1667,* and *vrsA* promoter regions were amplified by PCR using *A. tumefaciens* C58 genomic DNA at a template, gene-specific primers, and *Taq* polymerase (Monserate Biotechnology Group). All oligonucleotides are listed in Table 3.6. Fragments were then separated on a 0.8% agarose gel, purified and end-labelled with [γ-^{32}P]ATP (PerkinElmer) and polynucleotide kinase (Monserate Biotechnology Group). Increasing amounts of rVtlR were added to DNA fragments in binding buffer, and subsequently incubated at room temperature for 30 minutes. In some gels, non-radiolabeled specific DNA (i.e. promoters of *abcR1* and *vrsA*) or non-radiolabeled non-specific DNA (i.e. promoter of *abcR2*) were added to reactions in 50 × molar concentrations. Binding reactions were separated on 6% native polyacrylamide

gels in 0.5 × TBE running buffer for 1 hour. Gels were dried onto 3 mm Whatman paper using a vacuum gel drier system and visualized by autoradiography.

RNA-sequencing.

(i) RNA extraction and precipitation

RNA extractions were carried out as previously described (Caswell *et al.*, 2012, Sheehan & Caswell, 2017, Sheehan *et al.*, 2015a). *Agrobacterium* strains were grown in triplicate to an OD_{600} nm of 1.0 with constant shaking at 28°C. An equal amount of 1:1 ethanol-acetone was added to cultures and stored at -80°C. For RNA isolation, the cell/ethanol-acetone mixtures were thawed and pelleted at 16,000 × *g* for 3 minutes. RNA was isolated from cells by use of TRIzol reagents (Invitrogen) followed by ethanol precipitation. Following RNA isolation, genomic DNA was removed with DNase I (2 U; Thermo Fisher Scientific), where 30 µg of RNA was incubated with DNase I for one hour at 37°C. Samples were then purified by phenol-chloroform extractions and subsequent ethanol precipitation. RNA samples were resuspended in nuclease-free H_2O and the purity of each sample was checked with a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific). All samples had an A_{260}/A_{280} ratio of ~2.0 and a concentration yield of ~1 µg/µl. RNA samples (10 µg total) were then submitted to the Bioinformatics Institute at Virginia Tech for RNA-seq analysis.

(ii) Stranded RNA Library Construction for Prokaryotic RNA-Seq

1 µg of total RNA with RIN ≥ 8.0 was depleted of rRNA using Illumina's Ribo-Zero rRNA Removal Kit (Gram-Positive and Gram-Negative Bacteria) (P/N MRZB12424, Illumina, CA). The depleted RNA was fragmented and converted to first strand cDNA using reverse transcriptase and random primers using using Illumina's TruSeq Stranded

mRNA HT Sample Prep Kit (Illumina, RS-122-2103). This is followed by second strand synthesis using polymerase I and RNAse H, and dNTPs that contain dUTP instead of dTTP. The cDNA fragments then go through end repair, addition of a single 'A' base, and then ligation of adapters and indexed individually. The products were then purified and the second strand digested with N-Glycosylase, thus resulting in stranded template. The template molecules with the adapters were enriched by 10 cycles of PCR to create the final cDNA library. The library generated was validated using Agilent 2100 Bioanalyzer and quantitated using Quant-iT dsDNA HS Kit (Invitrogen) and qPCR. 16 individually indexed cDNA libraries were pooled and sequenced on Illumina NextSeq to get a minimum of 25 million reads.

(iii) Illumina NextSeq Sequencing

The libraries were clustered and sequenced using, NextSeq 500/550 High Output kit V2 (150 cycles) (P/N FC-404-2002) to 2 x 75 cycles to generate ~50 million paired end reads. The Illumina NextSeq Control Software v2.1.0.32 with Real Time Analysis RTA v2.4.11.0 was used to provide the management and execution of the NextSeq 500 and to generate BCL files. The BCL files were converted to FASTQ files and demultiplexed using bcl2fastq Conversion Software v2.20.

(iv) RNA-Seq data processing and analysis

The *Agrobacterium tumefaciens* (strain C58) genes and genome sequences, as well as corresponding annotations from NCBI (https://www.ncbi.nlm.nih.gov/) were used as a reference. Raw reads were quality-controlled and filtered with FastqMcf (Aronesty, 2013), resulting in an average of 1,936 Mbp (1,703 to 2,329 Mbp) nucleotides. The remaining reads were mapped to the gene reference using BWA with default parameters

(Li & Durbin, 2009). Differential expression of genes was calculated using the edgeR package in R software (http://www.r-project.org/), with Benjamini–Hochberg adjusted P-values of 0.05 considered to be significant (Robinson *et al.*, 2010).

(v) RNA-Seq data processing

The *Agrobacterium tumefaciens* (strain C58) gene and genome sequences, as well as corresponding annotations from NCBI (https://www.ncbi.nlm.nih.gov/) were used as the reference. Raw reads were quality-controlled and filtered by FastqMcf (Aronesty, 2013), resulting in an average of 1,781 Mbp (1,497 to 2,130 Mbp) nucleotides (Figure 3.12). The remaining reads were mapped to the gene reference using BWA with default parameters (Li & Durbin, 2009). The differential expression of genes was calculated using the edgeR package in R software (http://www.r-project.org/), with Benjamini–Hochberg adjusted P-values of 0.05 considered to be significant (Robinson *et al.*, 2010).

Quantitative reverse transcriptase PCR (qRT-PCR).

Total RNA isolated from *A. tumefaciens* str. C58 and *A. tumefaciens* Δ*vtlR* for the performance of RNAseq analysis above was utilized to perform confirmatory qRT-PCR as previously stated (Sheehan *et al.*, 2015b). cDNA was generated from the final RNA preparation using a SuperScript III cDNA synthesis system (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol, and this cDNA was used for real-time PCR employing a SYBR green PCR supermix (Roche, Mannheim, Germany). For these experiments, primers for 16S rRNA were used as a control, while gene-specific primers were used for evaluating relative levels of *atu5161*, *avhB5*, *avhB11*, *atu5118*, *atu3253*, and *atu3368* mRNAs (Table 3.4). Parameters for PCR included a single denaturing step for 5 min at 95°C, followed by 40 cycles (denature for 15 s at 95°C, anneal for 15 s at

51°C, and extend for 15 s at 72°C) of amplification. Fluorescence from SYBR green incorporation into double-stranded DNA was measured with an iCycler machine (Bio-Rad), and the relative abundance of mRNA was determined using the Pfaffl equation (Pfaffl, 2001).

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References

Akakura, R. & S.C. Winans, (2002) Constitutive mutations of the OccR regulatory protein affect DNA bending in response to metabolites released from plant tumors. *The Journal of biological chemistry* **277**: 5866-5874.

Aronesty, E., (2013) Comparison of Sequencing Utility Programs. *The Open Bioinformatics Journal* **7**: 1-8.

Becker, A., A. Overlöper, J.-P. Schlüter & J. Reinkensmeier, (2014) Riboregulation in plant-associated α-proteobacteria. *RNA biology* **11**: 550-562.

Berger, B.R. & P.J. Christie, (1994) Genetic complementation analysis of the *Agrobacterium tumefaciens virB* operon: *virB2* through *virB11* are essential virulence genes. *J Bacteriol* **176**: 3646-3660.

Caswell, C.C., J.M. Gaines, P. Ciborowski, D. Smith, C.H. Borchers, C.M. Roux, K. Sayood, P.M. Dunman & R.M. Roop Ii, (2012) Identification of two small regulatory RNAs linked to virulence in *Brucella abortus* 2308. *Molecular microbiology* **85**: 345-360.

Chen, L., Y. Chen, D.W. Wood & E.W. Nester, (2002) A new type IV secretion system promotes conjugal transfer in *Agrobacterium tumefaciens*. *J Bacteriol* **184**: 4838-4845.

Craven, S.H., O.C. Ezezika, S. Haddad, R.A. Hall, C. Momany & E.L. Neidle, (2009) Inducer responses of BenM, a LysR-type transcriptional regulator from *Acinetobacter baylyi* ADP1. *Mol Microbiol* **72**: 881-894.

Dangel, A.W., J.L. Gibson, A.P. Janssen & F.R. Tabita, (2005) Residues that influence in vivo and in vitro CbbR function in *Rhodobacter sphaeroides* and identification of a specific region critical for co-inducer recognition. *Mol Microbiol* **57**: 1397-1414.

Eiamphungporn, W., K. Nakjarung, B. Prapagdee, P. Vattanaviboon & S. Mongkolsuk, (2003) Oxidant-inducible resistance to hydrogen peroxide killing in *Agrobacterium tumefaciens* requires the global peroxide sensor-regulator OxyR and KatA. *FEMS microbiology letters* **225**: 167-172.

Ezezika, O.C., L.S. Collier-Hyams, H.A. Dale, A.C. Burk & E.L. Neidle, (2006) CatM regulation of the *benABCDE* operon: functional divergence of two LysR-type paralogs in *Acinetobacter baylyi* ADP1. *Appl Environ Microbiol* **72**: 1749-1758.

Heindl, J.E., M.E. Hibbing, J. Xu, R. Natarajan, A.M. Buechlein & C. Fuqua, (2015) Discrete Responses to Limitation for Iron and Manganese in *Agrobacterium tumefaciens*: Influence on Attachment and Biofilm Formation. *J Bacteriol* **198**: 816-829.

Henikoff, S., G.W. Haughn, J.M. Calvo & J.C. Wallace, (1988) A large family of bacterial activator proteins. *Proc Natl Acad Sci U S A* **85**: 6602-6606.

Kemner, J.M., X. Liang & E.W. Nester, (1997) The *Agrobacterium tumefaciens* virulence gene *chvE* is part of a putative ABC-type sugar transport operon. *J Bacteriol* **179**: 2452-2458.

Khan, S.R., J. Gaines, R.M. Roop, 2nd & S.K. Farrand, (2008) Broad-host-range expression vectors with tightly regulated promoters and their use to examine the influence of TraR and TraM expression on Ti plasmid quorum sensing. *Appl Environ Microbiol* **74**: 5053-5062.

Kim, J., J.E. Heindl & C. Fuqua, (2013) Coordination of division and development influences complex multicellular behavior in *Agrobacterium tumefaciens*. *PLoS One* **8**: e56682.

Kovacikova, G., W. Lin & K. Skorupski, (2010) The LysR-type virulence activator AphB regulates the expression of genes in *Vibrio cholerae* in response to low pH and anaerobiosis. *J Bacteriol* **192**: 4181-4191.

Kullik, I., M.B. Toledano, L.A. Tartaglia & G. Storz, (1995) Mutational analysis of the redox-sensitive transcriptional regulator OxyR: regions important for oxidation and transcriptional activation. *J Bacteriol* **177**: 1275-1284.

Li, H. & R. Durbin, (2009) Fast and accurate short read alignment with Burrows-Wheeler transform. *Bioinformatics* **25**: 1754-1760.

Liu, Z., H. Wang, Z. Zhou, N. Naseer, F. Xiang, B. Kan, M. Goulian & J. Zhu, (2016) Differential Thiol-Based Switches Jump-Start *Vibrio cholerae* Pathogenesis. *Cell Rep* **14**: 347-354.

Liu, Z., M. Yang, G.L. Peterfreund, A.M. Tsou, N. Selamoglu, F. Daldal, Z. Zhong, B. Kan & J. Zhu, (2011) *Vibrio cholerae* anaerobic induction of virulence gene expression is controlled by thiol-based switches of virulence regulator AphB. *Proc Natl Acad Sci U S A* **108**: 810-815.

Lu, D., G. Tang, D. Wang & L. Luo, (2013) The *Sinorhizobium meliloti* LysR family transcriptional factor LsrB is involved in regulation of glutathione biosynthesis. *Acta Biochim Biophys Sin (Shanghai)* **45**: 882-888.

Luo, L., S.Y. Yao, A. Becker, S. Ruberg, G.Q. Yu, J.B. Zhu & H.P. Cheng, (2005) Two new *Sinorhizobium meliloti* LysR-type transcriptional regulators required for nodulation. *J Bacteriol* **187**: 4562-4572.

Maddocks, S.E. & P.C. Oyston, (2008) Structure and function of the LysR-type transcriptional regulator (LTTR) family proteins. *Microbiology* **154**: 3609-3623.

Matthysse, A.G., P. Jaeckel & C. Jeter, (2008) *attG* and *attC* mutations of *Agrobacterium tumefaciens* are dominant negative mutations that block attachment and virulence. *Can J Microbiol* **54**: 241-247.

Munch, R., K. Hiller, A. Grote, M. Scheer, J. Klein, M. Schobert & D. Jahn, (2005) Virtual Footprint and PRODORIC: an integrative framework for regulon prediction in prokaryotes. *Bioinformatics* **21**: 4187-4189.

Nakjarung, K., S. Mongkolsuk & P. Vattanaviboon, (2003) The *oxyR* from *Agrobacterium tumefaciens*: evaluation of its role in the regulation of catalase and peroxide responses. *Biochem Biophys Res Commun* **304**: 41-47.

Overloper, A., A. Kraus, R. Gurski, P.R. Wright, J. Georg, W.R. Hess & F. Narberhaus, (2014) Two separate modules of the conserved regulatory RNA AbcR1 address multiple target mRNAs in and outside of the translation initiation region. *RNA Biol* **11**: 624-640.

Pfaffl, M.W., (2001) A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res* **29**: e45.

Privett, B.R., M. Pellegrini, G. Kovacikova, R.K. Taylor, K. Skorupski, D. Mierke & F.J. Kull, (2017) Identification of a Small Molecule Activator for AphB, a LysR-Type Virulence Transcriptional Regulator in *Vibrio cholerae*. *Biochemistry* **56**: 3840-3849.

Robinson, M.D., D.J. McCarthy & G.K. Smyth, (2010) edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. *Bioinformatics* **26**: 139-140.

Ruangprasert, A., S.H. Craven, E.L. Neidle & C. Momany, (2010) Full-length structures of BenM and two variants reveal different oligomerization schemes for LysR-type transcriptional regulators. *J Mol Biol* **404**: 568-586.

Sheehan, L.M., J.A. Budnick, C. Blanchard, P.M. Dunman & C.C. Caswell, (2015a) A LysR-family transcriptional regulator required for virulence in *Brucella abortus* is highly conserved among the alpha-proteobacteria. *Molecular microbiology*.

Sheehan, L.M., J.A. Budnick, R.M. Roop, 2nd & C.C. Caswell, (2015b) Coordinated zinc homeostasis is essential for the wild-type virulence of *Brucella abortus*. *J Bacteriol* **197**: 1582-1591.

Sheehan, L.M. & C.C. Caswell, (2017) A 6-Nucleotide Regulatory Motif within the AbcR Small RNAs of *Brucella abortus* Mediates Host-Pathogen Interactions. *MBio* **8**.

Sheehan, L.M. & C.C. Caswell, (2018) An account of evolutionary specialization: the AbcR small RNAs in the *Rhizobiales*. *Mol Microbiol* **107**: 24-33.

Smith, S.A. & F.R. Tabita, (2002) Up-regulated expression of the cbb(I) and cbb(II) operons during photoheterotrophic growth of a ribulose 1,5-bisphosphate carboxylase-oxygenase deletion mutant of *Rhodobacter sphaeroides*. *J Bacteriol* **184**: 6721-6724.

Spratt, B.G., P.J. Hedge, S. te Heesen, A. Edelman & J.K. Broome-Smith, (1986) Kanamycin-resistant vectors that are analogues of plasmids pUC8, pUC9, pEMBL8 and pEMBL9. *Gene* **41**: 337-342.

Storz, G., J. Vogel & K.M. Wassarman, (2011) Regulation by small RNAs in bacteria: expanding frontiers. *Mol Cell* **43**: 880-891.

Tang, G., Q. Li, S. Xing, N. Li, Z. Tang, L. Yu, J. Yan, X. Li & L. Luo, (2018) The LsrB protein is required for *Agrobacterium tumefaciens* interaction with host plants. *Molecular plant-microbe interactions : MPMI*.

Tang, G., Y. Wang & L. Luo, (2014) Transcriptional regulator LsrB of *Sinorhizobium meliloti* positively regulates the expression of genes involved in lipopolysaccharide biosynthesis. *Applied and environmental microbiology* **80**: 5265-5273.

Tang, G., S. Xing, S. Wang, L. Yu, X. Li, C. Staehelin, M. Yang & L. Luo, (2017) Regulation of cysteine residues in LsrB proteins from *Sinorhizobium meliloti* under free-living and symbiotic oxidative stress. *Environ Microbiol* **19**: 5130-5145.

Taylor, J.L., R.S. De Silva, G. Kovacikova, W. Lin, R.K. Taylor, K. Skorupski & F.J. Kull, (2012) The crystal structure of AphB, a virulence gene activator from *Vibrio cholerae*, reveals residues that influence its response to oxygen and pH. *Mol Microbiol* **83**: 457-470.

Tichi, M.A. & F.R. Tabita, (2002) Metabolic signals that lead to control of CBB gene expression in *Rhodobacter capsulatus*. *J Bacteriol* **184**: 1905-1915.

Torres-Quesada, O., V. Millan, R. Nisa-Martinez, F. Bardou, M. Crespi, N. Toro & J.I. Jimenez-Zurdo, (2013) Independent activity of the homologous small regulatory

RNAs AbcR1 and AbcR2 in the legume symbiont *Sinorhizobium meliloti*. *PLoS One* **8**: e68147.

Torres-Quesada, O., J. Reinkensmeier, J.P. Schluter, M. Robledo, A. Peregrina, R. Giegerich, N. Toro, A. Becker & J.I. Jimenez-Zurdo, (2014) Genome-wide profiling of Hfq-binding RNAs uncovers extensive post-transcriptional rewiring of major stress response and symbiotic regulons in *Sinorhizobium meliloti*. *RNA Biol* **11**: 563-579.

Wang, L., J.D. Helmann & S.C. Winans, (1992) The *A. tumefaciens* transcriptional activator OccR causes a bend at a target promoter, which is partially relaxed by a plant tumor metabolite. *Cell* **69**: 659-667.

Wilms, I., A. Overloper, M. Nowrousian, C.M. Sharma & F. Narberhaus, (2012) Deep sequencing uncovers numerous small RNAs on all four replicons of the plant pathogen *Agrobacterium tumefaciens*. *RNA Biol* **9**: 446-457.

Wilms, I., B. Voss, W.R. Hess, L.I. Leichert & F. Narberhaus, (2011) Small RNA-mediated control of the *Agrobacterium tumefaciens* GABA binding protein. *Mol Microbiol* **80**: 492-506.

Zheng, M., F. Aslund & G. Storz, (1998) Activation of the OxyR transcription factor by reversible disulfide bond formation. *Science* **279**: 1718-1721.

Tables

Table 3.1: Differential gene expression in *A. tumefaciens* str. C58::Δ*abcR1.*

Gene Designation	Description	Log_2 fold change (Δ*abcR1* vs. C58) in gene expression
	Transport Systems and Membrane Proteins	
Atu0126	membrane lipoprotein	1.5
Atu0157	ABC transporter, substrate binding protein	2.8
Atu0158	ABC transporter, membrane spanning protein	2.7
Atu0159	ABC transporter, nucleotide binding/ATPase protein	2.2
Atu1201	ABC transporter, nucleotide binding/ATPase protein	1.8
Atu1202	ABC transporter, substrate binding protein	2.3
Atu1879	ABC transporter, substrate binding protein (amino acid)	2.2
Atu2143	ABC transporter, membrane spanning protein (amino acid)	1.8
Atu2287	outer membrane heme receptor	-1.6
Atu2348	*chvE*, sugar binding protein	-1.5
Atu2391	ABC transporter, substrate binding protein (nitrate/sulfonate/taurine/bicarbonate)	2.8
Atu2422	ABC transporter, substrate binding protein (amino acid)	2.4
Atu3041	dipeptide ABC transporter substrate-binding protein	1.7
Atu3047	oligopeptide ABC transporter permease	1.9
Atu3048	oligopeptide ABC transporter permease	1.9
Atu3049	oligopeptide ABC transporter substrate-binding protein	1.6
Atu3053	peptide ABC transporter ATPase	2.4
Atu3063	ABC transporter permease	1.7
Atu3269	dipeptide ABC transporter substrate-binding protein	2.2
Atu3338	*thuE*, ABC transporter, substrate binding protein (trehalose/maltose)	-1.6
Atu3339	*thuF*, ABC transporter, membrane spanning protein	-1.7
Atu3340	*thuG*, trehalose/maltose ABC transporter permease	-1.7
Atu3341	*thuK*, trehalose/maltose ABC transporter ATPase	-1.7
Atu3342	*thuA*, trehalose utilization-like protein	-1.7
Atu3409	oligopeptide ABC transporter substrate-binding protein (atu2409)	1.7
Atu3800	*proX*, proline/glycine betaine ABC transporter substrate-ninding protein	2.2
Atu3801	*proW*, proline/glycine betaine ABC transporter permease	1.7

Atu3802	*proV*, proline/glycine betaine ABC transporter ATPase	1.7
Atu3821	*rbsB*, ribose ABC transporter substrate-binding protein	-1.8
Atu4031	sugar ABC transporter permease	-1.6
Atu4046	glycine betaine ABC transporter substrate-binding protein	1.5
Atu4233	amino acid ABC transporter substrate-binding protein	2.1
Atu4234	amino acid ABC transporter permease	2.1
Atu4235	amino acid ABC transporter ATPase/permease	1.5
Atu4284	amino acid ABC transporter substrate-binding protein	1.5
Atu4370	sugar ABC transporter ATPase	-1.9
Atu4371	sugar ABC transporter permease	-1.8
Atu4423	ABC transporter permease	-2.1
Atu4577	ABC transporter substrate binding protein	3.5
Atu4578	ABC transporter permease	2.8
Atu4579	ABC transporter permease	1.9
Atu4678	amino acid ABC transporter substrate-binding protein	1.6
Atu4695	oligopeptide ABC transporter substrate-binding protein	3.7
Atu4696	oligopeptide ABC transporter permease	3.4
Atu4697	oligopeptide ABC transporter permease	2.6
Atu4700	oligopeptide ABC transporter ATPase	2.3
Atu4701	oligopeptide ABC transporter ATPase	2.3
Atu4723	ABC transporter permease	1.6
Atu4755	amino acid ABC transporter substrate-binding protein	2.2
Atu5006	*socA*, Deoxyfructosyl-amino Acid Transporter Periplasmic Binding Protein	1.7
Atu5531	ABC transporter substrate binding protein	1.9
Atu6026	*nocQ*, ABC transporter, membrane spanning protein nopaline	1.6
Atu6027	*nocT*, ABC transporter, substrate binding protein (nopaline)	2.2
	Transcription and Translation	
Atu4718	GntR family transcriptional regulator	1.5
	Chemotaxis and Motility	
Atu3725	methyl-accepting chemotaxis protein	2.5
	Metabolism, Signaling, and Enzymatic Processes	
Atu0666	*glcE*, glycolate oxidase subunit	-2.1
Atu0667	*glcF*, glycolate oxidase iron-sulfur subunit	-1.8
Atu0946	dehydrogenase	2.4
Atu2127	*caiB*, L-carnitine dehydratase	2.1

Atu2128	*iivG*, acetolactate synthase 2 catalytic subunit	1.5
Atu2386	*dht*, dihydropyrimidinase	1.7
Atu2497	hydrolase	2.1
Atu2499	Isochorismatase	3.0
Atu2500	*rutA*, monooxygenase (pyrimidine utilization protein A)	3.2
Atu3046	oxidoreductase	1.7
Atu4153	aldehyde dehydrogenase	2.4
Atu4236	dehydrogenase	1.8
Atu4377	oxidoreductase	-3.1
Atu4379	*cy2*, cytochrome c2	-1.8
Atu4418	*gcdH*, glutaryl-CoA dehydrogenase	2.1
Atu4698	Catalyzes the deamination of cytosine to uracil and ammonia	2.2
Atu4699	*trzA*, N-ethylammeline chlorohydrolase	2.4
	Phage Related Genes	
Atu0954	*gp34*, phage head portal protein	-1.7
Atu0957	*gp36*, phage phi-C31 major capsid gp36-like protein	-2.0
	Hypothetical Proteins	
Atu0058	hypothetical protein	-2.7
Atu0945	hypothetical protein	1.5
Atu0958	hypothetical protein	-1.7
Atu0961	hypothetical protein	-1.5
Atu0964	hypothetical protein	-1.7
Atu0965	hypothetical protein	-1.7
Atu0967	hypothetical protein	-1.9
Atu2146	hypothetical protein	1.7
Atu2469	hypothetical protein	-1.7
Atu2471	hypothetical protein	-1.6
Atu2498	hypothetical protein	2.1
Atu2541	hypothetical protein	-2.7
Atu3942	hypothetical protein	-2.7
Atu4374	hypothetical protein	-1.8
Atu4375	hypothetical protein	-2.1
Atu4376	hypothetical protein	-2.9
Atu4378	hypothetical protein	-2.8
Atu4484	hypothetical protein	-1.5
Atu4580	hypothetical protein	1.9
Atu8141	hypothetical protein	-1.6
Atu8177	hypothetical protein	1.6

Table 3.1: Summarized *abcR1* RNA-seq dataset.
RNA-seq analysis was performed using total cellular RNA from *Agrobacterium* strains grown in LB medium to late exponential phase, and genes with expression ≥3-fold dysregulated are shown. Cells highlighted in grey represent genes also found in Table 3 (Δ*vtlR* RNA-seq dataset). Dataset sorted based on gene designation.

