Characterizing the AbcR/VtlR system in the *Rhizobiales*

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Abstract

Rhizobiales encompass a diverse group of microbes, ranging from free-living, soil-dwelling bacteria to disease-causing, intracellular pathogens. Although the lifestyle of these organisms vary, many genetic systems are well conserved. One system, named the AbcR/VtlR system, is found throughout rhizobiales, and even extends to bacteria in other orders within the *Alphaproteobacteria*.

The AbcR sRNAs are an example of sibling sRNAs, where two copies of the *abcR* gene are typically present in the genome. The AbcRs are involved in the negative regulation of ABC-type transport systems, which are important components for nutrient acquisition. Although the AbcRs share several features amongst organisms, major differences can be found in their functional and regulatory redundancy, the targets they regulate and how they regulate them. Specifically, one major difference in the AbcRs lies in the nucleotide sequences utilized by the sRNAs to bind mRNA targets. In the present studies, the regulatory mechanisms of the AbcR sRNAs were further characterized in the mammalian pathogen *Brucella abortus*, and the full regulatory profiles of the AbcRs were defined in the plant pathogen *Agrobacterium tumefaciens*.

As mentioned above, the AbcR sRNAs are important for the proper regulation of nutrient-acquiring transport systems in the *Rhizobiales*. Since these sRNAs are critical to the lifestyle of a bacterium, proper regulation of this system is key to survival. A LysR-

type transcriptional regulator, named VtlR, was found to be the bonefide transcriptional activator of *abcR1* in *B. abortus*. Furthermore, VtlR has been shown to be a key component in host interactions in several rhizobiales. The preset work has shed light on the evolutionary divergence of this regulator in bacteria, and further defined the regulatory capacity of VtlR in *Agrobacterium*.

Overall, the studies described here have made significant advances in our knowledge of the AbcR/VtlR-regulatory systems in the *Rhizobiales*, and have further defined this system as being a vital part of host-microbe interactions.

Characterizing the AbcR/VtlR system in the *Rhizobiales*

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General Audience Abstract

Understanding the genetic systems utilized by microbes to cause infection is key for developing therapeutics that can be administered to fight against them. Moreover, identifying and characterizing these essential microbial systems can be exploited for the development of drugs to target and shut down these systems, thus causing cell death. The present work took a basic molecular biology approach and characterized a highly conserved genetic system, named the AbcR/VtlR system, in two pathogenic bacteria: the plant pathogen *Agrobacterium* and the mammalian pathogen *Brucella*. Overall, the work described here shows this system to be an important component in acquiring nutrients for the microbe, and, most importantly, found the AbcR/VtlR system to be essential for host-microbial interactions.

Dedication

I dedicate this work to my loving parents, Isabel and Gary Sheehan, and my boyfriend, Earl Dodge.

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Chapter 1. General Information

The phylogeny of *Proteobacteria*

The phylum *Proteobacteria*

The class of *Alphaproteobacteria* is a very large and diverse group of Gram-negative organisms that inhabit a variety of environmental niches. This class is a part of the phylum *Proteobacteria*, which was originally named “purple bacteria and their relatives” by the American microbiologist Carl Woese in 1987 (Woese, 1987). Woese and his colleagues were the first to categorize this group of bacteria, and classified organisms to be associated with this phylum based on 16S rRNA analyses. In 1988, the class was renamed after Proteus, the Greek god of the sea whom was capable of assuming many shapes, because of the group’s diversity in both shape and physiology (Stackebrandt *et al*., 1988). This phylum is one of the largest groups in prokaryotes, and is comprised of a myriad of unique bacteria (Kersters *et al*., 2006).

Organisms classified in this phylum include pathogens, nitrogen-fixing bacteria, and free-living bacteria (Kersters *et al*., 2006). Many of the bacteria within this phylum move by use of a flagellum. However, some organisms completely lack a flagellum (e.g., some *Brucella* spp.), whereas others rely on alternative motility strategies such as gliding motility (e.g., *Myxococcus xanthus*). By far the greatest variation within this phylum is in their metabolism, which include bacteria that utilize light (phototrophs), organic substances (heterotrophs), or inorganic reduced compounds (chemolithotrophs). It is important to note that mitochondria are theorized to have originated from the *Proteobacteria*, specifically from the order *Rickettsiales*, (Andersson *et al*., 1998; Gupta, 2000; Vesteg and Krajcovic *et al*., 2008).