Table 3.2: Differential gene expression in *A. tumefaciens* str. C58::Δ*abcR2.*

Gene Designation	Description	Log_2 fold change (Δ*abcR2* vs. C58) in gene expression
Atu2109	*rnpB*, RNAase P RNA	-1.9
Atu2478	hypothetical protein	1.5
Atu3667	*panD*, aspartate alpha-decarboxylase	1.7
Atu3676	putative siderophore biosynthesis protein	1.6
Atu3752	hypothetical protein	2.0
Atu4088	transcriptional regulator	1.6
Atu4376	hypothetical protein	-1.5
Atu5115	hypothetical protein	1.5

Table 3.2: Summarized *abcR2* RNA-seq dataset.
RNA-seq analysis was performed using total cellular RNA from *Agrobacterium* strains grown in LB medium to late exponential phase, and genes with expression ≥3-fold dysregulated are shown.

Table 3.3: Differential gene expression in *A. tumefaciens* str. C58::Δ*vtlR*.

Gene Designation	Description	Log_2 fold change (Δ*vtlR* vs. C58) in gene expression
	Transport Systems and Membrane Proteins	
Atu0063	*frcB,* ABC transporter, substrate binding protein (sugar)	1.7
Atu0157	ABC transporter, substrate binding protein	1.9
Atu0158	ABC transporter, membrane spanning protein	1.6
Atu0159	ABC transporter, nucleotide binding/ATPase protein	1.7
Atu0394	ABC transporter, substrate binding protein (sugar)	-1.7
Atu0593	*aglG*, ABC transporter, membrane spanning protein	-2.1
Atu0843	P type cation (metal) transporter, ATPase component	-1.6
Atu0893	ABC transporter, nucleotide binding/ATPase protein	-1.7
Atu0894	ABC transporter, membrane spanning protein	-2.1
Atu1398	ABC transporter, membrane spanning protein (amino acid)	1.9
Atu1399	ABC transporter, substrate binding protein (amino acid)	2.6
Atu1403	ABC transporter, membrane spanning protein	1.9
Atu1579	ABC transporter, membrane spanning protein (amino acid)	-1.7
Atu1877	OmpA family protein	-1.5
Atu2287	outer membrane heme receptor	-1.5
Atu2346	*gguB*, ABC transporter, membrane spanning protein (sugar)	-3.1
Atu2347	*gguA*, ABC transporter, nucleotide binding/ATPase protein (sugar)	-2.3
Atu2348	*chvE*, sugar binding protein	-2.5
Atu2391	ABC transporter, substrate binding protein (nitrate/sulfonate/taurine/bicarbonate)	2.1
Atu2422	ABC transporter, substrate binding protein (amino acid)	1.5
Atu2492	*mtbA*, permease (Atu2492)	2.1
Atu2505	ABC transporter, substrate binding protein (sugar)	2.4
Atu2708	MFS permease	-3.7
Atu3102	sugar ABC transporter substrate-binding protein	1.8
Atu3113	sugar ABC transporter permease	1.7
Atu3114	sugar ABC transporter substrate-binding protein	1.7
Atu3151	sugar ABC transporter substrate-binding protein	1.5
Atu3165	sorbitol/mannitol ABC transporter substrate-binding protein	1.8
Atu3185	*ugpB*, ABC transporter, substrate binding protein (glycerol-3-phosphate)	2.9
Atu3187	*ugpE*, ABC-type sugar transport system, permease	1.7

	component	
Atu3198	ribose ABC transporter substrate-binding protein	2.2
Atu3200	ribose ABC transporter ATPase	1.9
Atu3202	RND multidrug efflux transporter	-3.1
Atu3203	RND multidrug efflux membrane permease	-2.9
Atu3222	ribose ABC transporter substrate-binding protein	2.8
Atu3236	sugar ABC transporter ATPase	1.7
Atu3237	sugar ABC transporter permease	1.6
Atu3238	sugar ABC transporter permease	1.7
Atu3239	sugar ABC transporter substrate-binding protein	2.7
Atu3253	ABC transporter substrate binding protein	4.5
Atu3254	dicarboxylate ABC transporter ATPase	3.3
Atu3255	dicarboxylate ABC transporter permease	3.2
Atu3298	*dctA*, C4-dicarboxylate transporter (Atu3298)	1.8
Atu3338	*thuE*, ABC transporter, substrate binding protein (trehalose/maltose)	1.9
Atu3352	sugar ABC transporter substrate-binding protein	2.2
Atu3368	periplasmic mannitol-binding protein	3.7
Atu3370	sugar ABC transporter permease	1.5
Atu3371	sugar ABC transporter ATPase	2.9
Atu3372	sugar ABC transporter substrate-binding protein	3.6
Atu3455	oligopeptide ABC transporter substrate-binding protein	1.9
Atu3533	sugar ABC transporter substrate-binding protein	2.0
Atu3576	*xylF*, ABC transporter, substrate binding protein (xylose)	3.3
Atu3804	permease	-1.7
Atu3881	sugar ABC transporter substrate-binding protein	1.8
Atu3893	sugarl ABC transporter permease	2.2
Atu3894	sugar ABC transporter permease	2.0
Atu3895	sugar ABC transporter ATPase	2.7
Atu3896	sugar ABC transporter ATPase	3.2
Atu4017	*kgtP*, MFS permease	-2.8
Atu4032	sugar ABC transporter ATPase	1.8
Atu4033	sugar ABC transporter substrate-binding protein	2.8
Atu4123	branched chain amino acid ABC transporter substrate-binding protein	2.4
Atu4124	ABC transporter ATP-binding protein	2.1
Atu4192	oligopeptide ABC transporter substrate-binding protein	2.4
Atu4423	ABC transporter permease	-1.8
Atu4468	*sitD*, ABC transporter, membrane spanning protein (iron transport)	-1.7
Atu4469	*sitC*, ABC transporter, membrane spanning protein	-1.6

	(iron)	
Atu4471	*sitA*, ABC transporter, substrate binding protein (iron	-1.6
Atu4534	amino acid ABC transporter substrate-binding protein	2.1
Atu4577	ABC transporter substrate binding protein	2.0
Atu4578	ABC transporter permease	2.0
Atu4626	oligopeptide ABC transporter substrate-binding protein	1.5
Atu4661	*agpA*, ABC transporter, substrate binding protein (alpha-galactoside)	2.0
Atu4710	MFS permease	2.5
Atu4784	*afuA*, ABC-type Fe3+ transport system, periplasmic component	2.1
Atu4842	sugar ABC transporter substrate-binding protein	2.0
Atu4843	sugar ABC transporter ATPase	2.1
Atu5005	*socB*, Deoxyfructosyl-amino acid ABC-type Membrane Transporter	1.9
Atu5006	*socA*, Deoxyfructosyl-amino Acid Transporter Periplasmic Binding Protein	3.2
Atu5126	*attA1*, ABC transporter, nucleotide binding/ATPase protein(putrescine)	-2.7
Atu5127	*attA2*, ABC transporter, membrane spanning protein (mannopine)	-3.2
Atu5128	*attB*, ABC transporter, membrane spanning protein (mannopine)	-3.1
Atu5129	*attC*, ABC transporter, substrate binding protein (Atu5129)	-3.6
Atu5130	*attD*, attachment protein	-3.0
Atu6027	*nocT*, ABC transporter, substrate binding protein (nopaline)	1.5
	Transcription and Translation	
Atu0484	two component response regulator	2.7
Atu0828	*betI*, transcriptional regulator	2.4
Atu1296	*divK*, two component response regulator	1.5
Atu2186	*vtlR*	-6.2
Atu2187	transcriptional regulator, ArsR family	1.7
Atu2350	*gbpR*, transcriptional regulator, LysR family	-1.6
Atu2384	transcriptional regulator, TetR family	1.7
Atu3252	*exuR*, transcriptional regulator, GntR family	1.6
Atu4782	*drrA*, two component response regulator	1.7
Atu5116	*rctB*, transcriptional regulator protein	-2.7
Atu5119	two component response regulator	-3.4
Atu5121	two component response regulator	-2.8

	Secretion Systems	
Atu5162	*avhB1*, type IV secretion protein AvhB1	-7.4
Atu5163	*avhB2*, type IV secretion protein AvhB2	-6.3
Atu5164	*avhB3*, type IV secretion protein AvhB3	-6.9
Atu5165	*avhB4*, type IV secretion protein AvhB4	-7.6
Atu5166	*avhB5*, type IV secretion protein AvhB5	-7.6
Atu5167	*avhB6*, type IV secretion protein AvhB6	-6.7
Atu5168	*avhB7*, type IV secretion protein AvhB7	-6.0
Atu5169	*avhB8*, type IV secretion protein AvhB8	-6.3
Atu5170	*avhB9*, type IV secretion protein AvhB9	-6.6
Atu5171	*avhB10*, type IV secretion protein AvhB10	-6.1
Atu5172	*avhB11*, type IV secretion protein AvhB11	-4.5
	Metabolism, Signaling, and Enzymatic Processes	
Atu0462	endolysin	-2.3
Atu0594	*aglA*, alpha-glucosidase	-1.9
Atu0666	*glcE*, glycolate oxidase subunit	-1.9
Atu0667	*glcF*, glycolate oxidase iron-sulfur subunit	-1.9
Atu0704	*dgoK*, 2-dehydro-3-deoxygalactonate kinase	-1.9
Atu0811	*mqo*, malate:quinone oxidoreductase	-3.6
Atu0830	*betA*, choline dehydrogenase	2.0
Atu0946	dehydrogenase	2.7
Atu1277	*nuoH*, NADH dehydrogenase subunit H	-1.9
Atu1432	*aceF*, branched-chain alpha-keto acid dehydrogenase subunit E2	-1.6
Atu1661	*sthA*, soluble pyridine nucleotide transhydrogenase	-1.7
Atu1956	*rpoB*, DNA-directed RNA polymerase subunit beta	-1.5
Atu2109	*rpnB*, RNAase P RNA	-1.6
Atu2127	*caiB*, L-carnitine dehydratase	2.9
Atu2128	*iivG*, acetolactate synthase 2 catalytic subunit	1.5
Atu2224	*aldA*, aldehyde dehydrogenase	3.8
Atu2497	hydrolase	1.6
Atu2499	Isochorismatase	1.9
Atu2500	*rutA*, monooxygenase (pyrimidine utilization protein A)	2.3
Atu2511	*dat*, D-amino acid aminotransferase	-1.7
Atu3137	C4-dicarboxylate-binding protein	2.5
Atu3256	zinc-binding dehydrogenase	1.7
Atu3354	pyrroloquinoline-quinone-dependent quinate dehydrogenase	-2.9
Atu3471	*bkdB*, branched-chain alpha-keto acid dehydrogenase subunit E2	3.0
Atu3472	*bkdA2*, 2-oxoisovalerate dehydrogenase beta subunit	3.8

Atu3473	*bkdA1*, 2-oxoisovalerate dehydrogenase alpha subunit	3.8
Atu3474	*mmgC*, acyl-CoA dehydrogenase	1.6
Atu3475	acetyl-CoA acetyltransferase	3.0
Atu3478	*mccB*, 3-methylcrotonoyl-CoA carboxylase beta subunit	2.5
Atu3479	*mccA*, 3-methylcrotonyl-CoA carboxylase alpha subunit	2.6
Atu3480	*hmgL*, hydroxymethylglutaryl-CoA lyase	2.0
Atu3667	*panD*, aspartate alpha-decarboxylase	2.2
Atu3736	*tktA*, transketolase	-1.7
Atu4153	aldehyde dehydrogenase	2.6
Atu4416	phosphopantetheinyl transferase	2.1
Atu4418	*gcdH*, glutaryl-CoA dehydrogenase	3.4
Atu4709	*fdsD*, NAD-dependent formate dehydrogenase delta subunit	2.0
Atu4740	zinc-binding dehydrogenase	-3.5
Atu4825	dehydrogenase	1.7
Atu5004	*socC*, deoxyfructose oxidoreductase	1.9
Atu5123	*atrC*, acetolactate synthase catalytic subunit	-5.8
Atu5124	*atrB*, glutamate-1-semialdehyde aminotransferase	-5.9
Atu5173	dehydrogenase	-2.1
Atu6000	*torf6*, agrocinopine synthase	1.7
Atu6007	*mas1*, Mannopine synthase	1.5
Atu6010	*tms2*, indole acetimide hydrolase	1.9
Atu6012	*ipt*, isopentenyl transferase	1.5
Atu6013	6a protein, Cytokinin glycosidase	1.6
Atu6179	*virC2*, virA/G regulated protein	1.6
	Phage Related Genes	
Atu0954	*gp34*, phage head portal protein	-1.7
Atu0956	*gp35*, phage prohead protease	-1.7
Atu0957	*gp36*, phage phi-C31 major capsid **gp36**-like protein	-3.6
Atu8126	putative phage tail protein I	-2.3
	Conjugation	
Atu5108	*traG*, conjugal transfer protein	-4.0
Atu5109	*traD*, conjugal transfer protein	-4.3
Atu5110	*traC*, conjugal transfer protein	-5.2
	Polysaccharide Biosynthesis	
Atu3326	*exoF*, exopolysaccharide production protein	-1.6
Atu3327	*exoY*, succinoglycan exopolysaccharide synthesis protein	-1.8
Atu4049	*exoP*, exopolysaccharide polymerization/transport	-2.0

	protein	
Atu4050	*exoN*, UTP-glucose-1-phosphate uridylyltransferase	-2.0
Atu4054	*exoL*, succinoglycan biosynthesis protein	-1.5
Atu4059	*exoV*, succinoglycan biosynthesis protein	-2.1
	Hypothetical Proteins	
Atu0056	hypothetical protein	2.2
Atu0058	hypothetical protein	-2.3
Atu0104	hypothetical protein	1.7
Atu0115	hypothetical protein	1.7
Atu0240	hypothetical protein	1.9
Atu0250	hypothetical protein	1.5
Atu0277	hypothetical protein	-1.7
Atu0452	hypothetical protein	-1.6
Atu0453	hypothetical protein	-2.3
Atu0454	hypothetical protein	-2.1
Atu0463	hypothetical protein	-2.1
Atu0468	hypothetical protein	2.3
Atu0604	hypothetical protein	1.6
Atu0772	hypothetical protein	1.6
Atu0824	hypothetical protein	1.6
Atu0853	hypothetical protein	1.9
Atu0945	hypothetical protein	2.4
Atu0958	hypothetical protein	-3.0
Atu0961	hypothetical protein	-2.1
Atu0964	hypothetical protein	-2.7
Atu0965	hypothetical protein	-2.4
Atu0967	hypothetical protein	-2.9
Atu1009	hypothetical protein	1.7
Atu1031	hypothetical protein	-2.9
Atu1427	hypothetical protein	1.7
Atu1469	hypothetical protein	1.5
Atu1587	hypothetical protein	2.1
Atu1634	hypothetical protein	2.4
Atu1667	hypothetical protein	-4.3
Atu1716	hypothetical protein	1.8
Atu1727	hypothetical protein	1.5
Atu1766	hypothetical protein	2.8
Atu1773	hypothetical protein	-1.9
Atu2146	hypothetical protein	2.4
Atu2248	hypothetical protein	1.7

Atu2345	*gguC*, hypothetical protein	-2.5
Atu2433	hypothetical protein	1.7
Atu2469	hypothetical protein	1.9
Atu2478	hypothetical protein	2.9
Atu2498	hypothetical protein	1.8
Atu2524	hypothetical protein	1.8
Atu2541	hypothetical protein	-2.3
Atu2543	hypothetical protein	2.2
Atu2768	hypothetical protein	1.5
Atu3124	hypothetical protein	1.5
Atu3189	hypothetical protein	2.6
Atu3470	hypothetical protein	2.1
Atu3638	hypothetical protein	1.5
Atu3645	hypothetical protein	2.9
Atu3752	hypothetical protein	3.1
Atu3822	hypothetical protein	2.1
Atu3841	hypothetical protein	1.5
Atu3891	hypothetical protein	3.5
Atu3892	hypothetical protein	2.0
Atu3940	hypothetical protein	2.2
Atu3942	hypothetical protein	-2.3
Atu4020	hypothetical protein	-2.3
Atu4179	hypothetical protein	1.8
Atu4183	hypothetical protein	2.2
Atu4185	hypothetical protein	2.5
Atu4213	hypothetical protein	1.9
Atu4442	hypothetical protein	2.3
Atu4443	hypothetical protein	1.8
Atu4484	hypothetical protein	-1.7
Atu4527	hypothetical protein	1.9
Atu4670	hypothetical protein	-2.1
Atu4727	hypothetical protein	1.9
Atu4780	hypothetical protein	-1.8
Atu4820	hypothetical protein	1.5
Atu5008	hypothetical protein	1.6
Atu5037	hypothetical protein	1.8
Atu5117	hypothetical protein (VBP1- VirD2-binding protein) - NTPase that might energize the recruitment of T-complex to the transport site	-4.5
Atu5118	hypothetical protein	-8.3
Atu5161	hypothetical protein	-6.9
Atu6163	hypothetical protein	1.5

Atu8021	hypothetical protein	1.9
Atu8083	hypothetical protein	-1.5
Atu8120	hypothetical protein	1.9
Atu8127	hypothetical protein	-2.1
Atu8141	hypothetical protein	-1.9
Atu8161	hypothetical protein	-3.8
Atu8166	hypothetical protein	1.5
Atu8177	hypothetical protein	1.7
Atu8178	hypothetical protein	-1.6
Atu8201	hypothetical protein	1.6

Table 3.3: Summarized Δ*vtlR* RNA-seq dataset.
RNA-seq analysis was performed using total cellular RNA from *Agrobacterium* strains grown in LB medium to late exponential phase, and genes with expression ≥3-fold dysregulated are shown. Cells highlighted in grey represent genes also found in Table 3.1 (Δ*abcR1* RNA-seq dataset). Dataset sorted based on gene designation.

Table 3.4: qRT-PCR confirmation of gene dysregulation of several VtlR target genes.

	Strain		
Target gene	**2308**	**Δ*vtlR*-RNAseq**	**Δ*vtlR*-qRT-PCR**
16S rRNA	1.0	-0.5	1.0
atu5161	1.0	-6.8	-25.0
avhB5	1.0	-7.6	-2.7
avhB11	1.0	-4.5	-2.3
atu5118	1.0	-8.3	-30.3
atu3253	1.0	4.5	1.4
atu3368	1.0	3.6	5.1

Table 3.5: Differential gene expression of putative sRNAs identified in Δ*vtlR* from Wilms et al.

trans-encoded sRNAs				
Replicon	**flanking genes**	**Log_2 fold change (Δ*vtlR* vs. C58) in gene expression**	**Confirmed via northern blot?**	**Differentially expressed in Δ*vtlR*?**
Circular Chromosome	*atu0052-atu0053*	1.4	Yes	No
Circular Chromosome	*atu0523-atu0524*	1.6	No	-
Circular Chromosome	*atu0612-atu0613*	1.3	Yes	No
Circular Chromosome	*atu0654-atu8135*	1.7	No	-
Circular Chromosome	*atu0923-atu0924*	2.7	No	-
Circular Chromosome	*atu0985-atu0986*	-2.5	Yes	Yes
Circular Chromosome	*atu1061-atu1062*	1.6	Yes	No
Circular Chromosome	*atu2014-atu2015*	1.4	No	-
Circular Chromosome	*atu2108-atu2110*	-1.5	No	-
Circular Chromosome	*atu2122-atu2123*	3.3	No	-
Circular Chromosome	*atu2555-atu2557*	2.6	Yes	No
Circular Chromosome	*atu2770-atu2771*	1.3	No	-
Linear Chromosome	*atu3126-atu3127*	1.1	No	-
Linear Chromosome	*atu3367-atu3368*	2.8	No	-
Linear Chromosome	*atu4670-atu4671*	-2.8	No	-
Linear Chromosome	*atu4671-atu4673*	1.1	Yes	No
Linear Chromosome	*atu4727-atu4728*	2.1	No	-

cis-encoded sRNAs				
Replicon	**cis-encoded gene**	**Log_2 fold change (Δ*vtlR* vs. C58) in gene expression**	**Confirmed via northern blot?**	**Differentially expressed in Δ*vtlR?***
Circular Chromosome	*atu0468*	2.3	No	-
Circular Chromosome	*atu0604*	1.6	No	-
Circular Chromosome	*atu1727*	1.5	No	-
Circular Chromosome	*atu2478*	2.9	No	-
Circular Chromosome	*atu2524*	1.8	No	-
Linear Chromosome	*atu4670*	-2.1	Yes	Yes
At plasmid	*atu5117*	-4.5	No	No

Table 3.6: Oligonucleotide primers used in this study.

Primer name	**Sequence (5'->3')**
atu2186-Up-For	TAGGATCCTATTTCCATGTCTGCCCGCCAC
atu2186-Up-Rev	TGCCATGCCTATCCGGCCTTTC
atu2186-Dn-For	TTCTGACGTAAAAATAATCGACGTCG
atu2186-Dn-Rev	TAACTAGTTTTCTGGTCGGTTGGCGTAAT
atu2186-con-For	AACTGTTCAAGGGCAAGGTG
atu2186-con-Rev	AAAACCTCCAGAGGGGAACAGC
USP003	CGCCAGGGTTTTCCCAGTCACGAC
abcR1-Up-For	TAGGATCCAGTCGGCCATCAGCCGCCAGGTCAGC
abcR1-Up-Rev	TCAACTGAGAGCTATTTGGTGTTTTT
abcR1-Dn-For	TTTTTACCCGCAAGAATTTTCTCCA
abcR1-Dn-Rev	TAACTAGTATGCCAGATCTGCGCAACGATCTTGC
abcR2-Up-For	TAGGATCCACGACGGTTGGTCTCGGCCAGGGCTG
abcR2-Up-Rev	TGACAGCTAACTAAAATATGGGAGTG
abcR2-Dn-For	TTTTTTGGAGCCGCCTGCCCG
abcR2-Dn-Rev	TAACTAGTATATCGTCGATGGTGAGCGCGCGG
abcR-con-For	TTCCGCGACTTCATCGTCGCCAAGGC
abcR-con-Rev	ACGTCTCTGTGAGCATCGTGGCAAGC
AbcR-Northern	AAAACCTCCAGAGGGGAACAGCTG
VrsA-Northern-Rev	GTGTTACGTTCATGACATGACCCTTTCTGA
Atu1667-Northern	CGAGTTCGGTTACGGTCTGACGATACTTGC
comp-*atu2186*-For	TACATATGATGGCAATGCCATTGGACTGGGA
comp-*atu2186*-Rev	TAGCTAGCTCAGAAGTTCCAGTTCCGCGCCTT
comp-*BavtlR*-For	TAGAGCTCGTGGTCGCACCGCTTGACTGGGAT
comp-*BavtlR*-Rev	TAGCTAGCTCAATAGGTCCAATTGCGGGCCTT
comp-*SmlsrB*-For	TACATATGATGGGGGATTCTATGTCGCTGGA
comp-*SmlsrB*-For	TAGCTAGCTCAGAAGTTCCAGTTTCTCGCTT
abcR1-At-EMSA-For	GCGGAACTGGAACTTCTGACGTAA
abcR1-At-EMSA-Rev	GAACTGGGAGGAAAAGCCACCGTG
abcR2-At-EMSA-For	CGGCGGCCCTCTTTTTTTACC
abcR2-At-EMSA-Rev	GCCGTGGGCAAGCGTGACAGCT
atu5161-EMSA-For	CATCTTTCACTTAAATTGCTTGC
atu5161-EMSA-Rev	CTATGATCATTCGAAAGAAAAGG
avhB1-EMSA-For	GCAAAGGGTAAACGGGCGAT
avhB1-EMSA-Rev	CGGCGTCCGACGCAAGGAATG
chvE-EMSA-For	GACAGTTATATGGATTCAGA
chvE-EMSA-Rev	GTCCTGTGCGAAAGCCGGCGC
atrA-EMSA-For	GCTCGGAGCGGCTTTCAAGA
atrA-EMSA-Rev	CTGGCCTTGGGCGCCGGTAT

atrB-EMSA-For	GATATCAGGGAGGGTTACCGC
atrB-EMSA-Rev	CCGGTCGTTGGGACCTGTTAGC
atu0828-EMSA-For	TTCAAGCCGCCTCGCGTGCTT
atu0828-EMSA-Rev	GAAATAATGATGCGCAAGC
atu0484-EMSA-For	ATCGTTGCGCGTTGTGGCT
atu0484-EMSA-Rev	AAAGACCGCCTGTCCACAAG
atu2384-EMSA-For	CATGCCGGCCGCCTCGG
atu2384-EMSA-Rev	TGACCGGCTGGTAAACATT
atu2350-EMSA-For	ATAGAATATATCGGTTTCCATA
atu2350-EMSA-Rev	CGAGGCTCTCTGACAAAGCGGTG
atu2187-EMSA-For	CTTCAAACTTGTCAGGCCG
atu2187-EMSA-Rev	TGCGACAGGCCGCTGATCT
atu1296-EMSA-For	TTTTTGTCTCGCGCAGCATTTCT
atu1296-EMSA-Rev	GATGAGGTCCGGGCGGTGCT
atu5119-EMSA-For	CGGCTGGGCAACTTTGTC
atu5119-EMSA-Rev	ATAATACGCTCTGACTTCAAG
atu5116-EMSA-For	GATGACTCTCCCGTCGTCAA
atu5116-EMSA-Rev	CGTCGGATCGACGTTTTCGT
atu4782-EMSA-For	GATTTGCTCGTGTCCCAGTGTC
atu4782-EMSA-Rev	CAATCCATTGAATGGTTACCGA
atu3252-EMSA-For	CCGACCGGCTGAGCTGCCCTC
atu3252-EMSA-Rev	GAACGGTAGGGCGGGAAAC
atu5121-EMSA-For	GGTGTGGCTGACTATTCGC
atu5121-EMSA-Rev	GGAGAGGCCGAAGAAGTGGC
atu5118-EMSA-For	GCAGCCGGCGTAAGCGCCGTTC
atu5118-EMSA-Rev	GTCGGTCGACATTTCTCAGGTCC
vrsA-EMSA-For	CATTTACTTTTTTAAAGATATGA
vrsA-EMSA-Rev	GAGATTTTGTAAGTCCCATGG
atu2708-EMSA-For	GCTGCAACGCTTTGCGATCGAT
atu2708-EMSA-Rev	AATAACCGATGAAAAACAGCGA
atu0055-EMSA-For	AACGCGATTGGTGTGGTTATGTGC
atu0055-EMSA-Rev	GACGTTTCGGGCCTAAACCCAA
atu0157-EMSA-For	ATGCGCCTGGCTGTGTTATCCT
atu0157-EMSA-Rev	AGCAGACGAAACAAGCGTCGC
atu0463-EMSA-For	CCAAAACAGTTCAATGCCGAAGAA
atu0463-EMSA-Rev	TGAGCCTTGCGATTTGCTTGTGC
atu0323-EMSA-For	GCGTCTTGCCCAGCGCGTTAA
atu0323-EMSA-Rev	TTTCTGCAAAAACTCAGATCGG
atu3939-EMSA-For	GGCTGTAGCTCAGCTGGGAGAG
atu3939-EMSA-Rev	CTAAACCCAAAACACCTGGA
atu4669-EMSA-For	TTCTGGCGCCTTCACCACCGC

atu4669-EMSA-Rev	CAGTTCACGGGCGCTCATGCG
rAtu2186-For	TAGGATCCATGGCAATGCCATTGGACTGGGATA
rAtu2186-Rev	TACTCGAGTCAGAAGTTCCAGTTCCGCGCCTTG
rLsrB-For	ATGGGGGATTCTATGTCGCTGGA
rLsrB-Rev	TCAGAAGTTCCAGTTTCTCGCTT
atu0052-atu0053-Rev	CAAATCTCTCCAGGTCCCGCAAGCTTCGCCGCCCA
atu0654-atu8135-For	GTCGGGGGCGGTGCCGCATCGTGCCGTTACGGAAG
atu0654-atu8135-Rev	CTTCCGTAACGGCACGATGCGGCACCGCCCCCGAC
atu0923-atu0924-For	GGAACTTTGCTGCATCGCCTTAGTTTTCAACGGGT
atu0923-atu0924-Rev	ACCCGTTGAAAACTAAGGCGATGCAGCAAAGTTCC
atu2014-atu2015-For	ATCGGCGAGGGTTGAGACCGTTGCCACCCCCCTCT
atu2014-atu2015-Rev	AGAGGGGGGTGGCAACGGTCTCAACCCTCGCCGAT
atu2108-atu2110-For	CCTTGCCACCACTGTCACCAGCGGCGCGGTGGGCT CTTA
atu2108-atu2110-Rev	TAAGAGCCCACCGCGCCGCTGGTGACAGTGGTGGC AAGG
atu2122-atu2123-For	GTCGGTTTGGCGTTTTTGTTCGTAGCGATGATGCT
atu2122-atu2123-Rev	AGCATCATCGCTACGAACAAAAACGCCAAACCGA C
atu2770-atu2771-For	GAACTCCTCGATGAGGACTGTTGGCAGCTTATCGT
atu2770-atu2771-Rev	ACGATAAGCTGCCAACAGTCCTCATCGAGGAGTTC
atu3126-atu3127-For	GGACCAATGCTTCAGGCTGATTGCGCAACCATGAC
atu3126-atu3127-Rev	GTCATGGTTGCGCAATCAGCCTGAAGCATTGGTCC
atu3367-atu3368-For	TGAAGCCGGACAGTCACTGACCAGCCTGTCCGGTT
atu3367-atu3368-Rev	AACCGGACAGGCTGGTCAGTGACTGTCCGGCTTCA
atu4670-atu4671-For	CGGTAATGCGGGTGATCCTACTCCTCCTCCCAAAG
atu4670-atu4671-Rev	CTTTGGGAGGAGGAGTAGGATCACCCGCATTACCG
atu4671-atu4673-Rev	CGAAAGATGCTCCACGGTGACGTTCTCCGA
atu4727-atu4728-Rev	AGGGTCTTGATGAATGATGATGTCATGCAACGCCT
atu0523-atu0524probeFor	CTTCTTGAATGGAATGATTCCATCCCGAGGTATAAT CTCC
atu0523-atu0524probeRev	GGAGATTATACCTCGGGATGGAATCATTCCATTCA AGAAG
atu0612-atu0613probeRev	GAAAGGGGGGACAATCTCGCCACTTGGAAATTATC AATCC
atu0985-atu0986probeFor	TCAGCGCTCGCGGAAATCCGCAAAGACTGCCGAGG GACGC
atu1061-atu1062probeFor	GGCTTACGCTTCAGCATCAGGGCTGCGGCAGCAAC GGCCT
atu2555-atu2557probeFor	TATCAACATCAACGCAATCCTTGGTCTTGTGTCCTG GAAT
atu2555-atu2557probeRev	ATTCCAGGACACAAGACCAAGGATTGCGTTGATGT TGATA
atu4670-atu4671-For	CGGTAATGCGGGTGATCCTACTCCTCCTCCCAAAG
atu4670-atu4671-Rev	CTTTGGGAGGAGGAGTAGGATCACCCGCATTACCG

atu0468-For-Northern	CATTTTCCCAAGACTGCTCCTCCGCTTCAATTGAT
atu0468-Rev-Northern	ATCAATTGAAGCGGAGGAGCAGTCTTGGGAAAATG
atu0604-For-Northern	TGGAGGCTCTTAACAGGGCATGGGTCGGTTGAG
atu0604-Rev-Northern	CTCAACCGACCCATGCCCTGTTAAGAGCCTCCA
atu1727-For-Northern	CCGGCATTTGAGACGCCGGGCCTGTGGAACTTATG
atu1727-Rev-Northern	CATAAGTTCCACAGGCCCGGCGTCTCAAATGCCGG
atu2478-For-Northern	GCCTTCAAGATCGCAGCAGTCATGTTGCCAATGAA
atu2478-Rev-Northern	TTCATTGGCAACATGACTGCTGCGATCTTGAAGGC
atu2524-For-Northern	CCGAAATCAAACGCTGCCGCGAAGTCGCGGTAGCG
atu2524-Rev-Northern	CGCTACCGCGACTTCGCGGCAGCGTTTGATTTCGG
atu5117-For-Northern	GAACCATGCTCTCTCCCGATGGTGATCGCGAGGGA
atu5117-Rev-Northern	TCCCTCGCGATCACCATCGGGAGAGAGCATGGTTC
atu4670-Up-For	TAGGATCCGCAGACGTATTGCAAAAAGAAAC
atu4670-Up-Rev	TCCTGCCACAGGCAAAGGGC
atu4670-Dn-For	TCGTTGATGGTCTGACGATATTT
atu4670-Dn-Rev	TACTGCAGTGGCTTCGGAGACAAGCGAGG

*Underlined sequences depict a restriction endonuclease recognition site.

Table 3.7: Plasmids used in this study.

Plasmid name	Description	Reference
pNPTS138	Cloning vector; contains *sacB*; Kan^R	(M.R.K. Alley, unpublished)
pSRK-Km	pBBR1MCS-2-derived broad-host-range expression vector containing *lac* promoter and *lacI*^{q}, *lacZ*α^+, and Km^R	Khan, et al. 2008
pASK-IBA7	Recombinant protein expression vector; Amp^R	IBA
p*atu2186*	In-frame deletion of *atu2186* plus 1 kb of each flanking region in pNPTS138	This study
p*abcR1*	In-frame deletion of *abcR1* plus 1 kb of each flanking region in pNPTS138	This study
p*abcR2*	In-frame deletion of *abcR2* plus 1 kb of each flanking region in pNPTS138	This study
P*atu4670*	In-frame deletion of *atu4670* plus 1 kb of each flanking region in pNPTS138	This study
pSRK-Km-*AtvtlR*	Coding region of *atu2186* in pSRK-Km for complementation	This study
pSRK-Km-*BavtlR*	Coding region of *bab1_1517* in pSRK-Km for complementation	This study
pSRK-Km-*SmlsrB*	Coding region of *smc01225* in pSRK-Km for complementation	This study
pSRK-Km-*vrsA*	Coding region of *vrsA* in pSRK-Km for complementation	This study
prAtu2186	Coding region of *atu2186* in pASK-IBA7 for recombinant protein purification	This study
rVtlR-IBA7	Coding region of *bab1_1517* in pASK-IBA7 for recombinantly protein purification	Sheehan, et al. 2015
prLsrB	Coding region of *smc01225* in pASK-IBA7 for recombinantly protein purification	This study

Figures/Figure Legends

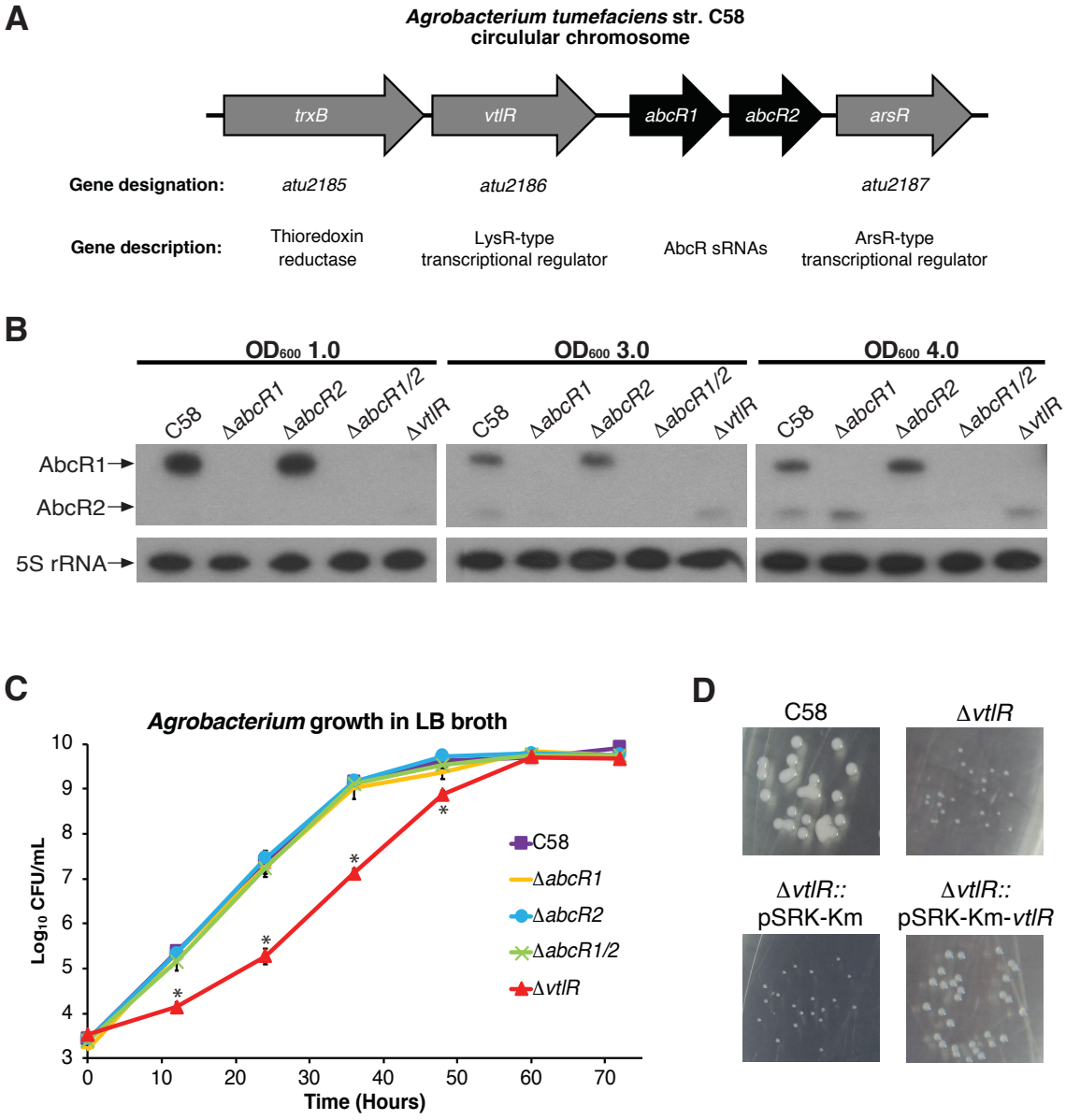

Figure 3.1: VtlR in *Agrobacterium tumefaciens* str. C58.
A. The *vtlR* gene (*atu2186*) is located on the *A. tumefaciens* circular chromosome, directly downstream of a thioredoxin reductase, *trxB* (*atu2185*). *vtlR* is upstream of the tandemly encoded AbcR sRNAs, *abcR1* and *abcR2*. Directly downstream of *abcR2* is an ArsR-type transcriptional regulator, *arsR* (*atu2187*).
B. Northern blot analyses for AbcR small RNAs. RNA was isolated from *A. tumefaciens* C58, Δ*vtlR*, Δ*abcR1*, Δ*abcR2*, and Δ*abcR1/2* cultures grown to optical densities (OD_{600}) of 1.0, 3.0, or 4.0, separated on a denaturing polyacrylamide gels, transferred to a nitrocellulose membranes, and probed with specific radiolabeled oligonucleotides. 5S ribosomal RNA was blotted for as a loading control.

C. In vitro growth kinetics of *A. tumefaciens* strains. The *A. tumefaciens* C58, Δ*abcR1*, Δ*abcR2*, Δ*abcR1/2*, and Δ*vtlR* strains were grown in LB broth, and at specified time points, samples from each culture were taken, serial diluted and plated on AT-agar plates to determine colony forming units (CFUs). Data represents average CFUs per ml ± the standard deviation of results from triplicate samples. Statistical significance is denoted by an asterisk (*) (*t*-test; $P < 0.05$).
D. Photographs of *A. tumefaciens* colonies on ATNG+kanamycin (45 μg/mL) agar after 72 hours of growth.

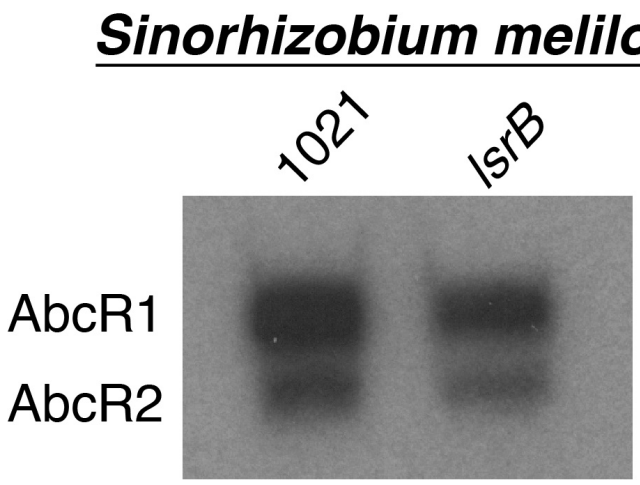

Figure 3.2: *S. meliloti* LsrB does not control expression of the AbcR sRNAs. Northern blot analysis was carried out with *S. meliloti* wild-type (1021) and a deletion of *lsrB* analyzing the expression of the AbcR sRNAs.

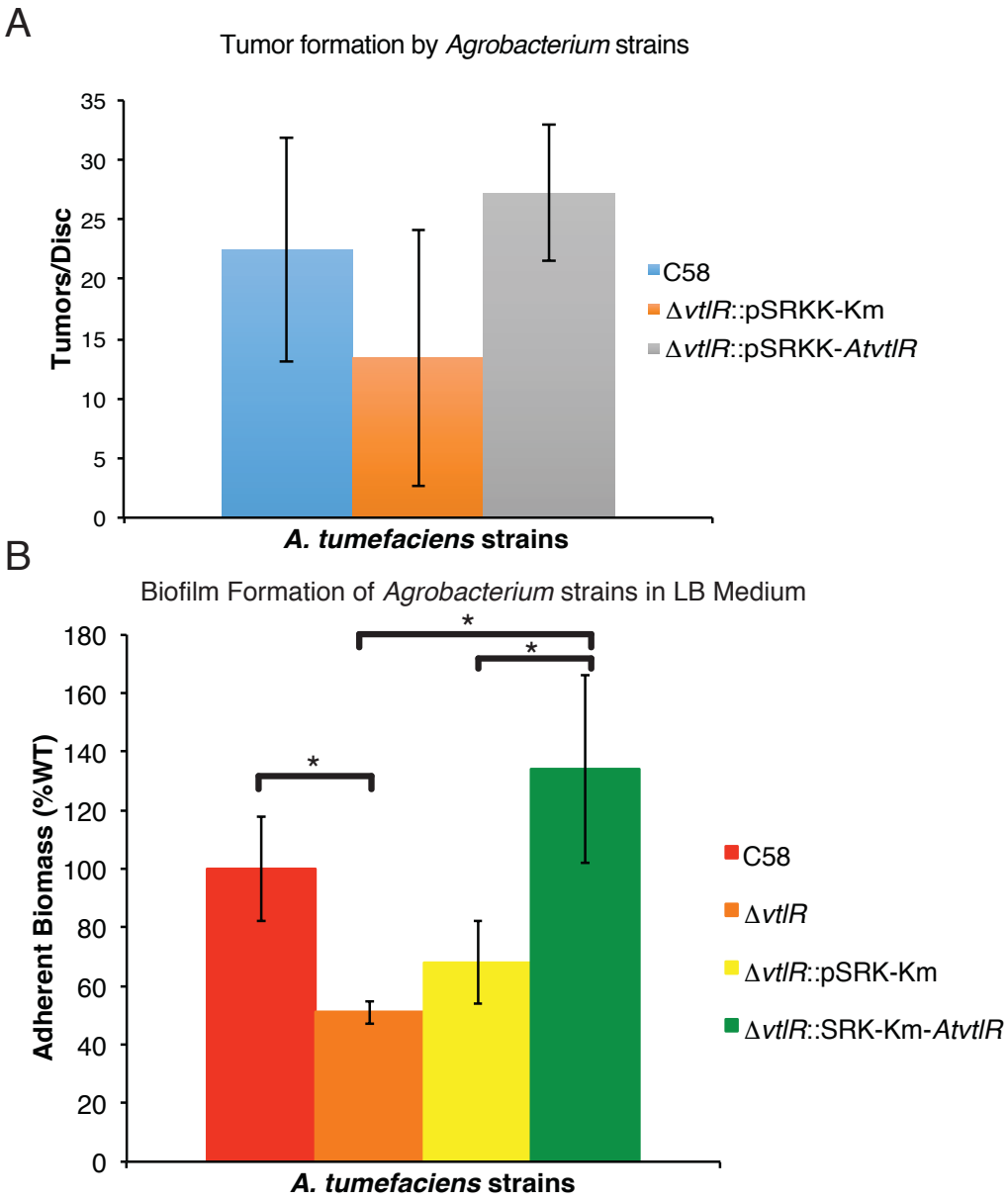

Figure 3.3: Contribution of *vtlR* to *A. tumefaciens*-mediated tumorigenesis and biofilm formation.

A. Tumor formation of *A. tumefaciens* str. C58 and Δ*vtlR* in experimentally infected potatoes. Sterile potato discs were inoculated with C58 or Δ*vtlR*, and the number of tumors was counted 21-days post infection.

B. Biofilm formation by *A. tumefaciens* mutants. Data are means and standard deviations from three separate experiments normalized to C58. Statistical significance is denoted by an asterisk (*) (*t*-test; $P < 0.05$).