There are five classes within the *Proteobacteria* phylum: *Alphaproteobacteria* (α-proteobacteria), *Betaproteobacteria* (β-proteobacteria), *Gammaproteobacteria* (γ-proteobacteria), *Deltaproteobacteria* (δ–proteobacteria), and *Epsilonproteobacteria* (ε-proteobacteria). Organisms have been classified into these subdivisions based on their 16S and 23S rRNA.

The class *Alphaproteobacteria*

The *Alphaproteobacteria* comprise a diverse genera of Gram-negative organisms, where some can cause serious diseases in plants or animals, some that can form symbiotic relationships with their hosts, and some that are free-living (Garrity *et al*., 2005). This class also encompasses the most marine bacteria, where 10% of the ocean microbial community is made of *Alphaproteobacteria* (Giovannoni *et al*., 2005). As mentioned in the previous section, it is hypothesized that this class of bacteria is the ancestral group for the origin of the mitochondria. Specifically, the mitochondrion is thought to have arisen from the *Rickettsiales*, although there are conflicting reports about this claim (Wu *et al*., 2004; Esser *et al*., 2004; Fitzpatrick *et al*., 2006). Organisms in this class vary in their metabolic activities (e.g., nitrogen fixation and photosynthesis), morphologies (e.g., spiral and stalked), and life styles (e.g., intracellular and extracellular) (Williams *et al*., 2007).

Two characteristics that unify this class of bacteria are: their ability to survive and thrive in low-nutrient environments; and the conservation of highly related genes and genetic systems. Although these organisms share some features, the loss or expansion of their genomes has been a major factor contributing to the divergence within this class

(Moreno, 1998). For example, gene loss has occurred in intracellular bacteria that associate with invertebrates, animals, and humans (e.g., *Rickettsia* and *Wolbachia*), whereas gene expansion has occurred in soil-growing, plant-associated bacteria (e.g., *Agrobacterium* and *Sinorhizobium*). The major genes that have been shown to have changed include those involved in regulation, transport, and small-molecule metabolism (Batut *et al*., 2004).

This class can be broken down into eight orders: the *Rickettsiales*, the *Acetobacteraceaea*, the *Rhodospirillaceae*, the *Sphingomonadales*, the *Kordiimonadales*, the *Rhodobacterales*, the *Hyphomonadales*, and the *Rhizobiales*.

The order *Rhizobiales*

The bacteria categorized in the order *Rhizobiales* are diverse in their ecological niches and lifestyles, but largely conserved in their genomic composition. Bacteria in this order include those capable of fixing atmospheric nitrogen during symbiosis with legumes, obligate and facultative intracellular bacteria, and soil-dwelling organisms. Interestingly, many of the proteins that are necessary for symbiosis and/or pathogenesis are conserved in all *Rhizobiales* (Guerrero *et al*., 2005).

The order *Rhizobiales* can be broken down into 15 distinct families: the *Aurantimonadaceae*, the *Bartonellaceae*, the *Beijerinckiaseae*, the *Bradyrhizobiaceae*, the *Brucellaceae*, the *Cohaesibacteraceae*, the *Hyphomicrobiaceae*, the *Labriaceae*, the *Methylobacteriaceae*, the *Methylocystaceae*, the *Methylopilaceae*, the *Phyllobacteriaeceae*, the *Rhizobiaceae*, the *Rhodobiaceae*, and the *Xanthobacterazeae*.

The family *Brucellaceae*

The family *Brucellaceae* was named after the Scottish microbiologist Sir David Bruce. This family includes the following genera: *Brucella*, *Crabtreella*, *Daeguia*, *Mycoplana*, *Ochrobacterum*, *Paenochrobactrum*, and *Pseudochrobactrum* (Kämpfer *et al*., 2014). All of the organisms are classified as chemoorganotrophs, are capable of aerobic respiration, and have a G+C content of 49.7 to 65.3%. This family contains pathogens of animals and humans, opportunistic pathogens associated with human infections, and free-living organisms that can be isolated from sludge, soil, and/or water (Berg et al., 2005). *Brucellaceae* have rod-shaped morphologies and can sometimes be motile (Kämpfer *et al*., 2014).