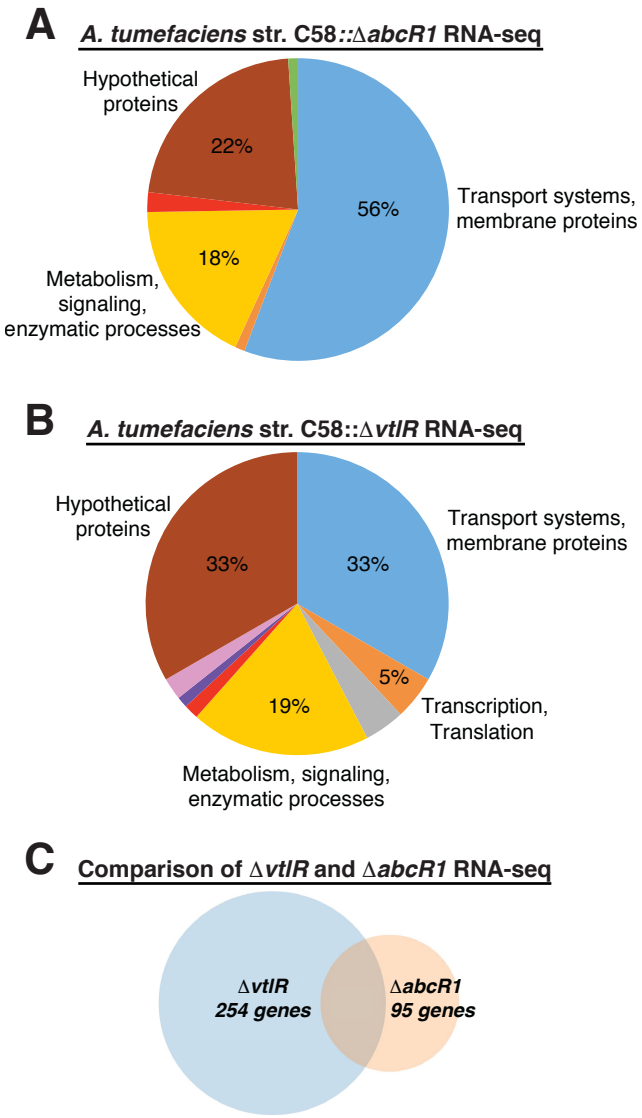

Figure 3.4: Overview of RNA-sequencing analyses of *A. tumefaciens* Δ*abcR1* and Δ*vtlR*.

A. Pie chart summarizing genes differentially expressed (≥3-fold) in *A. tumefaciens* Δ*abcR1*. Blue: Transport systems and membrane proteins (56%); Brown: Hypothetical proteins (22%); Yellow: Metabolism, signaling, enzymatic processes (18%); Red: Phage-related (2%); Orange: Transcription, translation (1%); Green: Chemotaxis, motility (1%).

B. Pie chart summarizing genes differentially expressed (≥3-fold) in *A. tumefaciens* Δ*vtlR*. Blue: Transport systems and membrane proteins (33%); Brown: Hypothetical proteins (33%); Yellow: Metabolism, signaling, enzymatic processes (19%); Orange: Transcription, translation (5%); Grey: Secretion systems (4%); Pink: Polysaccharide biosynthesis (3%); Red: Phage-related (2%); Purple: Conjugation (1%).

C. Venn diagram comparing genes differentially expressed in *A. tumefaciens* Δ*vtlR* (254 genes) and *A. tumefaciens* Δ*abcR1* (95 genes) deletion strains.

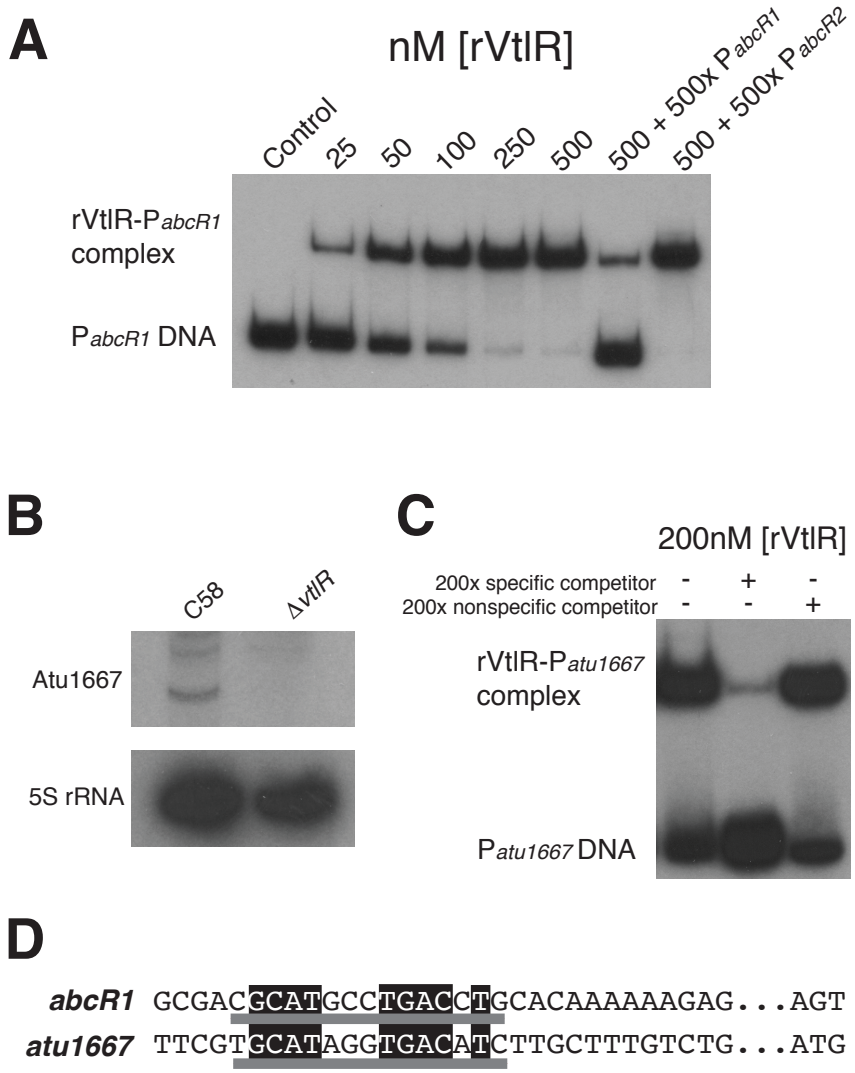

Figure 3.5: *A. tumefaciens* VtlR directly regulates *abcR1*, encoding a small RNA, and *atu1667*, encoding a small hypothetical protein.

A. Electrophoretic mobility shift assays (EMSAs) were carried out with recombinantly purified *A. tumefaciens* VtlR (rVtlR) and ^{32}P-radiolabeled *abcR1* promoter region (P_{abcR1}). Increasing concentrations of rVtlR were added to P_{abcR1}, and the binding reactions were incubated at room temperature for 30 minutes. To determine specificity of binding, specific (unlabeled P_{abcR1}) and non-specific (unlabeled P_{abcR2}) competitors were added to some binding reactions.

B. Northern blot analysis confirming VtlR activation of *atu1667*. RNA from *A. tumefaciens* C58 and Δ*vtlR* was isolated from cultures grown in LB broth to OD_{600} 0.6, separated on a denaturing polyacrylamide gel, transferred to a nitrocellulose membrane,

and probed with radiolabeled oligonucleotides. 5S ribosomal RNA was used as a loading control.
C. EMSAs were performed with rVtlR and ^{32}P-radiolabeled *atu1667* promoter region ($P_{atu1667}$). To determine specificity of binding, specific (unlabeled $P_{atu1667}$) and non-specific (unlabeled P_{abcR2}) competitors were added to some binding reactions.
D. Nucleotide alignment of the promoter regions of *abcR1* and *atu1667*. A consensus sequence is underlined in grey, with 100% identity of nucleotides highlighted in black.

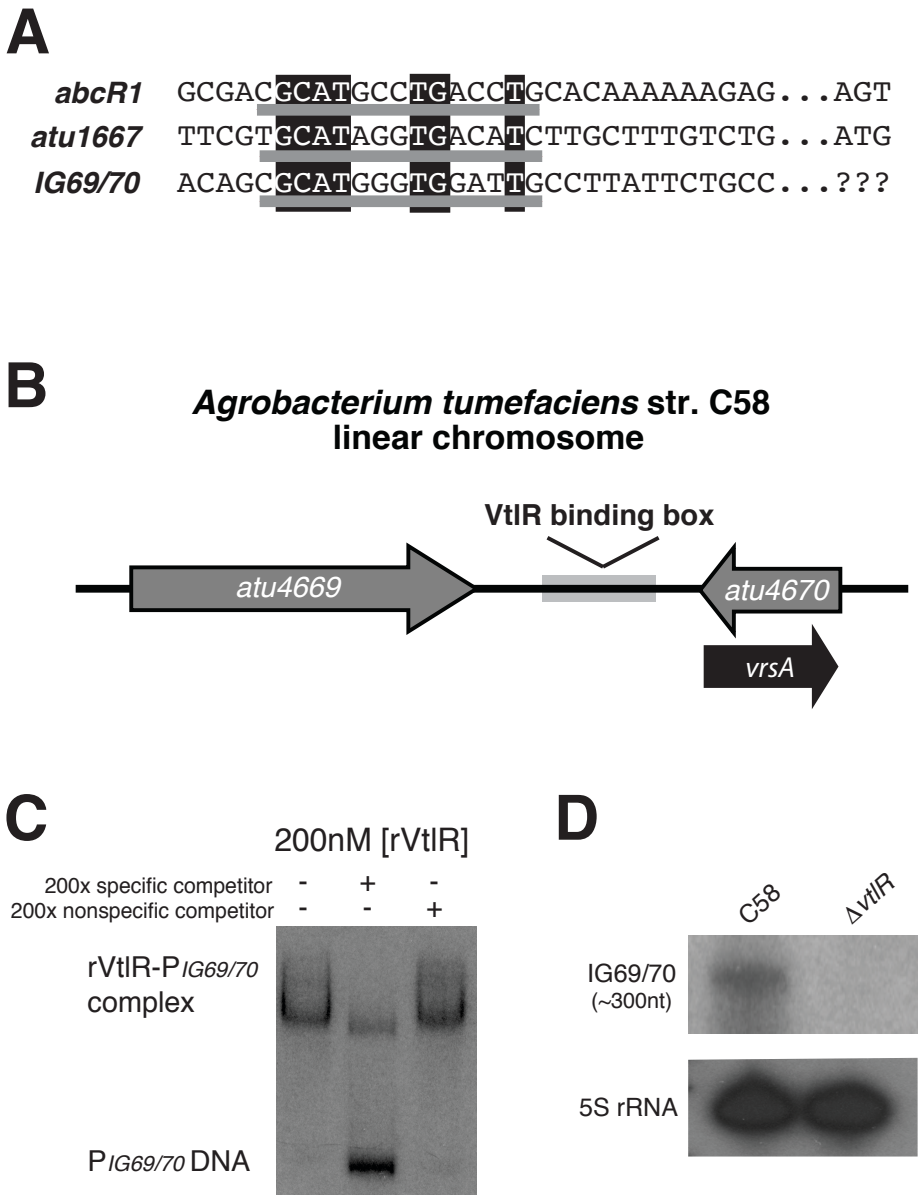

Figure 3.6: VtlR activates a novel transcript in *A. tumefaciens.*
A. Virtual footprinter (Münch *et al*., 2005) revealed a novel VtlR-binding site in *A. tumefaciens* in the intergenic region of *atu4669* and *atu4670* (called *vrsA*) Nucleotide alignment of the promoter regions of *abcR1*, *atu1667*, and *vrsA*. A consensus sequence is underlined in grey, with 100% identity of nucleotides highlighted in black.
B. The new VtlR-binding site is located on the linear chromosome in the intergenic region of two genes encoding for hypothetical proteins (*atu4669* and *atu4670*). The VtlR-binding box is depicted by a light grey box, and the putative transcript is depicted by the black arrow antisense to *atu4670*.
C. EMSAs were carried out with rVtlR and ^{32}P-radiolabeled *vrsA* promoter region. To determine specificity of binding, specific (unlabeled P_{vrsA}) and non-specific (unlabeled P_{abcR2}) competitors were added to some binding reactions.

D. Northern blot analysis was employed to determine if VtlR regulates the putative transcript. RNA from *A. tumefaciens* C58 and Δ*vtlR* was isolated from cultures grown in LB broth to OD_{600} 0.6, separated on a denaturing polyacrylamide gel, transferred to a nitrocellulose membrane, and probed with radiolabeled oligonucleotides. 5S ribosomal RNA was used as a loading control.

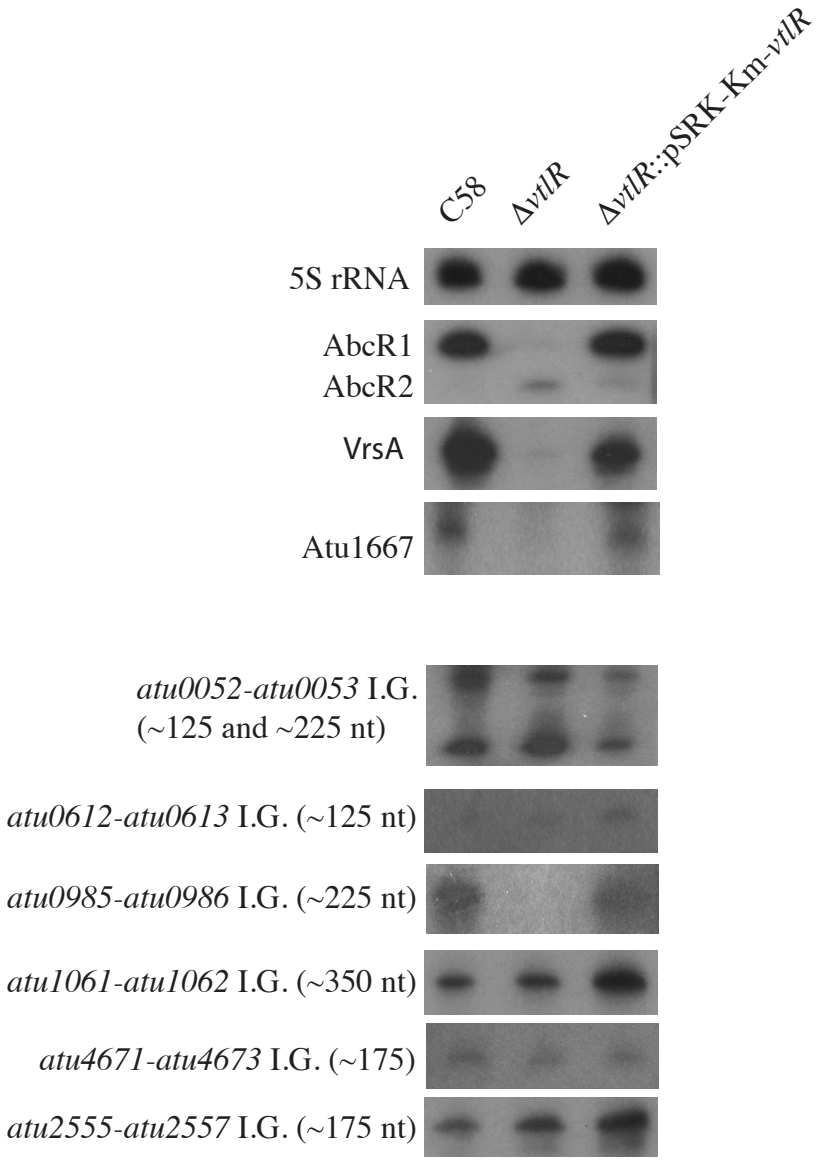

Figure 3.7: Expression of novel sRNAs in *A. tumefaciens* str. C58, Δ*vtlR*, and Δ*vtlR*-pSRK-Km-*vtlR*.
Northern blot analysis to measure expression of AbcR1, AbcR2, VrsA, Atu1667, and novel trans- and cis-encoded sRNAs identified to be putatively differentially expressed in Table 3.2 in *A. tumefaciens* str. C58, Δ*vtlR*, and Δ*vtlR*-pSRK-Km-*vtlR*.

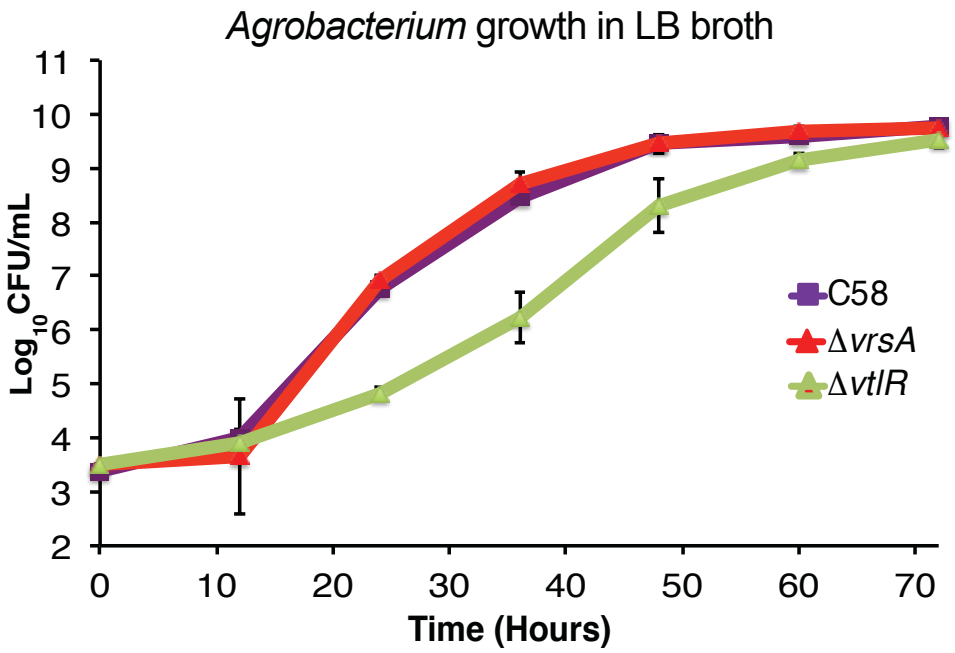

Figure 3.8: Characterization of *A. tumefaciens* Δ*vrsA*.
In vitro growth kinetics of *A. tumefaciens* strains. The *A. tumefaciens* C58, Δ*vrsA*, and Δ*vtlR* strains were grown in LB broth, and at specified time points, samples from each culture were taken, serial diluted and plated on AT-agar plates to determine colony forming units (CFUs). Data represents average CFUs per ml ± the standard deviation of results from triplicate samples.

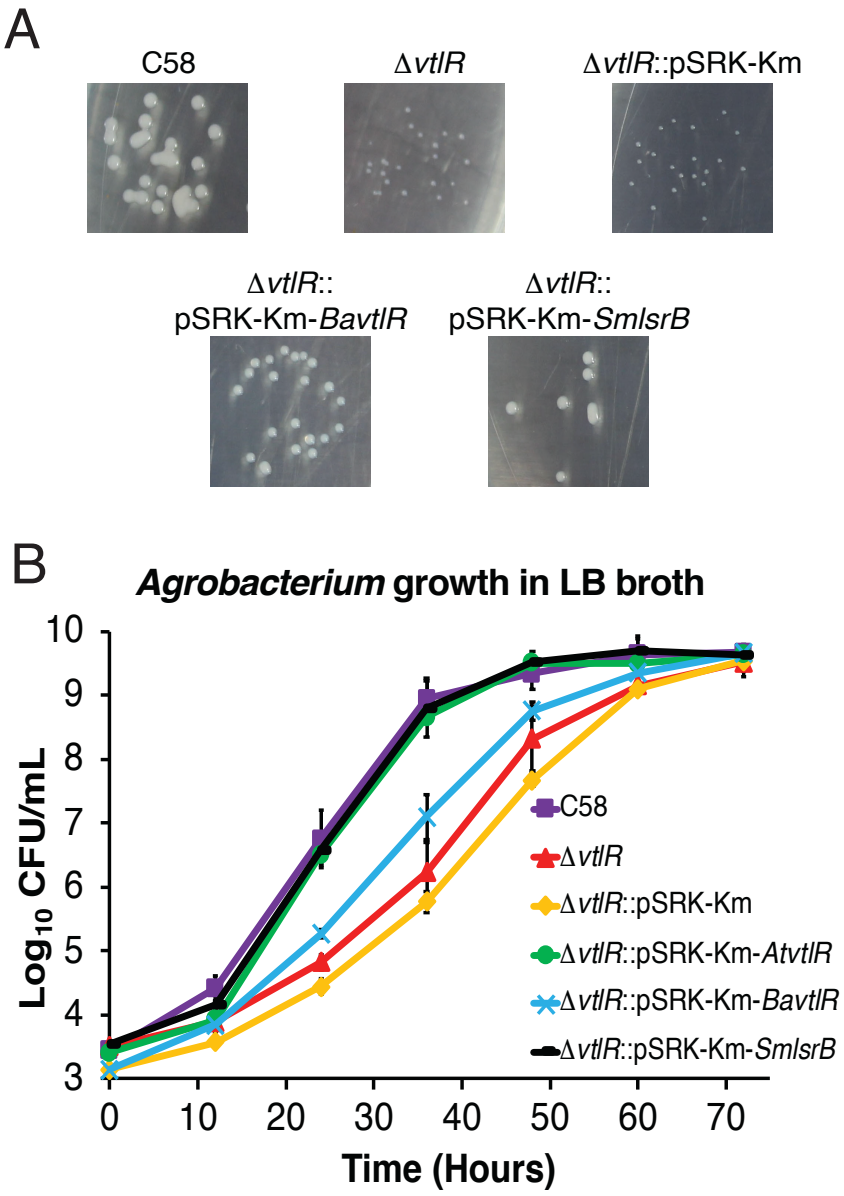

Figure 3.9: Heterologous complementation of *A. tumefaciens* ΔvtlR with *S. meliloti lsrB* and *B. abortus vtlR.*

A. Photographs of *A. tumefaciens* colonies on AT-agar after 72 hours of growth.

B. The *A. tumefaciens* strains C58, D*vtlR*, and *vtlR*-complement strains were grown in LB broth with constant shaking at 28°C. At 12-hour intervals, samples from each culture were taken, serial diluted and plated out on AT-agar plates to determine colony-forming units (CFUs). Data represents average CFU per ml from each *A. tumefaciens* strain ± the standard deviation of results from triplicate samples. *Ba=Brucella abortus*; *Sm=Sinorhizobium meliloti*

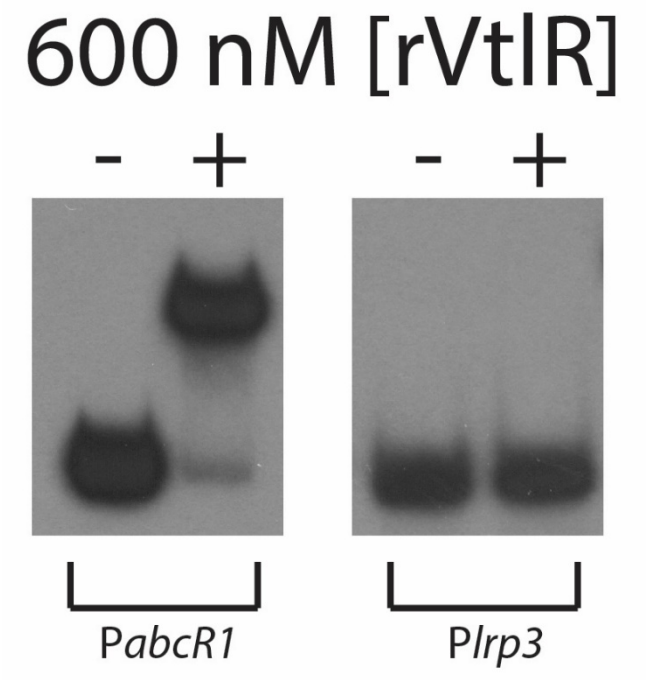

Figure 3.10: *A. tumefaciens* VtlR does not bind to the promoter region of *lrp3*.
Since it was previously shown how LsrB bound to the promoter region of *lrp3* in *S. meliloti*, we sought to test the ability of VtlR to bind *lrp3* in *A. tumefaciens*. Electrophoretic mobility shift assays (EMSAs) were carried out with recombinantly purified VtlR (rVtlR) and ^{32}P-radiolabeled *lrp3* promoter region (P*lrp3*). A (+) represents the addition of 600 nM rVtlR, where a (–) represents no protein added to the binding reaction mixture. The promoter region of *abcR1* was used as a control for rVtlR activity (P*abcR1*).