The genus *Brucella* is one of the most studied groups in the *Brucellaceae*, and the brucellae are responsible for causing disease in both animals and humans. Of importance, *Brucella* spp. are very closely related to other *Rhizobiales* such as *Agrobacterium*, *Sinorhizobium*, *Rhizobium*, *Mesorhizobium,* and *Bartonella*.

Brucella

History and general description

Although *Brucella* spp. are commonly thought to have first been isolated and described in the late 1800s, the presence of this pathogen was noted long before this. In 1600 BC, it is hypothesized that the fifth plague of Egypt (the disease of livestock) could have been caused by *Brucella* spp. (Pappas *et al*., 2006a). However, there are a handful of other pathogens, such as *Bacillus anthracis*, that are speculated to have resulted in this plague. Brucellosis, the disease caused by *Brucella* spp., first appeared in the writings of Hippocrates in 450 BC (Spink, 1948). The next noted appearance of *Brucella* occurred during the time of Jesus. Paul the Apostle is reported to have healed the chief official Publius' father on the Island of Malta. The man was said to be suffering from a chronic, undulating fever, which is now known to be one of the most common symptoms of brucellosis (Bible, Acts 28:7-10; Barrett and Stanberry, 2009).

The genus *Brucella* was first identified and described by the British army physician Sir David Scott on the Island of Malta in 1887 (Bruce, 1887; Godfroid *et al*., 2005). Sir David Bruce originally named this organism *Micrococcus melitensis*, and isolated the bacterium from the spleen of an infected soldier. Concurrently, Bernard Bang was unknowingly studying the same genus of bacteria that were infecting cattle in Denmark (Mochmann and Köhler, 1988). Bang noted that the organism, which he named *Bacillus abortus*, lead to abortion in cattle, and thus was called 'contagious abortion.' In 1905, Bruce, along with Maltese scientist Themistocles Zammit, determined that goats harbor the bacteria, and moreover, that goat's milk was the prominent source of human infection (Zammit, 1905; Godfroid *et al*., 2005). Following this discovery, American microbiologist

Alice Evans considered this organism to be the sole microbe responsible for human brucellosis (Mochmann and Köhler, 1988).

To avoid further confusion regarding the name of the pathogen, Karl Meyer, a veterinarian at the Hooper Foundation, suggested naming the genus "*Brucella*" in honor of Sir David Bruce in 1920 (Gessner, 2016). Although the disease caused by *Brucella* spp. is known as 'brucellosis', it is also sometimes referred to as one of the following: Malta Fever, due to the prevalence of the disease on the Island of Malta in the late 1800s; undulant fever, due to the bacterium causing a relapsing fever in patients; and Bang's disease, named after Bernard Bang. A general timeline detailing the history of *Brucella* is shown below in Figure 1.