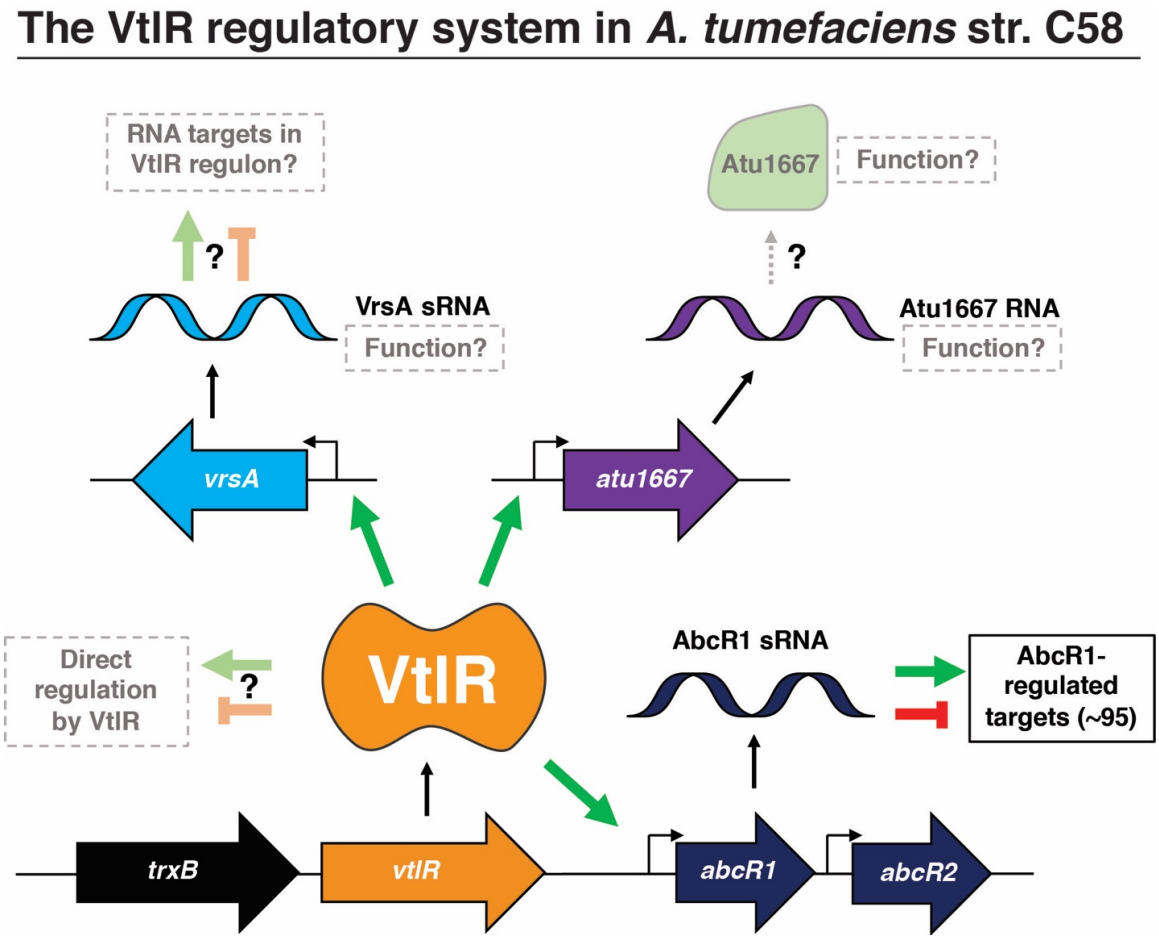

Figure 3.11: Working model of VtlR regulation in *A. tumefaciens*.
vtlR (orange) is located downstream of the thioredoxin reductase, *trxB* (black) and upstream of the tandemly encoded sRNAs, *abcR1* and *abcR2*. VtlR directly activates *abcR1*, *vrsA* (blue) and *atu1667* (purple). AbcR1 regulated ~95 target independent of VtlR. The regulatory capacity of VrsA and Atu1667 are currently unknown.

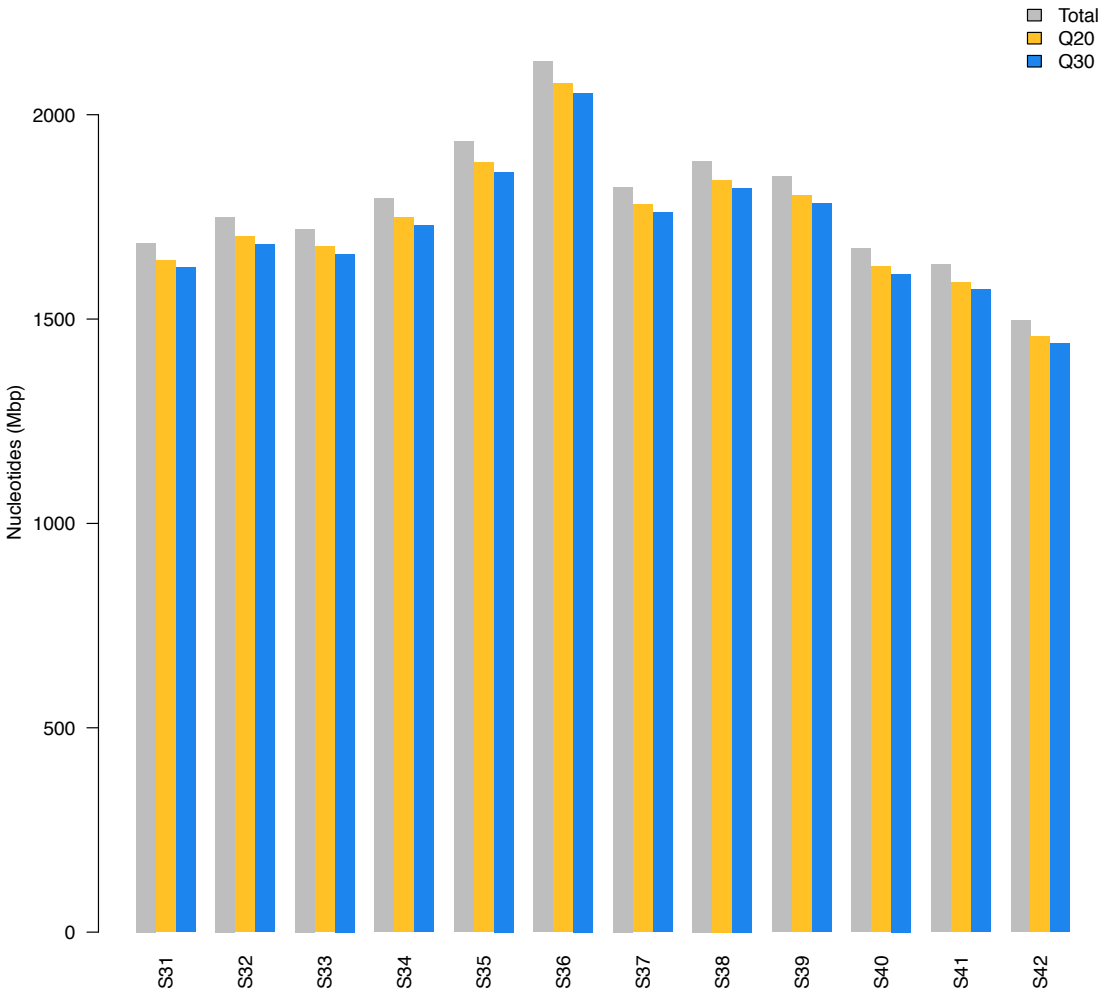

Figure 3.12: Sequencing summary after quality control.
Bars in different colors represent nucleotides from all (Total, grey) sequencing quality greater than or equal to 20 (Q20, yellow), and sequencing quality greater than or equal to 30 (Q30, blue). Samples S31-S33: 3 replicates of *A. tumefaciens* str. C58 cultured in LB broth; S34-S36: 3 replicates of *A. tumefaciens* str. C58::Δ*abcR1* cultured in LB broth; S37-39: 3 replicates of *A. tumefaciens* str. C58::Δ*abcR2* cultured in LB broth; S40-42: 3 replicates of *A. tumefaciens* str. C58::Δ*vtlR* cultured in LB broth.

Chapter 4: The role of GABA as a communication molecule during host-*Brucella* interactions

Budnick, J.A., Sheehan, L.M., Pitzer, J., Dunman, P.M., Roop, R.M. II, and Caswell, C.C.

Abstract

The neurotransmitter gamma-aminobutyric acid (GABA) is the most abundant inhibitory neurotransmitter in the human brain; however, it is becoming more evident that this non-proteinogenic amino acid plays multiple functional roles in biology. In the present study, the transport and function of GABA is characterized in the highly infectious intracellular bacterium *Brucella abortus*. The data show that ^{3}H-GABA is imported by *B. abortus* under nutrient limiting conditions and that the small RNAs AbcR1 and AbcR2 negatively regulate this transport. Similar assays revealed that ^{3}H-GABA transport is uninhibited by the 20 standard proteinogenic amino acids, representing preference for the transport of ^{3}H-GABA. Metabolic studies did not show any potential metabolic utilization of GABA by *B. abortus* as a carbon or nitrogen source, but microarray analysis determined several loci with elevated expression in *B. abortus* exposed to GABA, including putative nitrate and nitric oxide reductases, a putative type I secretion system, and a putative periplasmic binding protein with homology to a GABA specific periplasmic binding protein in *Agrobacterium tumefaciens*. This study provides evidence for GABA sensing during host-*Brucella* interactions and outlines a divergence of GABA transport between *B. abortus* and the phylogenetically related bacterium *A. tumefaciens*.

Introduction

Gamma-aminobutyric acid (GABA) is a non-proteinogenic amino acid that is commonly associated as an important inhibitory neurotransmitter in the vertebrate brain (Mittal *et al.*, 2017). However, understanding of the biological function of GABA has evolved over the years to include neurobiology, immunology, and bacteriology. With regards to metabolism, the GABA shunt is utilized by both prokaryotes and eukaryotes to metabolize GABA to succinate, which can then be supplied into the TCA cycle (Feehily & Karatzas, 2013, Shelp *et al.*, 1999). This is achieved by transport of exogenous GABA or conversion of glutamate to GABA by the enzyme glutamate decarboxylase (GAD). GAD is an important enzyme for both the production of GABA and deacidification of the intracellular environment. If the pH of the cell becomes unfavorably low, GAD can convert glutamate to GABA with the attachment of a proton, then export it out of the cell, which will result in increasing the intracellular pH to safer neutral conditions (De Biase & Pennacchietti, 2012, Pennacchietti *et al.*, 2018).

In plants, several studies have revealed the necessity for GABA during metabolism and developmental growth (Palanivelu *et al.*, 2003, Bown & Shelp, 2016, Michaeli & Fromm, 2015), but GABA is also an important modulator of immunity against pathogenic organisms, including insects, fungi, and bacteria. Upon plant cell damage, the pH of the plant intracellular environment will decrease, activating the GAD system, producing an excess of secreted GABA surrounding the damaged area of the plant (Shelp *et al.*, 1999). In insects, increased environmental GABA concentrations have been shown to lead to decreased larvae growth rate, survival, and feeding by pests on tobacco plants (Ramputh & Bown, 1996, Shelp *et al.*, 2006, MacGregor *et al.*, 2003).

Exogenous GABA also has a negative effect on bacterial pathogenesis of plants. A deletion of the GABA transaminase, responsible for the conversion of GABA to succinic semialdehyde during the GABA shunt, in *Pseudomonas syringae* led to decreased expression of a type III secretion system required for full virulence of the bacterium (Park *et al.*, 2010). This deletion strain displayed significant differences in virulence *in planta* when compared to the parental strain, which was attributed to the decreased expression of the type III secretion system (Park *et al.*, 2010). Decreased virulence by exogenous GABA has also been shown in the bacterial plant pathogen *Agrobacterium tumefaciens*. *A. tumefaciens* encodes two ABC transport systems, Bra and Gts, that import exogenous GABA (Planamente *et al.*, 2010, Planamente *et al.*, 2013). Once GABA is transported into *A. tumefaciens*, it is catabolized via the GABA shunt and byproducts of the shunt induce the expression of AttM, a lactonase (Wang *et al.*, 2006). This lactonase will quench quorum signaling molecules expressed by *A. tumefaciens* leading to a decrease in the expression of virulence related genes (Planamente *et al.*, 2010, Planamente *et al.*, 2013). By increasing the expression and secretion of GABA by plants, studies have shown that tobacco plant susceptibility to *A. tumefaciens* can be decreased. Mutating the GAD system in a plant, however, led to increased T-DNA transfer, a major virulence factor of *A. tumefaciens*, from *A. tumefaciens* to a tomato plant model (Chevrot *et al.*, 2006, Nonaka *et al.*, 2017). Alternatively, increasing GABA transaminase activity in *A. tumefaciens,* causing a decrease in intracellular GABA concentrations, led to higher rates of T-DNA transfer and transformation of tomato plants, further characterizing the inhibitory role of GABA in *A. tumefaciens* virulence (Nonaka *et al.*, 2017).

More recently, GABA has been observed to be an immunomodulator in mammalian systems and several studies have shown GABA to activate immune cells and play a role in the antimicrobial activity of macrophages. Bhat et al. showed that immune cells (dendritic cells and macrophages) can synthesize and catabolize GABA and the presence of GABAergic agents led to a decrease in inflammation (Bhat *et al.*, 2010). The authors hypothesize that GABA could potentially be utilized as a signaling molecule between immune cells to modulate inflammation. Interestingly, GABAergic signaling has also been shown to enhance phagosomal maturation in macrophages and inhibition of this signaling led to increased intracellular concentrations of bacteria within the macrophage (Kim *et al.*, 2018).

Brucella spp. are pathogenic intracellular bacteria within the Order *Rhizobiales* in the Class *Alphaproteobacteria.* The brucellae infect a variety of mammalian species both wild and domesticated in which brucellosis primarily effects reproductive health in these animals and chronic infection can lead to multiple organ complications (Hull & Schumaker, 2018, von Bargen *et al.*, 2012). Several *Brucella* spp. also have the capacity to cause infection in humans via direct contact with contaminated animal products and is one of the most prevalent zoonoses worldwide (Pappas *et al.*, 2006). Human brucellosis primarily presents as flu-like symptoms including an undulating fever, and chronic infection can also cause damage to multiple organs (de Figueiredo *et al.*, 2015, Dean *et al.*, 2012). *Brucella* spp. are stealth pathogens that contain few Classical virulence factors and primarily evade the host immune system by adaptation and formation of a replicative niche within primary immune cells (dendritic cells and macrophages) of the host (Roop *et al.*, 2009, He, 2012).

An understudied virulence factor of the brucellae are small regulatory RNAs (sRNAs), which can swiftly regulate gene function post-transcriptionally to adapt to changing environmental conditions (Waters & Storz, 2009). While characterizing the role of the sRNAs AbcR1 and AbcR2 in *B. abortus* pathogenesis, Caswell et al. showed that these small RNAs primarily function as negative regulators of several ABC type transport systems in *B. abortus* (Caswell et al., 2012). One system in particular, a locus including *bab1_1792-bab1_1799*, encodes proteins with high amino acid identity to one of the GABA ABC transport systems, Bra, mentioned above in *Agrobacterium tumefaciens* (Figure 4.1). Specifically, BAB1_1794 and BAB1_1799 are overexpressed in the absence of the small RNA chaperone Hfq or the sRNAs AbcR1 and AbcR2 (Caswell *et al.*, 2012). Similarly to *B. abortus*, the homologous transport system in *A. tumefaciens* has also been shown to be negatively regulated by AbcR1 (Wilms *et al.*, 2011). No previous studies have explored the function of GABA within the brucellae, with the exception of the GAD system. Interestingly, the functionality of the GAD system differs between species of *Brucella*. The "Classical" species of *Brucella* (*B. melitensis*, *B. abortus*, *B. suis*, *B. canis*, *B. neotomae*, and *B. ovis*) do not possess a functional GAD system due to point or frame-shift mutations in *gadB* and *gadC* genes (Damiano *et al.*, 2015). Thus the potential role for GABA utilization by the "Classical" species of *Brucella* is unknown.

The following study will focus on characterizing the potential import of GABA into *B. abortus* and elucidate the functional role of GABA in *Brucella* pathogenesis. The results revealed that GABA is transported under nutrient limiting conditions and GABA transport is regulated by AbcR1 and AbcR2 in *B. abortus*. The data also showed no

metabolic potential for GABA by *B. abortus in vitro* but the presence of GABA did induce transcriptional changes in several genetic loci.

Results

The transport of ^{3}H-GABA is inhibited by the presence of glutamate during incubation.

A radiolabeled transport assay was utilized to assess the ability of *B. abortus s*trains to import tritium labeled GABA (^{3}H-GABA) grown under several growth conditions. Cultures of *B. abortus* were inoculated with ^{3}H-GABA and subsequently collected via filtration through a syringe filter. The radioactivity of the filter was measured to quantify the amount of radiation imported by the brucellae collected. If ^{3}H-GABA is imported by *B. abortus*, then the filter will measure high radioactivity above background; however, if ^{3}H-GABA is not imported by *B. abortus*, then the ^{3}H-GABA will passed through the filter and the filter will not measure high radioactivity above background.

GMM is a commonly used minimal medium to mimic a nutrient-limiting environment. *Brucella* growth is sustained in this medium, but will not reach high concentrations compared to growth in nutrient rich medium, such as brucella broth. This medium specifically contains the amino acid glutamate as a carbon and nitrogen source. The above transport study was utilized to determine 1) if *B. abortus* would import ^{3}H-GABA and 2) if glutamate in the medium would inhibit the uptake of ^{3}H-GABA. The experiment was conducted with GMM containing glutamate (GMM) and GMM without the addition of glutamate (GMM-Glu). As a control, 1,000-fold excess non-radiolabeled GABA was added to the cultures simultaneously with ^{3}H-GABA to inhibit any observed ^{3}H-GABA import.

Firstly, the assay revealed that *B. abortus* 2308 imported GABA in both GMM and GMM-Glu and this transport was inhibited by the addition of excess non-

radiolabeled GABA in both culture media (Figure 4.2). However, when glutamate was present in the culture medium, the amount of ^{3}H-GABA imported by *B. abortus* 2308 decreased by over 95%. This indicated that transport of ^{3}H-GABA was increased in the absence of the known carbon and nitrogen source glutamate.

Due to the inhibitory effect of glutamate on the import of ^{3}H-GABA, all further ^{3}H-GABA uptake experiments were conducted in GMM-Glu. Although glutamate inhibited the amount of GABA imported when present in the medium, glutamate does not competitively inhibit GABA transport.

The presence of proteinogenic amino acids does not significantly inhibit the import of ^{3}H-GABA into *B. abortus*.

The above ^{3}H-GABA transport assay was again utilized to assess whether other amino acids could inhibit ^{3}H-GABA import in *B. abortus*. The assay was repeated with the addition of no inhibitor or 100 μM individual amino acids in combination with ^{3}H-GABA, resulting in a ratio of 1:1,000 ^{3}H-GABA:nonradiolabeled amino acid. As a control, the data showed ^{3}H-GABA uptake was almost completely inhibited by the presence of 1,000-fold excess nonradiolabled GABA (Figure 4.3). The import of ^{3}H-GABA was not significantly inhibited by the presence of any other nonradiolabled amino acid (Figure 4.3). Combined with the previous experiment, glutamate may not competitively inhibit the transport of GABA via interactions with the putative transport system, but rather this transporter may be induced in the absence of glutamate in an environment of extremely limited nutrient availability. This experiment also indicates that the mechanism responsible for the transport of GABA would preferentially transport

GABA prior to the transport of other amino acids, if it can transport other amino acids at all.

^{3}H-GABA import is inhibited by the sRNAs AbcR1 and AbcR2.

The regulation of GABA transport is mediated by the sRNA AbcR1 in *A. tumefaciens,* and a deletion of *abcR1* results in increased import of radiolabeled GABA in *A. tumefaciens* (Wilms *et al.*, 2011). *B. abortus* 2308 encodes the sRNAs AbcR1 and AbcR2, homologs of AbcR1, which have been shown to regulate ABC-type transport systems, including the homologous GABA transport in *A. tumefaciens* (Figure 4.1) (Caswell *et al.*, 2012). Therefore, it was hypothesized that a deletion of *abcR1* and *abcR2* in *B. abortus* would result in increased GABA transport.

To test this hypothesis, the above mentioned ^{3}H-GABA transport assay was utilized to assess the import of ^{3}H-GABA by *B. abortus* 2308 (2308) or *B. abortus* 2308::Δ*abcR1*Δ*abcR2* (Δ*abcR1/2*) (Figure 4.4). The results indicated that ^{3}H-GABA import was increased by almost 50% in Δ*abcR1/2* compared to the parental strain. This indicated that GABA transport was negatively regulated by the sRNAs AbcR1 and AbcR2, similarly to what has been observed in *A. tumefaciens*.

GABA is not utilized as a nitrogen or carbon source by *Brucella*.

To elucidate the biological role of GABA in the brucellae, two options were considered, GABA is either acting as a source of carbon and nitrogen or GABA as a signaling molecule to induce changes in gene expression. The hypothesis that GABA is a metabolite was first examined. As mentioned before, GMM is often utilized as a defined medium to mimic a nutrient-limiting environment. This medium was developed in 1958 by Philipp Gerhardt and contains several sources of carbon; including lactic acid,

glycerol, and glutamate; and glutamate as the sole nitrogen source (Gerhardt, 1958). Growth curves were utilized to test the ability for GABA to be utilized as a nitrogen source for the brucellae via replacement of glutamate. *B. abortus* 2308 was grown overnight in brucella broth to late exponential phase, pelleted and washed, and then used to inoculate GMM with glutamate (GMM), GMM without glutamate (GMM-Glu), or GMM without glutamate but supplemented with GABA (0.15%) (GMM-Glu+GABA). Growth of the bacterium was measured in each culture for 175 hours (Figure 4.5A). Initially, all cultures showed growth, due to residual nutrients from the nutrient rich brucella broth. However, *B. abortus* 2308 in GMM-Glu revealed a decrease in concentration in comparison to *B. abortus* 2308 in GMM over time. *B. abortus* grown in GMM-Glu+GABA showed similar growth to brucellae grown in the absence of glutamate (GMM-Glu). This indicated that GABA could not be utilized as a nitrogen source in place of glutamate for sustained *B. abortus* growth.

A respirometry assay was utilized to assess GABA as a potential carbon source utilized by *B. abortus*. Oxygen concentrations of *B. abortus* cultures were measured via oxygraph machine as a means to measure respiration in response to different carbon sources. Carbon sources tested included GABA, glutamate, or erythritol. The metabolic role of glutamate is discussed above. Erythritol is a sugar alcohol found in the reproductive tracts of animals susceptible to brucellosis and has been shown to be a preferred carbon source for brucellae growth as well as an inducer of virulence related genes (Keppie *et al.*, 1965, Petersen *et al.*, 2013). Oxygen is consumed during aerobic respiration, thus if the bacterium is actively utilizing the presented carbon source, then respiration will increase, and oxygen concentrations of the culture medium will decrease.

In the presence of erythritol, a preferred carbon source of *B. abortus*, respiration occurred at a high rate, and oxygen levels decreased rapidly (Figure 4.5B). In the presence of glutamate, a suitable carbon and nitrogen source for *B. abortus*, but not preferred over erythritol, respiration occurred at a slower rate, but oxygen consumption still occurred. In the presence of GABA, however, the change in oxygen concentration over time was negligible, indicating that GABA was not utilized as a carbon source by *B. abortus* (Figure 4.5B).

GABA induces the expression of a secretion system, nitrate and nitric oxide reductase systems, and genes encoded in the putative GABA transporter

Microarray analysis was performed to assess the potential role of GABA as a signaling molecule. RNA was isolated from cultures of *B. abortus* 2308 grown aerobically in GMM in the presence of absence of 100 nM GABA and analyzed via microarray analysis.

Four loci were upregulated in the presence of GABA (Table 4.1). These included a secretion system (*bab2_0771*, *bab2_0773*, and *bab2_0774*) and genes associated with the putative GABA transporter (*bab1_1793* and *bab1_1792*). Interestingly, both nitrate (*bab2_0909*, *bab2_0908*, *bab2_0907*, *bab2_0906*, and *bab2_0905*) and nitric oxide (bab2_0955 and bab2_0954) reductase systems were also induced in the presence of GABA.

***bab1_1794* is necessary for chronic infection of a mouse model of brucellosis.**

An *in vitro* naïve macrophage and *in vivo* BALB/c mouse infection model were utilized to assess the role of *bab1_1794*, a putative periplasmic GABA-binding protein, in *B. abortus* virulence. A strain containing an unmarked in-frame gene deletion of *bab1_1794*

from the *B. abortus* genome was constructed and utilized for the following *in vitro* and *in vivo* studies. Naïve macrophages were isolated from the peritoneal cavity as previously described and infected with either *B. abortus* 2308 or *B. abortus* 2308::Δ*bab1_1794* (Δ*bab1_1794*) at an MOI of 100. Infected macrophages were lysed 2, 24, and 48 hours post-infection and serial diluted to calculated CFU brucellae/well. A deletion of *bab1_1794* did not affect the ability of *B. abortus* to survive and replicate within macrophages when compared to the parental strain *B. abortus* 2308 (Figure 4.6A).

BALB/c mice were infected intraperitoneally with 10^5 CFU of *B. abortus* 2308 or Δ*bab1_1794* and infection was monitored 4 and 8 weeks post-infection. After either 4 or 8 weeks, the mice were sacrificed, spleens removed and homogenized, and homogenate was serial diluted to assess CFU brucellae/spleen (Figure 4.6B). The ability of *B. abortus* Δ*bab1_1794* strain was significantly decreased in colonization of spleens 4 and 8 weeks post-infection when compared to the parental strain.

Deletion of both putative binding periplasmic binding proteins, *bab1_1794* and *bab1_1792*, encompassed in the putative GABA ABC transport system loci had no effect on ^{3}H-GABA transport *in vitro*.

The ^{3}H-GABA uptake assay was utilized to assess the role of *bab1_1792* and *bab1_1794* as the sole periplasmic GABA binding proteins in *B. abortus*. To test this hypothesis, *B. abortus* 2308, the isogenic *bab1_1794* deletion strain (Δ*bab1_1794*), and a double deletion strain of *bab1_1794* and *bab1_1792* (Δ*bab1_1794*Δ*bab1_1792*) were utilized for the aforementioned assay. As a control, non-radiolabled GABA was added in excess to cultures and all ^{3}H-GABA uptake measurements were normalized to *B. abortus* 2308. The assay revealed that ^{3}H-GABA uptake was not significantly different in the isogenic

deletion strain of *bab1_1794* nor the double deletion strain of *bab1_1794* and *bab1_1792* compared to the parental strain (Figure 4.7).

Discussion

In this study, the transport and biological function of GABA was analyzed in the intracellular pathogen *Brucella abortus*. The data presented revealed that ^{3}H-GABA is transported under nutrient limiting conditions, transport was regulated by the sRNAs AbcR1 and AbcR2, and import of GABA led to transcriptional changes in *B. abortus*. While deletion of the putative periplasmic binding proteins had no effect on the transport of ^{3}H-GABA *in vitro*, deletion of one of these putative GABA-binding proteins is necessary for full virulence of *B. abortus* 2308 *in vivo*.

Brucella spp. and *Agrobacterium* spp. are both members of the Order *Rhizobiales* and are considered phylogenetically related. However, the above ^{3}H-GABA transport studies revealed a significant difference between the transport systems. In *A. tumefaciens*, ^{3}H-GABA uptake was competitively inhibited by short lateral chain amino acids as well as proline (Planamente *et al.*, 2010). Dissimilar from *A. tumefaciens*, ^{3}H-GABA transport in *B. abortus* was uninhibited by the presence of other amino acids under the conditions tested. This is a significant divergence between the two related organisms with regards to GABA transport. The import of GABA is actually detrimental to *A. tumefaciens* pathogenesis (Chevrot *et al.*, 2006, Nonaka *et al.*, 2017). Thus, one theory for this divergence between *A. tumefaciens* and *B. abortus* is that a mutation occurred in the *A. tumefaciens* genome that gave rise to a strain that preferentially imports proline over GABA, allowing this mutant to outcompete the GABA-specific *A. tumefaciens* strain during infection. This could account for the prevalence of a non-specific GABA transporter in *A. tumefaciens* among natural strains.

Important insights into the biological role of GABA can be learned by understanding the processing of imported GABA, and the *Brucella* genome may provide clues to this processing. The GABA shunt can be utilized to form succinate, a substrate utilized during the TCA cycle (Feehily & Karatzas, 2013, Shelp *et al.*, 1999). This process occurs by converting GABA to succinic semialdehyde by the enzyme GABA-transaminase (GabT), followed by the reaction from succinic semialdehyde to succinate by succinic semialdehyde dehydrogenase (SSDH) (Feehily & Karatzas, 2013). Although the metabolic studies presented in this study do not reveal any metabolic utilization of GABA by *B. abortus*, the *Brucella* genome does contain genes encoding putative GabT (BAB2_0285) and SSDH (GabD, BAB1_1655). The function of these genes has not been characterized, but if functional, one or both could be important in the conversion of GABA to a utilizable carbon substrate. Utilization of mass spectrometry analyses of imported radiolabeled GABA could identify how GABA is modified by *B. abortus* and could lead to the formation of knew hypotheses regarding the processing of this molecule by the brucellae.

Interestingly, deletion of the putative periplasmic binding proteins *bab1_1794* and *bab1_1792* in the putative GABA transport system in *B. abortus* showed no difference in the ability of the bacterium to transport ^{3}H-GABA when compared to the parental strain (Figure 4.7). However, this observation does not prove that this system is not responsible for the transport of GABA by *B. abortus* due to several hypotheses. One explanation involves the presence of another yet-to-be identified ABC transporter that can also import GABA or other amino acids into *B. abortus*. *A. tumefaciens* encodes two ABC transport systems responsible for the import of exogenous GABA (Planamente *et al.*, 2010,

Planamente *et al.*, 2013). Orthologs of the Bra system are found throughout the bacterial kingdom including *Alphaproteobacteria*, *Klebsiella* spp., *Pseudomonas* spp., and *B. abortus* (Figure 4.1). The second ABC transporter in *A. tumefaciens*, Gts, is not as widespread, and orthologs are only found within *Burkholderia* spp. and a few *Alphaproteobacteria*, excluding *Brucella* spp. (Planamente *et al.*, 2013). This co-occurrence of GABA transport systems contributes to a second hypothesis that *B. abortus* may encode a second, unidentified GABA transport system. A second transporter could explain why a deletion of *bab1_1792* and *bab1_1794* did not significantly decrease ^{3}H-GABA import by *B. abortus*. As mentioned in Chapter 2, the existence of orphan periplasmic binding proteins in bacterial genomes can rescue the function of specific periplasmic binding proteins in ABC transport systems (Thomas, 2010), which presents another hypothesis as to why Δ*bab1_1794*Δ*bab1_1792* had no effect on 3H-GABA transport by *B. abortus*. Structure-function analysis, similar to studies performed in *A. tumefaciens*, may be required to demonstrate the binding affinity of BAB1_1794 and BAB1_1792 to GABA and possibly other amino acid ligands to show potential as periplasmic binding proteins (Planamente *et al.*, 2010).

Microarray analysis revealed the activation of several loci in *B. abortus* when exposed to GABA. These included the putative periplasmic GABA binding protein BAB1_1792, nitrate and nitric oxide reductase systems, and a putative secretion system. The induction of genes involved in denitrification is intriguing. In the intramacrophagic environment, the brucellae will encounter damaging reactive nitrogen species, low nutrient concentrations, and low oxygen concentrations (Roop *et al.*, 2009). Induction of genes involved in denitrification of nitric oxide allow *Brucella* spp. to survive in this

environment by detoxifying nitric oxide with the added benefit of utilizing nitrogen as an energy source in low oxygen conditions (Haine *et al.*, 2006). If the brucellae are encountering exogenous GABA prior to or upon entry into the macrophage, then induction of these genes could prime the bacterium for the intracellular environment. Further studies are necessary to understand the effect of GABA on denitrification and utilization of nitrogen for metabolism during anaerobic growth.

The import of GABA is inhibitory to the pathogenesis of *A. tumefaciens* by decreasing the induction of virulence related genes and transfer of T-DNA into the host cell. It could be hypothesized then that suppression of this transport could result in hypervirulence. It could also be hypothesized that the effect of GABA on *A. tumefaciens* could have a similar effect on closely related organisms, like *B. abortus*. Our data, however, suggested that instead of an inhibitory effect, GABA transport may actually enhance the virulence of *B. abortus*, as indicated by the microarray and infection data. Intriguingly, there is a paradox for the function of GABA between plant- and animal-associated microbial pathogens. It is well documented that GABA plays a role in plant immunity and plants under duress secrete GABA into the environment to suppress the ability of pathogens to cause infection (Yang *et al.*, 2017). However, in the mammalian host, our data and data in *P. aeruginosa* supports the hypothesis that GABA does not inhibit bacterial pathogenesis but instead increases the virulence of animal associated bacterial pathogens. This paradox is perhaps indicative of another evolutionary divergence between plant- and animal- associated bacterial pathogens.

It has recently been shown that GABA signaling induces maturation of phagosomal compartments in macrophages infected with intracellular bacterial

pathogens, which increases the ability of the macrophage to kill intracellular parasites (Kim *et al.*, 2018). Under the conditions test, GABA signaling did not induce robust transcriptional changes in *B. abortus* nor did *B. abortus* metabolize GABA. Accordingly, instead of GABA inducing virulence in *Brucella*, it is possible that the import of GABA by *B. abortus* could instead act as a mechanism of masking induction of GABA-macrophage signaling. By hiding exogenous GABA from the macrophage, *Brucella* could decrease phagosomal maturation during infection. Evasion of phagolysosomal maturation is crucial to the survival and replication of the brucellae during infection and experiments are necessary to understand the effect of GABA on phagosomal maturation during *Brucella* infection.

Overall, the presented study characterizes the transport of the ubiquitous non-proteinogenic amino acid GABA by the intracellular bacterial pathogen *B. abortus*. While transport assays provided novel insights into the transport of this molecule by *B. abortus*, the data revealed no metabolic potential for GABA under the conditions tested and lackluster transcriptional changes in the presence of GABA. This warrants further studies to understand the biological role of GABA during *Brucella* pathogenesis.

Materials and Methods

Bacterial strains and growth conditions.

Brucella abortus 2308 and derivative strains were routinely grown on Schaedler blood agar (SBA), which is composed of Schaedler agar (BD, Franklin Lakes, NJ) containing 5% defibrinated bovine blood (Quad Five, Ryegate, MT). Cultures were routinely grown in brucella broth (BD), tryptic soy broth (TSB), or in Gerhardt's Minimal Medium (GMM) (Gerhardt, 1958). For cloning, *Escherichia coli* strain DH5α was grown on tryptic soy agar (BD) or in Luria-Bertani (LB) broth. When appropriate, growth media were supplemented with kanamycin (45 μg/ml).

Construction of *Brucella abortus* deletion strains.

bab1_1794 and *bab1_1792* in *Brucella abortus* 2308 were deleted using a nonpolar, unmarked gene excision strategy as described previously (Budnick *et al.*, 2018). Briefly, an approximately 1-kb fragment of the upstream region of each gene extending to the second codon of the coding region was amplified by PCR using primers *bab1_1794*-Up-For and *bab1_1794*-Up-Rev and genomic DNA from *Brucella abortus* 2308 as a template. Similarly, a fragment containing the last two codons of the coding region and extending to approximately 1 kb downstream of the *bab1_1794* open reading frame (ORF) was amplified with primers *bab1_1794*-Down-For and *bab1_1794*-Down-Rev. The sequences of all oligonucleotide primers used in this study can be found in Table 4.2, and the plasmids used in the study are listed in Table 4.3. The upstream fragment was digested with BamHI, the downstream fragment was digested with EcoRI, and both fragments were treated with polynucleotide kinase in the presence of ATP. Both of the DNA fragments were included in a single ligation mix with BamHI/EcoRI-digested

pNTPS138 (M. R. K. Alley, unpublished data) and T4 DNA ligase (Monserate Biotechnology Group, San Diego, CA). The resulting plasmid (pΔ*bab1_1794*) was introduced into *B. abortus* 2308, and merodiploid transformants were obtained by selection on SBA plus kanamycin. A single kanamycin-resistant clone was grown for >6 h in brucella broth and then plated onto SBA containing 10% sucrose. Genomic DNA was isolated from sucrose resistant, kanamycin-sensitive colonies and screened by PCR for loss of the *bab1_1794* gene. The method described above was used to construct a double deletion of *bab1_1794* and *bab1_1792* by ligating the 1 kb fragment upstream of *bab1_1794* with a 1 kb downstream fragment of *bab1_1792* into the BamHI/PstI-digested pNTPS138 plasmid and screened accordingly.

^{3}H-GABA uptake assays.

A radiolabeled transport assay was utilized to assess the ability of *B. abortus s*trains to import tritium labelled GABA (^{3}H-GABA) grown under several growth conditions. Gerhardt's Minimal Media (GMM) was inoculated with *Brucella* strains at a concentration of 10^{9} CFU brucellae/ml and incubated for 20 minutes at 37°C with shaking. The cultures were then inoculated with ^{3}H-GABA at a final concentration of 100 nM and incubated for another 20 minutes at 37°C with shaking. The bacteria were collected via filtration through a filter, washed three times with GMM, and the radioactivity of the filter was measured to quantify the amount of radiation imported by the brucellae collected on the filter. If ^{3}H-GABA is imported by *B. abortus*, then the filter will measure high radioactivity above background; however, if ^{3}H-GABA is not imported by *B. abortus*, then the ^{3}H-GABA will pass through the filter and the filter will not measure high radioactivity above background.

Respirometry assay.

Culture tubes of 5 mL of TSB were inoculated with *B. abortus* 2308 at a final concentration of 10^7 CFU/mL and either 10 mM erythritol, glutamic acid, or GABA. The cultures were grown overnight at 37°C with shaking. The following day, the brucellae were pelleted, supernatant removed, and pellet resuspended in PBS at a final concentration of 10^2 CFU/mL. Samples were then loaded into an oxygraph and oxygen concentrations were subsequently measured. After 300 seconds, erythritol, glutamic acid, or GABA were added to the corresponding culture tube at a final concentration of 100 mM and culture oxygen concentrations were measured for 2000 seconds.

Microarray analysis.

RNA was isolated from *B. abortus* 2308 cultures grown in GMM and GMM+100 nm GABA following growth to the late exponential phase in GMM (Caswell *et al.*, 2012), and contaminating genomic DNA was removed by treatment with RNase-free DNase I (Caswell *et al.*, 2012). Ten micrograms of each RNA sample from GMM and GMM+100 nM GABA was reverse transcribed, fragmented, and subjected to 3′ biotinylation as previously described (Beenken *et al.*, 2004). The labeled cDNA (1.5 μg) was hybridized to custom-made *B. abortus* GeneChips (PMD2308a520698F) according to the manufacturer's recommendations for antisense prokaryotic arrays (Affymetrix, Santa Clara, CA). Signal intensities were normalized to the median signal intensity value for each GeneChip, subjected to averaging, and analyzed with GeneSpring X software. RNA species exhibiting a ≥2-fold change in expression between GMM and GMM+100 nM GABA, as determined by Affymetrix algorithms to be statistically differentially expressed (*t* test; $P < 0.05$), were identified. The microarrays used in this study were

developed based on *B. melitensis* biovar *abortus* 2308 and on all of the *B. abortus* GenBank entries that were available at the time of design.

Virulence of *Brucella* strains in cultured murine macrophages and experimentally infected mice.

Experiments to test the virulence of *Brucella* strains in primary murine peritoneal macrophages were carried out as described previously (Gee *et al.*, 2005). Briefly, resident peritoneal macrophages were isolated from BALB/c mice and seeded in 96-well plates in Dulbecco's modified Eagle's medium with 5% fetal bovine serum, and the following day, the macrophages were infected with opsonized brucellae at an MOI of 100:1. After 2 h of infection, extracellular bacteria were killed by treatment with gentamicin (50 g/ml). For the 2-h time point, the macrophages were then lysed with 0.1% deoxycholate–PBS, and serial dilutions were plated on Schaedler blood agar (SBA). For the 24- and 48-h time points, the cells were washed with PBS following gentamicin treatment, and fresh cell culture medium containing gentamicin (20 g/ml) was added to the monolayer. At the indicated time point, the macrophages were lysed, and serial dilutions were plated on SBA. Triplicate wells were used for each *Brucella* strain tested. Infection and colonization of mice by *Brucella* strains were performed as described previously by Gee et al. (Gee *et al.*, 2005). BALB/c mice (5 per *Brucella* strain) were infected intraperitoneally with 10^5 CFU of each *Brucella* strain in sterile PBS. The mice were sacrificed at 4 and 8 weeks post-infection, and serial dilutions of spleen homogenates were plated on SBA to determine CFU counts of brucellae/spleen.

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References

Beenken, K.E., P.M. Dunman, F. McAleese, D. Macapagal, E. Murphy, S.J. Projan, J.S. Blevins & M.S. Smeltzer, (2004) Global gene expression in *Staphylococcus aureus* biofilms. *J Bacteriol* **186**: 4665-4684.

Bhat, R., R. Axtell, A. Mitra, M. Miranda, C. Lock, R.W. Tsien & L. Steinman, (2010) Inhibitory role for GABA in autoimmune inflammation. *Proc Natl Acad Sci U S A* **107**: 2580-2585.

Bown, A.W. & B.J. Shelp, (2016) Plant GABA: Not Just a Metabolite. *Trends Plant Sci* **21**: 811-813.

Budnick, J.A., L.M. Sheehan, J.M. Colquhoun, P.M. Dunman, G.C. Walker, R.M. Roop, 2nd & C.C. Caswell, (2018) The endoribonuclease YbeY is linked to proper cellular morphology and virulence in *Brucella abortus*. *J Bacteriol*.

Caswell, C.C., J.M. Gaines, P. Ciborowski, D. Smith, C.H. Borchers, C.M. Roux, K. Sayood, P.M. Dunman & R.M. Roop Ii, (2012) Identification of two small regulatory RNAs linked to virulence in *Brucella abortus* 2308. *Mol Microbiol* **85**: 345-360.

Chevrot, R., R. Rosen, E. Haudecoeur, A. Cirou, B.J. Shelp, E. Ron & D. Faure, (2006) GABA controls the level of quorum-sensing signal in *Agrobacterium tumefaciens*. *Proc Natl Acad Sci U S A* **103**: 7460-7464.

Damiano, M.A., D. Bastianelli, S. Al Dahouk, S. Kohler, A. Cloeckaert, D. De Biase & A. Occhialini, (2015) Glutamate decarboxylase-dependent acid resistance in *Brucella* spp.: distribution and contribution to fitness under extremely acidic conditions. *Appl Environ Microbiol* **81**: 578-586.

De Biase, D. & E. Pennacchietti, (2012) Glutamate decarboxylase-dependent acid resistance in orally acquired bacteria: function, distribution and biomedical implications of the *gadBC* operon. *Mol Microbiol* **86**: 770-786.

de Figueiredo, P., T.A. Ficht, A. Rice-Ficht, C.A. Rossetti & L.G. Adams, (2015) Pathogenesis and immunobiology of brucellosis: review of *Brucella*-host interactions. *Am J Pathol* **185**: 1505-1517.

Dean, A.S., L. Crump, H. Greter, J. Hattendorf, E. Schelling & J. Zinsstag, (2012) Clinical manifestations of human brucellosis: a systematic review and meta-analysis. *PLoS Negl Trop Dis* **6**: e1929.

Feehily, C. & K.A. Karatzas, (2013) Role of glutamate metabolism in bacterial responses towards acid and other stresses. *J Appl Microbiol* **114**: 11-24.

Gee, J.M., M.W. Valderas, M.E. Kovach, V.K. Grippe, G.T. Robertson, W.L. Ng, J.M. Richardson, M.E. Winkler & R.M. Roop, 2nd, (2005) 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. *Infect Immun* **73**: 2873-2880.

Gerhardt, P., (1958) The nutrition of brucellae. *Bacteriol Rev* **22**: 81-98.

Haine, V., M. Dozot, J. Dornand, J.J. Letesson & X. De Bolle, (2006) NnrA is required for full virulence and regulates several *Brucella melitensis* denitrification genes. *J Bacteriol* **188**: 1615-1619.

He, Y., (2012) Analyses of *Brucella* pathogenesis, host immunity, and vaccine targets using systems biology and bioinformatics. *Front Cell Infect Microbiol* **2**: 2.

Hull, N.C. & B.A. Schumaker, (2018) Comparisons of brucellosis between human and veterinary medicine. *Infect Ecol Epidemiol* **8**: 1500846.

Keppie, J., A.E. Williams, K. Witt & H. Smith, (1965) The Role of Erythritol in the Tissue Localization of the Brucellae. *Br J Exp Pathol* **46**: 104-108.

Kim, J.K., Y.S. Kim, H.M. Lee, H.S. Jin, C. Neupane, S. Kim, S.H. Lee, J.J. Min, M. Sasai, J.H. Jeong, S.K. Choe, J.M. Kim, M. Yamamoto, H.E. Choy, J.B. Park & E.K. Jo, (2018) GABAergic signaling linked to autophagy enhances host protection against intracellular bacterial infections. *Nat Commun* **9**: 4184.

MacGregor, K.B., B.J. Shelp, S. Peiris & A.W. Bown, (2003) Overexpression of glutamate decarboxylase in transgenic tobacco plants deters feeding by phytophagous insect larvae. *J Chem Ecol* **29**: 2177-2182.

Michaeli, S. & H. Fromm, (2015) Closing the loop on the GABA shunt in plants: are GABA metabolism and signaling entwined? *Front Plant Sci* **6**: 419.

Mittal, R., L.H. Debs, A.P. Patel, D. Nguyen, K. Patel, G. O'Connor, M. Grati, J. Mittal, D. Yan, A.A. Eshraghi, S.K. Deo, S. Daunert & X.Z. Liu, (2017) Neurotransmitters: The Critical Modulators Regulating Gut-Brain Axis. *J Cell Physiol* **232**: 2359-2372.

Nonaka, S., T. Someya, S. Zhou, M. Takayama, K. Nakamura & H. Ezura, (2017) An *Agrobacterium tumefaciens* Strain with Gamma-Aminobutyric Acid Transaminase Activity Shows an Enhanced Genetic Transformation Ability in Plants. *Sci Rep* **7**: 42649.

Palanivelu, R., L. Brass, A.F. Edlund & D. Preuss, (2003) Pollen tube growth and guidance is regulated by POP2, an *Arabidopsis* gene that controls GABA levels. *Cell* **114**: 47-59.