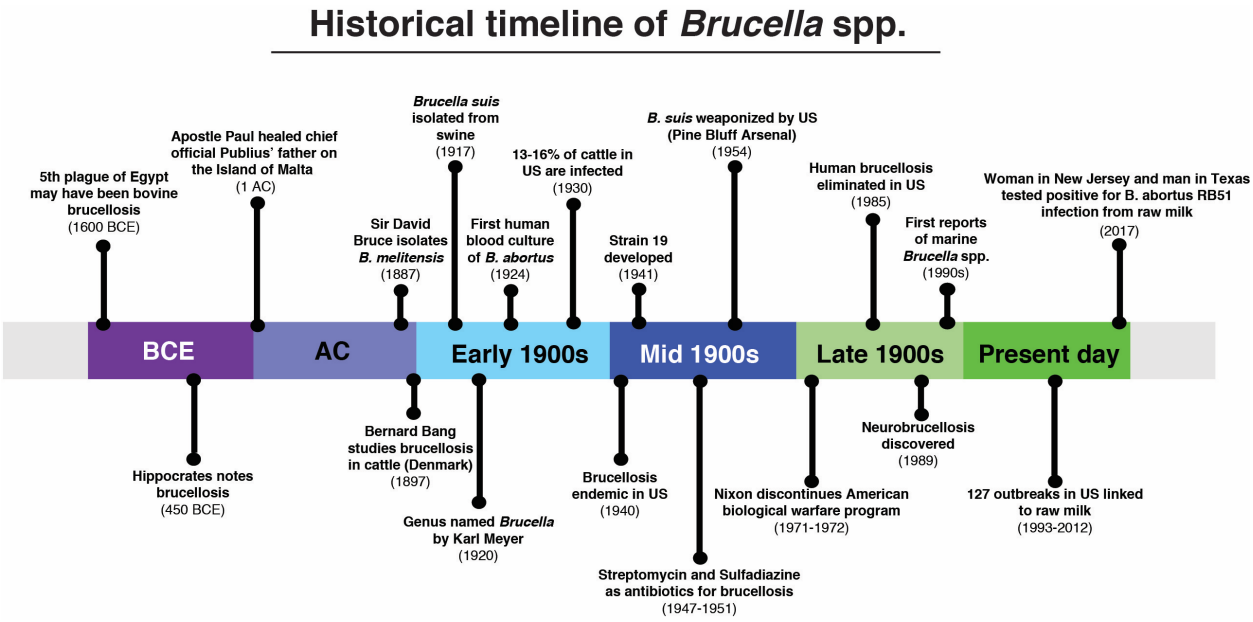

Figure 1.1. Events throughout history involving *Brucella* spp.

The genus *Brucella* encompasses species that are typically defined by the host(s) they preferentially infect (Table 1). Importantly, not all species of *Brucella* are zoonotic (i.e., transmissible to humans). In addition to having preferred hosts, *Brucella* spp. can cause various symptoms depending on the animal infected. In cattle, *B. abortus* causes spontaneous abortions, typically in the third trimester of pregnancy (Corbel, 1997; Pappas *et al*., 2006b). In sheep, *B. ovis* can cause sterility and epididymitis in rams, and abortion in ewes (Cerri *et al*., 1999). In dolphins, *B. ceti* has been reported to cause neurobrucellosis (Hernández-Mora *et al*., 2008). In humans, brucellosis is characterized by a chronic, debilitating fever accompanied by flu-like symptoms. Transmission to humans can occur by several means, including the following: consumption of unpasteurized dairy products such as milk and soft cheeses; direct contact of a skin abrasion with an infected animal; and laboratory exposure (Pappas *et al*., 2005). By far, consumption of infected dairy products is the most common method of transmission. Additionally, brucellosis is the most common laboratory-acquired infection worldwide (Harding *et al*., 2000). Brucellosis remains one the most zoonotic diseases worldwide, with an estimated 500,000 human cases per year (Seleem *et al*., 2010; Pappas *et al*., 2006b).

Classical *Brucella* spp.			
Name	**Preferential host(s)**	**First isolated from**	**Human infections?**
B. abortus	Cattle	Udder of a cow	Yes
B. canis	Dog	Beagle	Yes
B. melitensis	Goat/Sheep	Human spleen	Yes
B. neotomae	Desert wood rat	Rodent	Yes
B. ovis	Sheep	Sheep	No
B. suis	Swine	Swine	Yes
Non-classical *Brucella* spp.			
Name	**Preferential host(s)**	**First isolated from**	**Human infection?**
B. ceti	Cetaceans	Marine mammals	Unknown
B. inopinata	Human	Breast implant	Yes
B. microti	Common vole	Rodent	Unknown
B. papionis	Baboon	Cervix of baboon	Unknown
B. pinnipidialis	Seals	Marine mammals	Unknown
B. vulpis	Fox	Red fox	Unknown

Table 1.1. List of *Brucella* spp., their preferential host(s), where the bacterium was first isolated, and if the species has been documented to cause infection in humans.