Pappas, G., P. Papadimitriou, N. Akritidis, L. Christou & E.V. Tsianos, (2006) The new global map of human brucellosis. *Lancet Infect Dis* **6**: 91-99.

Park, D.H., R. Mirabella, P.A. Bronstein, G.M. Preston, M.A. Haring, C.K. Lim, A. Collmer & R.C. Schuurink, (2010) Mutations in gamma-aminobutyric acid (GABA) transaminase genes in plants or *Pseudomonas syringae* reduce bacterial virulence. *Plant J* **64**: 318-330.

Pennacchietti, E., C. D'Alonzo, L. Freddi, A. Occhialini & D. De Biase, (2018) The Glutaminase-Dependent Acid Resistance System: Qualitative and Quantitative Assays and Analysis of Its Distribution in Enteric Bacteria. *Frontiers in microbiology* **9**: 2869.

Petersen, E., G. Rajashekara, N. Sanakkayala, L. Eskra, J. Harms & G. Splitter, (2013) Erythritol triggers expression of virulence traits in *Brucella melitensis*. *Microbes Infect* **15**: 440-449.

Planamente, S., S. Morera & D. Faure, (2013) In planta fitness-cost of the Atu4232-regulon encoding for a selective GABA-binding sensor in *Agrobacterium*. *Commun Integr Biol* **6**: e23692.

Planamente, S., A. Vigouroux, S. Mondy, M. Nicaise, D. Faure & S. Morera, (2010) A conserved mechanism of GABA binding and antagonism is revealed by structure-function analysis of the periplasmic binding protein Atu2422 in *Agrobacterium tumefaciens*. *J Biol Chem* **285**: 30294-30303.

Ramputh, A.I. & A.W. Bown, (1996) Rapid [gamma]-Aminobutyric Acid Synthesis and the Inhibition of the Growth and Development of Oblique-Banded Leaf-Roller Larvae. *Plant Physiol* **111**: 1349-1352.

Roop, R.M., 2nd, J.M. Gaines, E.S. Anderson, C.C. Caswell & D.W. Martin, (2009) Survival of the fittest: how *Brucella* strains adapt to their intracellular niche in the host. *Med Microbiol Immunol* **198**: 221-238.

Shelp, B.J., A.W. Bown & D. Faure, (2006) Extracellular gamma-aminobutyrate mediates communication between plants and other organisms. *Plant Physiol* **142**: 1350-1352.

Shelp, B.J., A.W. Bown & M.D. McLean, (1999) Metabolism and functions of gamma-aminobutyric acid. *Trends Plant Sci* **4**: 446-452.

Thomas, G.H., (2010) Homes for the orphans: utilization of multiple substrate-binding proteins by ABC transporters. *Molecular microbiology* **75**: 6-9.

von Bargen, K., J.P. Gorvel & S.P. Salcedo, (2012) Internal affairs: investigating the *Brucella* intracellular lifestyle. *FEMS Microbiol Rev* **36**: 533-562.

Wang, C., H.B. Zhang, L.H. Wang & L.H. Zhang, (2006) Succinic semialdehyde couples stress response to quorum-sensing signal decay in *Agrobacterium tumefaciens*. *Mol Microbiol* **62**: 45-56.

Waters, L.S. & G. Storz, (2009) Regulatory RNAs in bacteria. *Cell* **136**: 615-628.

Wilms, I., B. Voss, W.R. Hess, L.I. Leichert & F. Narberhaus, (2011) Small RNA-mediated control of the *Agrobacterium tumefaciens* GABA binding protein. *Mol Microbiol* **80**: 492-506.

Yang, J., C. Sun, Y. Zhang, D. Fu, X. Zheng & T. Yu, (2017) Induced resistance in tomato fruit by gamma-aminobutyric acid for the control of alternaria rot caused by *Alternaria alternata*. *Food Chem* **221**: 1014-1020.

Tables

Table 4.1: Differential gene expression of *B. abortus* 2308 in GMM + 100 nM GABA vs. GMM.

BAB Designation	BAB_RS Designation	Description	Fold Change (GMM+GABA vs. GMM)
BAB2_0909	BAB_RS30605	hypothetical protein	2.65
BAB2_0773	BAB_RS30020	hemolysin secretion protein D	2.31
BAB2_0774	BAB_RS30025	tetR family transcriptional regulator	2.18
BAB2_0908	BAB_RS30600	peptidyl-prolyl cis-trans isomerase	2.17
BAB2_0906	BAB_RS30590	nitrate reductase subunit delta	2.14
BAB2_0907	BAB_RS30595	nitrate reductase	2.12
BAB2_0955	BAB_RS30825	cytochrome C	2.09
BAB2_0771	BAB_RS30005	mannose-1-phosphate guaniltransferase	1.97
BAB1_1793	N/A	hypothetical protein	1.95
BAB2_0954	BAB_RS30820	nitric oxide reductase	1.91
BAB1_1792	BAB_RS24455	Leu/Ile/Val-binding protein homolog 2	1.82
BAB2_0905	BAB_RS30585	narH, nitrate reductase A subunit beta	1.79

Table 4.2: Oligonucleotide primers used in this study.

Primer name	**Sequence (5'->3')**
bab1_1794-Up-For	TAGGATCCTGTTCCCGCGTCTGAAGGAGC
bab1_1794-Up-Rev	GAAGGCGATGACTGCAGCAAGAG
bab1_1794-Down-For	TACAAGTGGGAAAAGGGTGC
bab1_1794-Down-Rev	TAGAATTCTTGCAACCGAGATGCCCTGC
bab1_1792-Down-For	TACTTCCAGAAGTAAATTGCC
bab1_1792-Down-Rev	GACTGCAGACGCTCAAAAAGATGGACCG

*Underlined sequences depict a restriction endonuclease recognition site.

Table 4.3: Plasmids used in this study.

Plasmid name	Description	Reference
pNPTS138	Cloning vector; contains *sacB*; Kan^R	(M.R.K. Alley, unpublished)
p∆*bab1_1794*	In-frame deletion of *bab1_1794* plus 1 kb of each flanking region in pNPTS138	This study
p∆*bab1_1794*∆*bab1_1792*	In-frame deletion of *bab1_1794 and bab1_1792 locus* plus 1 kb of each flanking region in pNPTS138	This study

Figures/Figure Legends

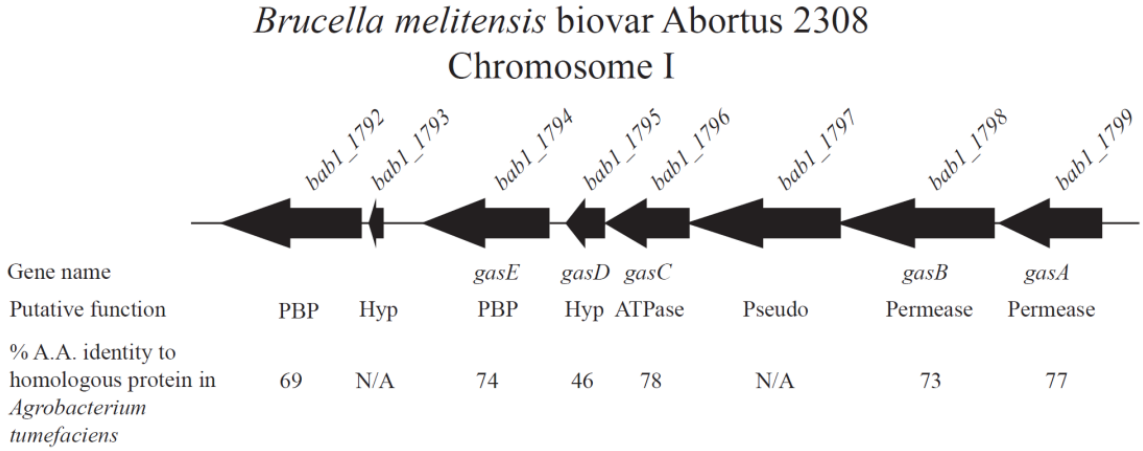

Figure 4.1: Organization of a putative GABA ABC-type transport system in *B. abortus* 2308 and homology to one of the GABA transport systems in *A. tumefaciens* str. C58.

Genetic organization of *bab1 1792-bab1 1799* (*bab rs24455-bab rs24485*) located on chromosome I of *Brucella melitensis* biovar Abortus 2308. Putative functions for each gene is located below the gene and together this locus encodes a putative ABC transport system. Proteins encoded from this locus exhibit high amino acid identity to the *bra* locus in *A. tumefaciens* str. C58.

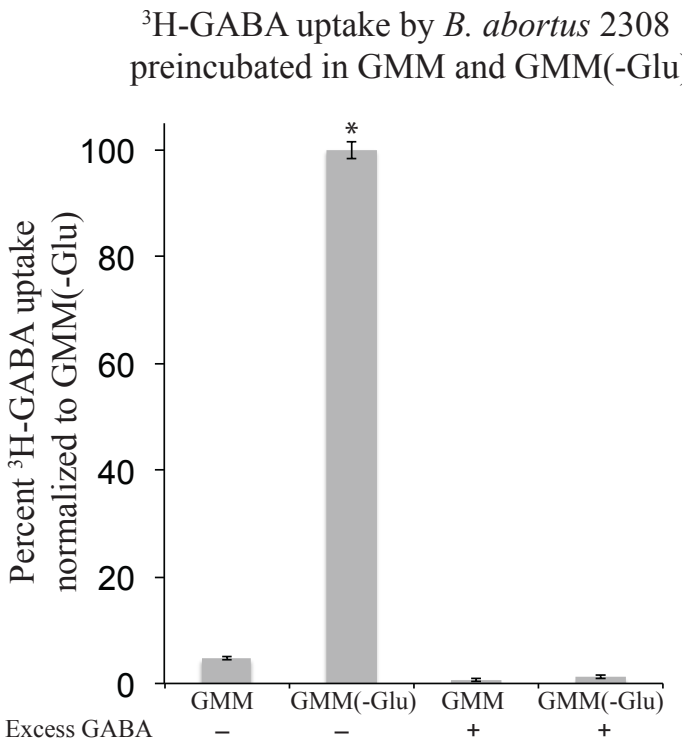

Figure 4.2: ^{3}H-GABA import is induced under nutrient limiting conditions.
^{3}H-GABA uptake by *B. abortus* 2308 was assessed in minimal medium with (GMM) and without (-Glu) the addition of glutamate to the medium. Controls include the addition of excess nonradiolabled GABA to competitively inhibit ^{3}H-GABA uptake. The asterisk denotes a statistically significant difference ($P<0.05$; Student's t test) in uptake between *B. abortus* 2308 incubated in GMM(-Glu) compared to *B. abortus* 2308 incubated in GMM.

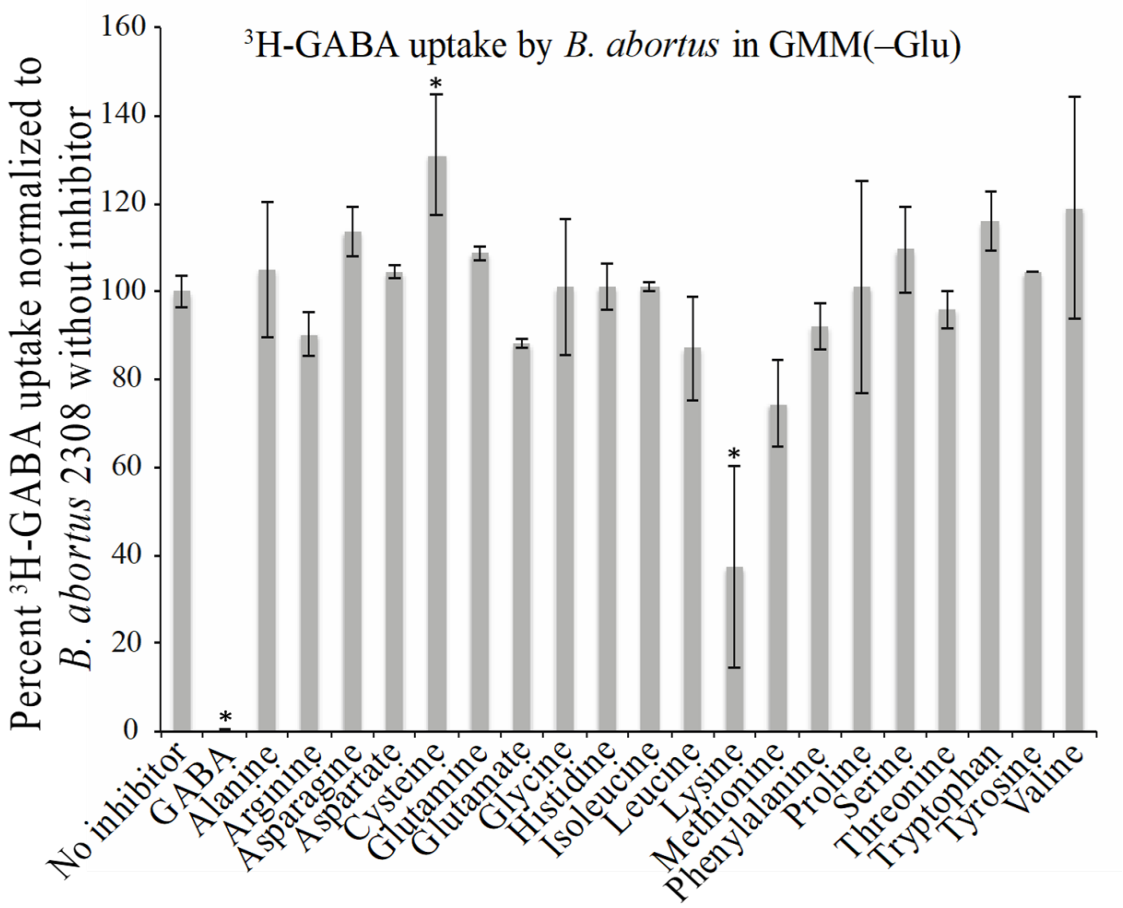

Figure 4.3: ^{3}H-GABA import by *B. abortus* 2308 is uninhibited by the presence of other amino acids *in vitro*.
^{3}H-GABA uptake by *B. abortus* 2308 was assessed uninhibited and in the presence of 1,000-fold excess 20 proteinogenic amino acids. A control for uptake inhibition includes the addition of excess nonradiolabled GABA to competitively inhibit ^{3}H-GABA uptake. The asterisk denotes a statistically significant difference ($P<0.05$; Student's t test) in uptake of ^{3}H-GABA between *B. abortus* 2308 uninhibited and in the presence of excess nonradiolabled GABA, cysteine, and lysine.

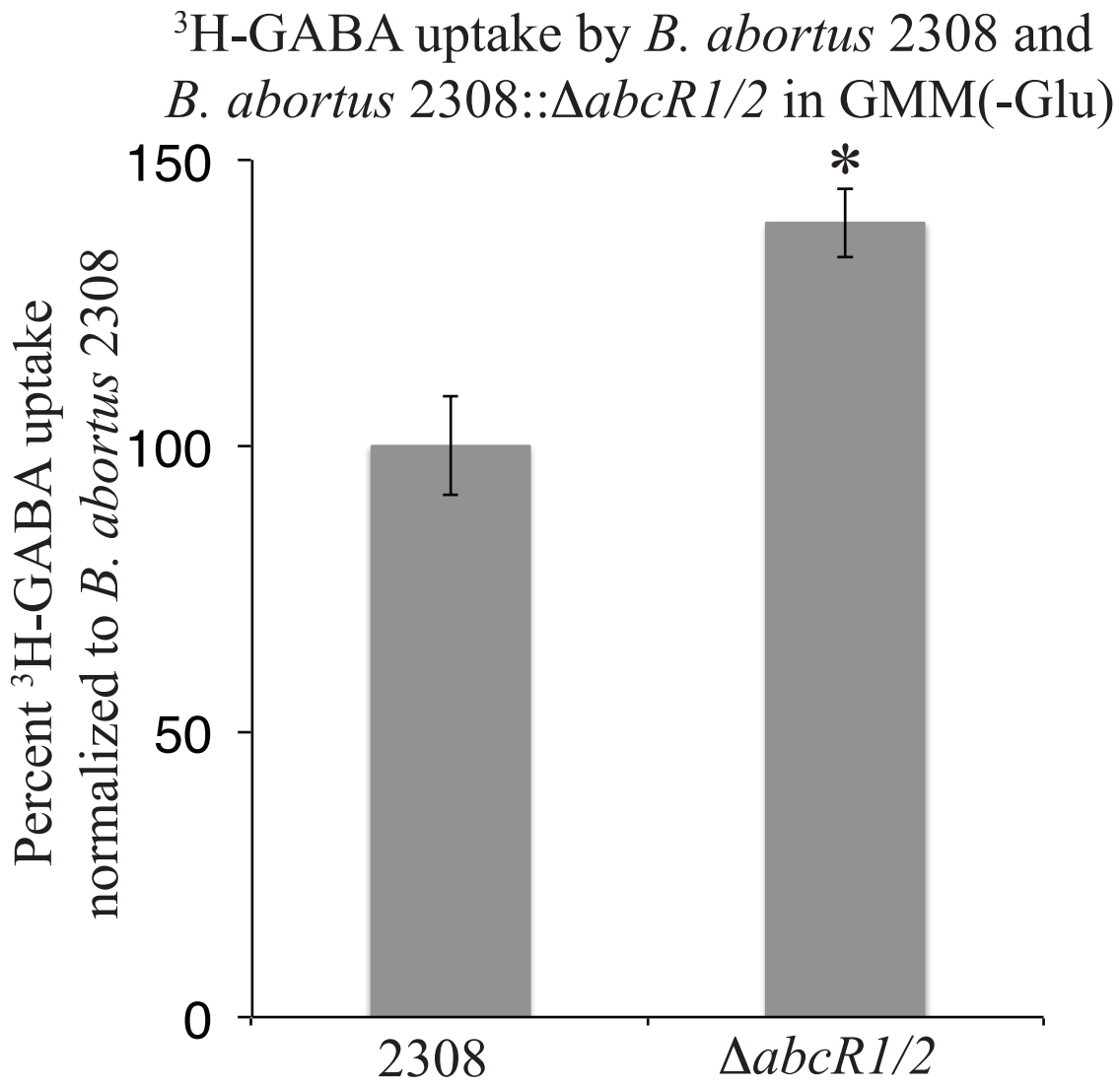

Figure 4.4: ^{3}H-GABA import is negatively regulated by the sRNAs AbcR1 and AbcR2 in *B. abortus*.

^{3}H-GABA uptake by *B. abortus* 2308 and *B. abortus* 2308::Δ*abcR1/2* was assessed in minimal medium, GMM(-Glu). The asterisk denotes a statistically significant difference ($P<0.05$; Student's t test) in uptake between *B. abortus* 2308::Δ*abcR1/2* and the parental strain, *B. abortus* 2308.

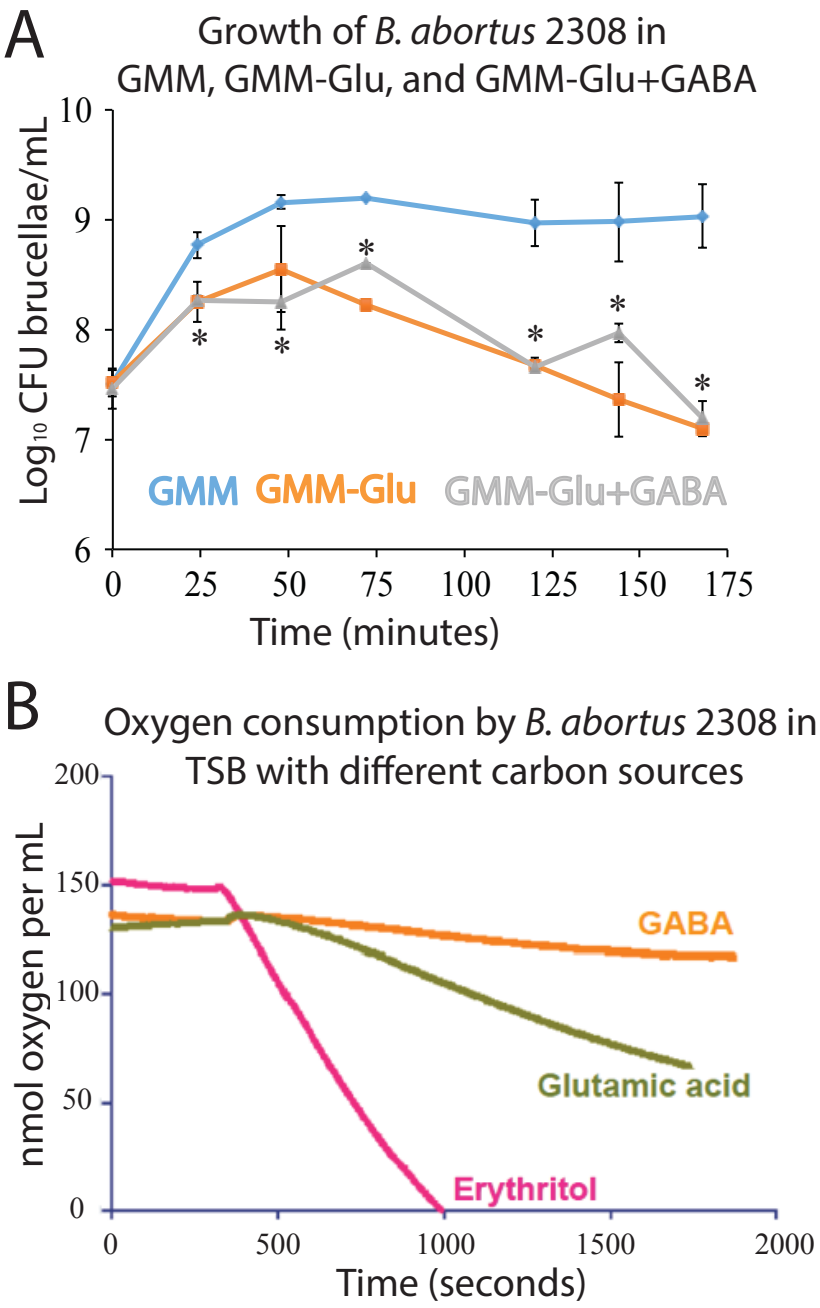

Figure 4.5: GABA is not utilized as a metabolite by B. abortus 2308 *in vitro*.
A. Growth of *B. abortus* 2308 in minimal medium (GMM), minimal medium lacking glutamate (GMM-Glu), and minimal medium lacking glutamate with the addition of GABA (0.15%) (GMM-Glu+GABA). The asterisk denotes a statistically significant difference ($P<0.05$; Student's t test) in uptake between *B. abortus* 2308 grown in GMM compared to *B. abortus* 2308 grown in either GMM-Glu or GMM-Glu+GABA.
B. Oxygen consumption by *B. abortus* 2308 grown in TSB with the addition of either GABA, glutamate, or erythritol 300 seconds after inoculation measured via oxygraph machine.

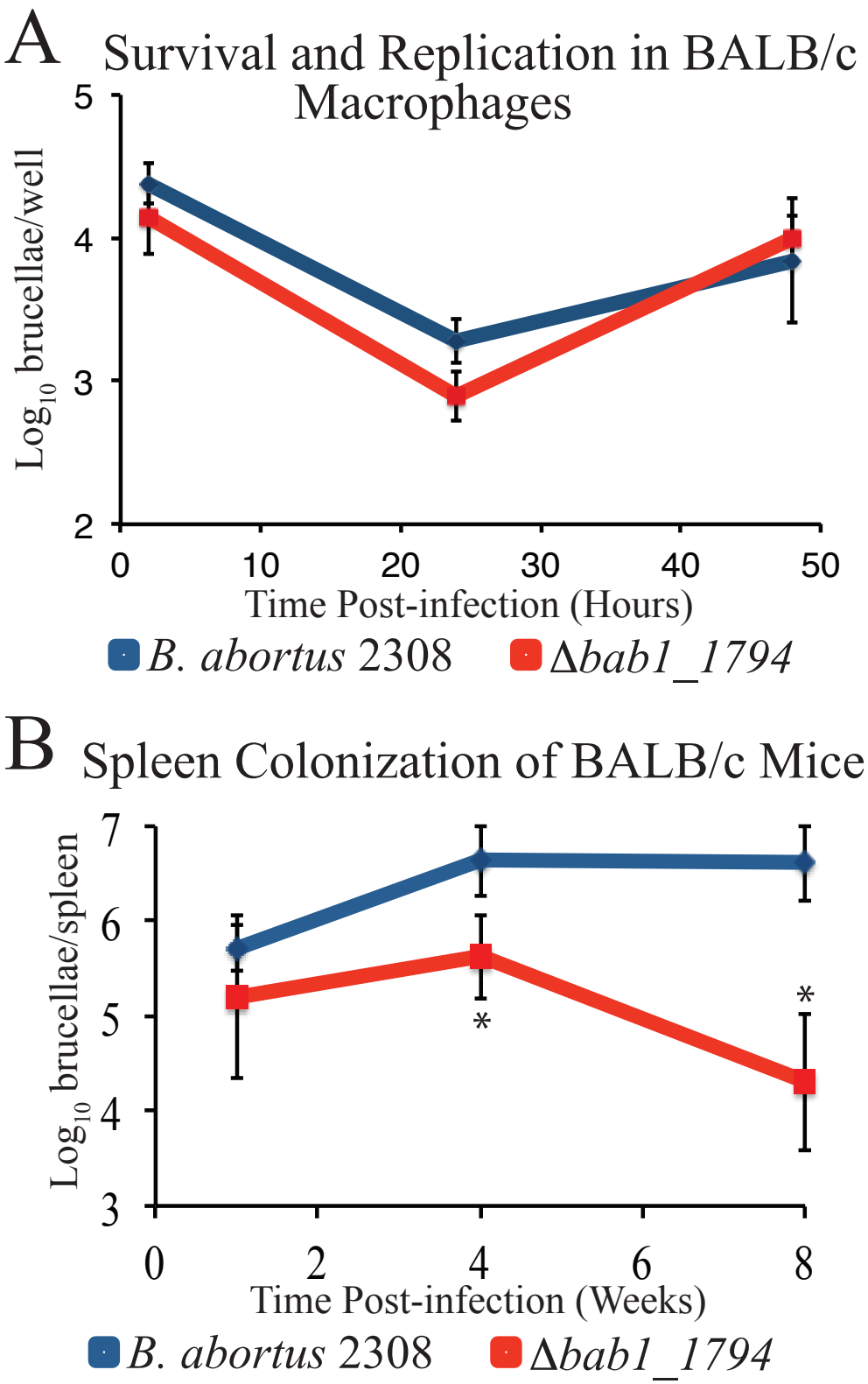
A
Survival and Replication in BALB/c Macrophages
Log10 brucellae/well
5
4
3
2
0
10
20
30
40
50
Time Post-infection (Hours)
B. abortus 2308
Δbab1_1794
B
Spleen Colonization of BALB/c Mice
Log10 brucellae/spleen
7
6
5
4
3
0
2
4
6
8
*
*
Time Post-infection (Weeks)
B. abortus 2308
Δbab1_1794

Figure 4.6: Virulence of *B. abortus* 2308 and Δ*bab1_1794* in peritoneally derived macrophages and BALB/c mice.

A. Macrophage survival and replication experiments. Cultured peritoneal macrophages from BALB/c mice were infected with *B. abortus* 2308, the isogenic *bab1_1794* (Δ*bab1 1794*). At the indicated times post-infection, macrophages were lysed, and the number of intracellular brucellae present in these phagocytes was determined by serial dilution and plating on agar medium.

B. Mouse infection experiments. BALB/c mice (5 per strain) were infected intraperitoneally with *B. abortus* 2308 and with the isogenic *bab1_1794* deletion strain (Δ*bab1 1794*). Mice were sacrificed 1, 4, and 8 weeks post-infection, and Log10 brucellae/spleen were calculated. The data are presented as average numbers of brucellae ± standard deviations of results from the 5 mice colonized with a specific *Brucella* strain at each time point. The asterisk denotes a statistically significant difference ($P < 0.05$; Student's *t* test) between Δ*bab1_1794* and the parental strain 2308 at 4 and 8 weeks post-infection.

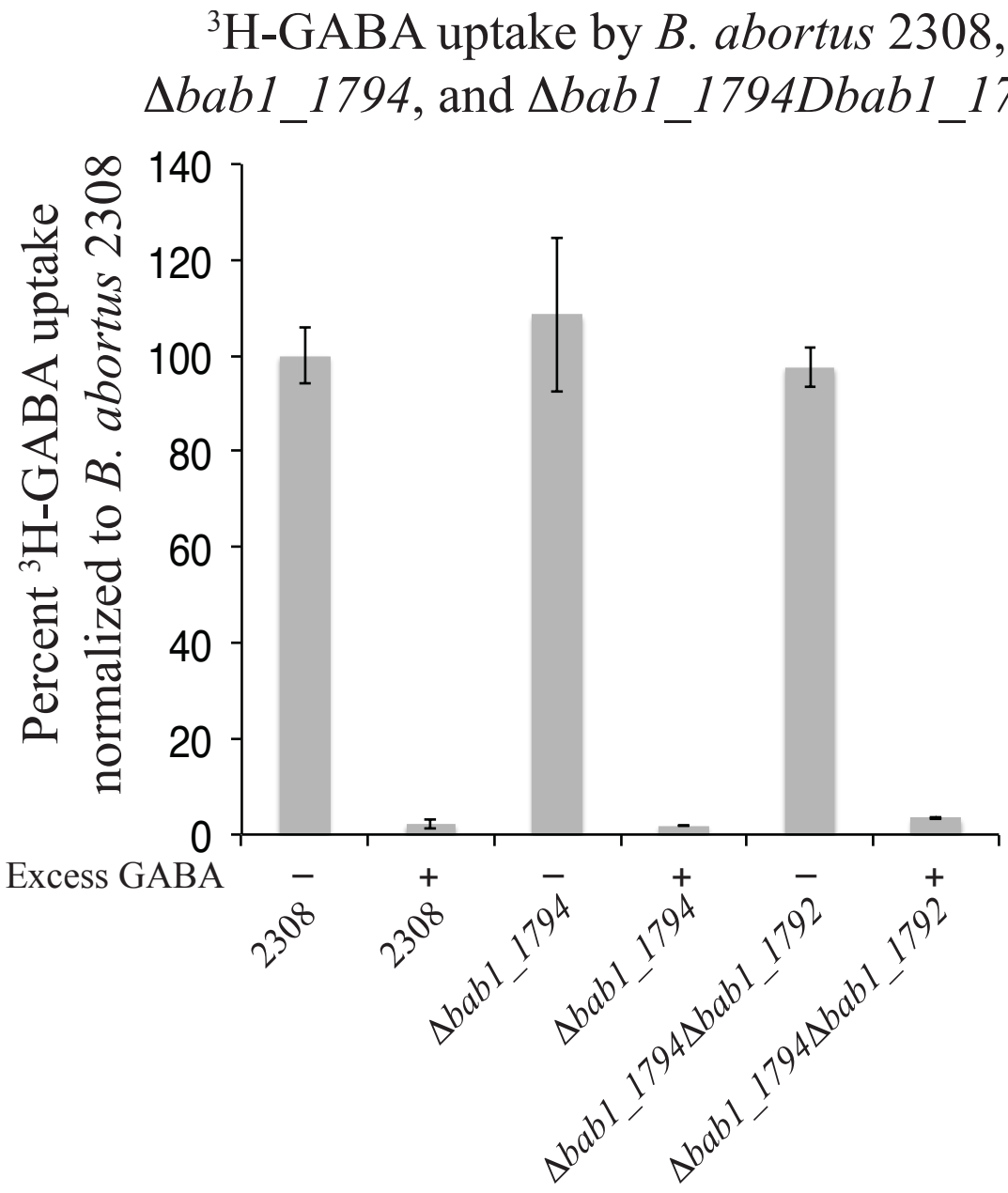

Figure 4.7: Deletion of *bab1_1794* and *bab1_1792* has no effect on ^{3}H-GABA transport in *B. abortus* 2308.

^{3}H-GABA uptake by *B. abortus* 2308, *B. abortus* 2308::Δ*bab1_1794,* and *B. abortus* 2308::Δ*bab1_1794*Δ*bab1_1792* was assessed in minimal medium, GMM(-Glu). A control for uptake includes the addition of excess nonradiolabled GABA to competitively inhibit ^{3}H-GABA uptake.

Chapter 5: Conclusions and Further Discussion

Conclusions and Further Discussion

The works presented in this dissertation have focused on characterizing the functional role of genes both directly and indirectly regulated by the transcriptional regulator VtlR in the bacterial pathogens *Brucella abortus* and *Agrobacterium tumefaciens*. This has led to the discovery of a system involved in the putative transport and metabolism of the sugar fucose, as well as the utilization of GABA as a host-derived signaling molecule during *Brucella* pathogenesis. Within these Chapters several interesting findings have been observed either directly or indirectly from the objectives examined. While there are similarities in these systems, several results highlight the robust differences between these organisms that occupy very different environmental niches.

VtlR as a gauge for the expression of transport systems

Chapter 2 focused on characterizing the functional role and virulence-association of the three small proteins BAB1_0914, BAB2_0512, and BAB2_0574 in *B. abortus*. An interesting discovery from this study included the observation that the redundantly functioning sRNAs AbcR1 and AbcR2 are differentially expressed under different growth conditions. AbcR1 was constitutively expressed under all growth conditions tested, including growth in nutrient rich broth, low pH, nutrient limitations, and exposure to oxidative stress or bile acid; however, AbcR2 was primarily expressed during the stationary phase of growth, under low pH conditions, and when exposed to oxidative stress (Budnick *et al.*, 2018). One conclusion from these results is that activation of the VtlR regulon is primarily in response to biologically relevant stresses. The results also displayed that AbcR2 expression was minimal during exponential phase of growth and under nutrient limiting conditions. Thus, another hypothesis for the function of VtlR, is

that during rapid growth in nutrient rich medium or growth during periods of starvation, VtlR does not activate the expression of AbcR2, allowing for the subsequent expression of ABC transport systems to acquire nutrients from the surrounding environment.

Microarray analysis of *B. abortus* Δ*vtlR* showed that AbcR1- and AbcR2-dependent ABC transport systems are slightly over-expressed in Δ*vtlR* (Sheehan *et al.*, 2015). Combined with the data discussed above, under rapid growth and nutrient limiting conditions, VtlR may be acting as a fine tuner of expression of these transport systems during the life cycle of *Brucella*. It would be interesting to measure the expression of the AbcRs *in vitro* and *in vivo* to understand how their expression changes over time during pathogenesis. I would hypothesize that during periods of slow growth and adaptation to the host environment, the AbcRs would be highly expressed, and repression of transport systems would occur so as not to expend excessive energy. However, during periods of replication and starvation, specifically after the formation of the rBCV near the endoplasmic reticulum, AbcR expression would be reduced to allow for the expression of these transport systems for the acquisition of metabolites and molecules for replication (Deghelt *et al.*, 2014).

When does *Brucella* encounter GABA during pathogenesis?

In Chapter 4, we outline GABA transport and utilization in *B. abortus* and compare and contrast these data to the homologous systems in *A. tumefaciens*. This study left many questions unanswered as to the mechanism by which this transport occurs and why GABA is utilized by *B. abortus*, both of which are currently being studied in our lab. Another overarching question is where does this molecule play a role in *Brucella* pathogenesis? GABA is found throughout nature, so it is possible that *Brucella* would

encounter it throughout the host environment, but several recent studies have revealed some interesting clues to help answer this question.

As mentioned in the introduction and Chapter 4, several studies have focused on characterizing GABA production and catabolism by primary immune cells (Bhat *et al.*, 2010, Kim *et al.*, 2018). Since the brucellae form replicative niches within these cells, this would be an obvious location for GABA transport and signaling. If this is the case, however, it is not known whether or not this signaling would occur outside of the immune cell, inside of the immune cell during *Brucella* trafficking, or both.

Another prominent location for GABA in the animal body is the brain; where GABA is the most abundant inhibitory neurotransmitter (McCormick, 1989). Although rare, neurobrucellosis is associated with human and marine mammal infections (Ceran *et al.*, 2011, McDonald *et al.*, 2006). The mechanisms of this are still not well understood but recent studies have shown that *Brucella* can in fact traverse the blood brain barrier, and environment with a large concentration of GABA, underlining potential mechanisms for the use of GABA by *B. abortus* during neurobrucellosis infection (Miraglia *et al.*, 2018, Drevets *et al.*, 2004, Miraglia *et al.*, 2016).

Prokaryotes and eukaryotes both utilize the GAD system to cope with acid stress by exporting protons to increase the pH of the intracellular environment (Feehily & Karatzas, 2013). In the gut, this system can be vital for the survival of bacteria in the acidic stomach, and activation of the GAD system would lead to export of excess GABA in this setting. Recently, several studies have focused on the role of the microbiome on neurotransmitter modulation and interplay between host microbiome and disease, specifically on GABA production by enteric organisms (Strandwitz *et al.*, 2018,

Strandwitz, 2018). Therefore, the gut is another potential location for *Brucella* to encounter high GABA concentrations since ingestion is a major route of infection for brucellosis (Godfroid, 2017, Atluri *et al.*, 2011).

Similarly to what was proposed in Chapter 2 for fucose utilization, another important question with regards to GABA transport is whether import and sensing of GABA in these locations affords *B. abortus* a competitive advantage over other organisms within the host. As discussed in Chapter 4 and previously in this discussion, the transport of GABA is regulated by the AbcRs in *B. abortus* and *A. tumefaciens*, thus, by better understanding the expression of the AbcRs temporally, hypotheses can be formed as to if an when GABA may be an important signaling molecule during pathogenesis.

Evolution of the *Brucella* replicon

The vast differences in the VtlR and AbcR regulons are also indicative of the evolution of the *Brucella* genome over time. Different ecological niches have shaped organisms gradually as they evolve to better fit their environmental alcove. This can be observed by the range in genome sizes between host-associated and free-living organisms as bacterial genomes that have changed over time to selectively keep essential genes and remove superfluous genetic elements. On average, host-associated parasites have relatively small genomes (0.6-1.5 Mbp) compared to other free-living organisms (1.5-10 Mbp) (Moran, 2002). This is evident in the replicons of the *Alphaproteobacteria* that have undergone expansion and reduction from their common ancestor to better fit their specific environmental niches (Ettema & Andersson, 2009). The *Brucella* genome, >3 Mbp, although relatively large compared to other parasitic bacteria, is significantly smaller than

the replicons of the phylogenetically related free-living organisms *Agrobacterium tumefaciens*, >5 Mbp, and *Sinorhizobium meliloti*, >6 Mbp. Bioinformatic comparisons have shown large genetic similarities between the *Rhizobiales* and *Brucella* with regards to metabolism, nutrient acquisition, and virulence (Paulsen *et al.*, 2002). These studies provide myriad evidence that *Brucella* has evolved from plant-associated bacteria within the *Rhizobiales*. Although, over time, the brucellae replicon has been reduced to save energy and better survive the threats of the intramacrophagic environment.

Proteomic and transcriptomic analysis of the deletion strains *A. tumefaciens* Δ*abcR1* and *B. abortus* Δ*abcR1/2* is suggestive of this evolution. In *A. tumefaciens*, 32 putative ABC transport systems were differentially regulated in Δ*abcR1*. Of these systems, only 9 orthologous systems were also found in the *B. abortus* genome and many of these orthologous systems are differentially regulated in *B. abortus* Δ*abcR1/2* (Caswell *et al.*, 2012). Loss of the other 23 putative transport systems dysregulated in *A. tumefaciens* Δ*abcR1* from the *B. abortus* genome may be indicative of an evolutionary shift and reduction of unnecessary genes for infection of an animal host. Further characterization of transport systems specifically conserved in the brucellae may be apparent of systems necessary for an animal-associated intracellular bacterium (i.e. *B. abortus*) compared to a free-living organism (i.e. *S. meliloti* or *A. tumefaciens*). Evolution of genetic systems can even be observed within the genera *Brucella*, and these differences may be attributed to host specificity between the *Brucella* species (Tsolis *et al.*, 2009, Paulsen *et al.*, 2002).

Overall, this dissertation has focused on characterizing the function and contribution to virulence of genetic systems in the intracellular animal pathogen *B.*

abortus and plant-associated pathogen *A. tumefaciens*. Many of these systems are interconnected with the regulation of the small regulatory RNAs AbcR1 and AbcR2 by the transcriptional regulator VtlR, and these studies present a complex network of genetic regulation in both *B. abortus* and *A. tumefaciens*. By understanding similarities and difference between the genetic regulatory systems of these organisms, we can better comprehend how environmental pressures have affected the evolution of these vastly different pathogens.

References

Atluri, V.L., M.N. Xavier, M.F. de Jong, A.B. den Hartigh & R.M. Tsolis, (2011) Interactions of the human pathogenic *Brucella* species with their hosts. *Annu Rev Microbiol* **65**: 523-541.

Bhat, R., R. Axtell, A. Mitra, M. Miranda, C. Lock, R.W. Tsien & L. Steinman, (2010) Inhibitory role for GABA in autoimmune inflammation. *Proceedings of the National Academy of Sciences of the United States of America* **107**: 2580-2585.

Budnick, J.A., L.M. Sheehan, L. Kang, P. Michalak & C.C. Caswell, (2018) Characterization of Three Small Proteins in *Brucella abortus* Linked to Fucose Utilization. *J Bacteriol* **200**.

Caswell, C.C., J.M. Gaines, P. Ciborowski, D. Smith, C.H. Borchers, C.M. Roux, K. Sayood, P.M. Dunman & R.M. Roop Ii, (2012) Identification of two small regulatory RNAs linked to virulence in *Brucella abortus* 2308. *Molecular microbiology* **85**: 345-360.

Ceran, N., R. Turkoglu, I. Erdem, A. Inan, D. Engin, H. Tireli & P. Goktas, (2011) Neurobrucellosis: clinical, diagnostic, therapeutic features and outcome. Unusual clinical presentations in an endemic region. *Braz J Infect Dis* **15**: 52-59.

Deghelt, M., C. Mullier, J.F. Sternon, N. Francis, G. Laloux, D. Dotreppe, C. Van der Henst, C. Jacobs-Wagner, J.J. Letesson & X. De Bolle, (2014) G1-arrested newborn cells are the predominant infectious form of the pathogen *Brucella abortus*. *Nat Commun* **5**: 4366.

Drevets, D.A., P.J. Leenen & R.A. Greenfield, (2004) Invasion of the central nervous system by intracellular bacteria. *Clin Microbiol Rev* **17**: 323-347.

Ettema, T.J. & S.G. Andersson, (2009) The alpha-proteobacteria: the Darwin finches of the bacterial world. *Biol Lett* **5**: 429-432.

Feehily, C. & K.A. Karatzas, (2013) Role of glutamate metabolism in bacterial responses towards acid and other stresses. *Journal of applied microbiology* **114**: 11-24.

Godfroid, J., (2017) Brucellosis in livestock and wildlife: zoonotic diseases without pandemic potential in need of innovative one health approaches. *Arch Public Health* **75**: 34.

Kim, J.K., Y.S. Kim, H.M. Lee, H.S. Jin, C. Neupane, S. Kim, S.H. Lee, J.J. Min, M. Sasai, J.H. Jeong, S.K. Choe, J.M. Kim, M. Yamamoto, H.E. Choy, J.B. Park & E.K. Jo, (2018) GABAergic signaling linked to autophagy enhances host protection against intracellular bacterial infections. *Nat Commun* **9**: 4184.

McCormick, D.A., (1989) GABA as an inhibitory neurotransmitter in human cerebral cortex. *Journal of neurophysiology* **62**: 1018-1027.

McDonald, W.L., R. Jamaludin, G. Mackereth, M. Hansen, S. Humphrey, P. Short, T. Taylor, J. Swingler, C.E. Dawson, A.M. Whatmore, E. Stubberfield, L.L. Perrett & G. Simmons, (2006) Characterization of a *Brucella* sp. strain as a marine-mammal type despite isolation from a patient with spinal osteomyelitis in New Zealand. *Journal of clinical microbiology* **44**: 4363-4370.

Miraglia, M.C., M.M. Costa Franco, A.M. Rodriguez, P.M. Bellozi, C.C. Ferrari, M.I. Farias, V.A. Dennis, P. Barrionuevo, A.C. de Oliveira, F. Pitossi, K.S. Kim, M.V. Delpino, S.C. Oliveira & G.H. Giambartolomei, (2016) Glial Cell-Elicited Activation of Brain Microvasculature in Response to *Brucella abortus* Infection

Requires ASC Inflammasome-Dependent IL-1beta Production. *J Immunol* **196**: 3794-3805.

Miraglia, M.C., A.M. Rodriguez, P. Barrionuevo, J. Rodriguez, K.S. Kim, V.A. Dennis, M.V. Delpino & G.H. Giambartolomei, (2018) *Brucella abortus* Traverses Brain Microvascular Endothelial Cells Using Infected Monocytes as a Trojan Horse. *Frontiers in cellular and infection microbiology* **8**: 200.

Moran, N.A., (2002) Microbial minimalism: genome reduction in bacterial pathogens. *Cell* **108**: 583-586.

Paulsen, I.T., R. Seshadri, K.E. Nelson, J.A. Eisen, J.F. Heidelberg, T.D. Read, R.J. Dodson, L. Umayam, L.M. Brinkac, M.J. Beanan, S.C. Daugherty, R.T. Deboy, A.S. Durkin, J.F. Kolonay, R. Madupu, W.C. Nelson, B. Ayodeji, M. Kraul, J. Shetty, J. Malek, S.E. Van Aken, S. Riedmuller, H. Tettelin, S.R. Gill, O. White, S.L. Salzberg, D.L. Hoover, L.E. Lindler, S.M. Halling, S.M. Boyle & C.M. Fraser, (2002) The *Brucella suis* genome reveals fundamental similarities between animal and plant pathogens and symbionts. *Proc Natl Acad Sci U S A* **99**: 13148-13153.

Sheehan, L.M., J.A. Budnick, C. Blanchard, P.M. Dunman & C.C. Caswell, (2015) A LysR-family transcriptional regulator required for virulence in *Brucella abortus* is highly conserved among the alpha-proteobacteria. *Mol Microbiol* **98**: 318-328.

Strandwitz, P., (2018) Neurotransmitter modulation by the gut microbiota. *Brain Res* **1693**: 128-133.

Strandwitz, P., K.H. Kim, D. Terekhova, J.K. Liu, A. Sharma, J. Levering, D. McDonald, D. Dietrich, T.R. Ramadhar, A. Lekbua, N. Mroue, C. Liston, E.J. Stewart, M.J. Dubin, K. Zengler, R. Knight, J.A. Gilbert, J. Clardy & K. Lewis, (2018) GABA-modulating bacteria of the human gut microbiota. *Nat Microbiol*.

Tsolis, R.M., R. Seshadri, R.L. Santos, F.J. Sangari, J.M. Lobo, M.F. de Jong, Q. Ren, G. Myers, L.M. Brinkac, W.C. Nelson, R.T. Deboy, S. Angiuoli, H. Khouri, G. Dimitrov, J.R. Robinson, S. Mulligan, R.L. Walker, P.E. Elzer, K.A. Hassan & I.T. Paulsen, (2009) Genome degradation in *Brucella ovis* corresponds with narrowing of its host range and tissue tropism. *PLoS One* **4**: e5519.