Monotherapy (treatment with one antibiotic) was deemed a failure for treating human brucellosis and was found to lead to a high incidence of relapse (Alavi and Alavi, 2013). Today, treatment for humans includes a regimen of two antibiotics, typically rifampicin (dosage between 600-900 milligrams/day) and doxycycline (dosage of 200 milligrams/day), given for a minimum of six weeks (Food and Agriculture Organization-World Health Organization, 1986). Other commonly used antibiotics include streptomycin and tetracycline. In complicated cases of brucellosis (meningoencephalitis and/or endocarditis), patients may be prescribed additional antibiotics (Solera, 2000). In terms of preventing disease in humans, there are currently no vaccines licensed for use in humans. In addition, diagnosis of brucellosis can prove to be challenging, since many diagnostics utilized to test for *Brucella* spp. are cross-reactive with other organisms (Corbel *et al*., 1984). However, because humans become infected with the bacteria from animals, we must primarily focus on eliminating the spread of animal brucellosis.

Epidemiology

As described above, brucellosis is a disease found in both humans and animals. Documentation of both types of brucellosis vary in countries due to the lack of surveillance and reporting of cases. Brucellosis is widely dispersed and can be found in Middle Eastern countries, the Mediterranean region, Western Asia, Africa, and Latin America (Pappas *et al*., 2006b). Recent reemergence in the areas of Malta and Oman have been documented (Doganay and Aygen, 2003). Additionally, since surveillance can be poor, the prevalence of brucellosis in many areas is unknown. Of importance, there have been several recent

cases of humans contracting brucellosis in the United States (Cossaboom *et al*., 2018). One case was a woman in New Jersey who consumed unpasteurized dairy products and became infected with the bovine vaccine strain *Brucella abortus* RB51. Similarly, a man in Texas became sick with brucellosis after consumption of unpasteurized dairy products. Regarding animal brucellosis, bovine brucellosis is currently a huge problem in Yellowstone National Park because of the interaction of infected wildlife such as bison and elk with cattle on farms close to the park (Rhyan *et al*., 2013). Swine brucellosis is also endemic in wild boar in parts of Florida and Texas, and can lead to hunters contracting the disease (Meng *et al*., 2009). To this point, although brucellosis is commonly referred to as a historical disease, we must be aware that this pathogen is still capable of causing disease in any part of the world.

Intracellular life cycle

Brucella spp., invade the reticuloendothelial system of the host and are able to reside within both nonphagocytic and phagocytic cells. Following entry into the cell, the bacteria can be found inside an acidic vacuole termed the *Brucella*-containing vacuole (BCV) (Arenas *et al*., 2000; Celli 2006). Initially, *Brucella* must adapt to the harsh intracellular conditions characterized by an acidic pH, low oxygen tension, reactive oxygen and nitrogen species, and minimal nutrients (Aderem 2003; Roop *et al*., 2009). In addition, the brucellae must turn on important molecular components to avoid immune recognition (e.g., inhibition of TNF-α) and inhibit cellular apoptosis (Gross *et al*., 2000; Barquero-Calvo *et al*., 2007). The bacteria must also successfully hijack the host's cellular components, such as parts of the cytoskeleton, to multiply. However, this must be done

with discretion so as to not disrupt the normal cell cycle and function. *Brucella* is able to survive and thrive in this hostile environment by employing an arsenal of strategies to avoid degradation during this phase of intracellular trafficking.

Within the first few hours of infection, proteomic analyses of intracellular brucellae have demonstrated decreases in various transport systems, carbon metabolism and DNA/RNA/protein synthesis, and increases in stress-response proteins, DNA repair mechanisms and catabolic pathways (Chaves-Olarte *et al*., 2012). During these initial stages of infection, *Brucella* interacts transiently with the lysosome, leading to acidification of the BCV (Starr et al., 2008). This acidification, along with nutrient deprivation, has been shown to be an important signal for induction of the Type IV VirB secretion system (Boschiroli *et al*., 2002). Following this initial period of adaptation, the brucellae continue to traffic through the environment and eventually interact and reside in close contact with components of the endoplasmic reticulum (ER). Following association of the BCV with host ER, the conditions of this ER-derived vacuole become conducive for bacterial replication (Arenas *et al*., 2000; Celli 2006).

Survival mechanisms

As mentioned above, a major aspect of *Brucella* pathogenicity is the ability to avoid phagosomal degradation and ultimately reside and replicate in the permissive ER-associated vacuole. Unlike other pathogenic bacteria, *Brucella* does not encode classical virulence factors such as toxins, fimbriae, capsules, exopolysaccharide, or plasmids (Seleem *et al*., 2008). Instead, *Brucella* uses a number of mechanisms to avoid extensive immune stimulation and successfully traffic through the host environment. Some of the

molecular regulatory systems that have been characterized are the BvrR/BvrS two-component system, the Type IV VirB secretion system, and the quorum sensing VjbR system (Sola-Landa *et al*., 1998; Boschiroli *et al*., 2002; Delrue *et al*., 2005). These systems are involved in sensing the environmental conditions encountered during trafficking and eliciting a response, typically by altering gene expression, to allow for survival. Some stresses *Brucella* encounters intracellularly along with the strategies *Brucella* is known to employ to cope with them are described below.

Reactive Oxygen Species (ROS): One of the many stresses encountered by *Brucella* during trafficking are reactive oxygen species (ROS) generated directly or indirectly by NADPH oxidase (Flannagan *et al*., 2009). Two of the most common ROS include superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2). This bactericidal mechanism can cause significant damage to bacterial DNA, enzymes, double-chain fatty acids, and other biomolecules (Slauch, 2011). However, *Brucella* has a plethora of strategies to combat ROS and survive intracellularly (Roop, 2009).

Brucella encodes two superoxide dismutases, SodC and SodA, which are responsible for the detoxification of O_2^-. The periplasmic Cu,Zn superoxide dismutase SodC has been shown to be the main protein responsible for defending the bacteria against the respiratory burst of host macrophages (Stabel *et al*., 1994). Moreover, a *B. abortus sodC* mutant exhibits reduced intracellular survival within murine macrophages as well as experimentally infected mice, confirming its necessity during infection of the host (Gee *et al*., 2005). The cytoplasmic manganese-cofactored superoxide dismutase SodA is a major cytoplasmic antioxidant and is responsible for the protection of the

bacteria against endogenous O_2^- produced by respiratory metabolism (Martin *et al*., 2012; Sriranganathan *et al*., 1991).

The protein KatE is the sole monofunctional catalase in *Brucella* and plays the important role in detoxification of H_2O_2 when the ROS is present at extreme exogenous levels (Kim *et al*., 2000; Steele *et al*., 2010). This catalase is under the transcriptional control of the widespread LysR-type regulator OxyR. In *Brucella*, OxyR has been suggested to assist in protecting cells against prolonged exposure to H_2O_2 (Kim and Mayfield, 2000). In contrast to KatE, the peroxiredoxin AphC is the primary antioxidant for scavenging endogenous H_2O_2 generated by aerobic metabolism (Steele *et al*., 2010).

In addition to inorganic H_2O_2, *Brucella* has specific strategies for survival against organic H_2O_2, such as cumene hydroperoxide and tert-butyl hydroperoxide. The organic hydroperoxide resistance protein Ohr, along with its transcriptional MarR-type regulator OhrR, is responsible for protection against organic hydroperoxides (Caswell *et al*., 2012a).

Brucella may also have indirect mechanisms to cope with stress by ROS with use of its cytochrome oxidases. Since cytochrome *bd* ubiquinol oxidase as well as c33b-type cytochrome oxidase have high affinities for oxygen, they can be used to indirectly scavenge O_2 and prevent toxicity (Roop *et al*., 2009).

Reactive nitrogen species (RNS): For intracellular bacteria such as *Brucella* spp., the ability to grow under hypoxic conditions is critical for their survival. One mechanism to overcoming this stressor is by utilizing other molecules as terminal electron acceptors, such as nitric oxide (NO) (MacMicking *et al*., 1997). NO is an ‘antimicrobial’ component produced by macrophages, and is synthesized by the inducible NO synthase (iNOS). NO,

along with other RNS, are used by macrophages to kill intracellular pathogens (Ko *et al*., 2002). However, *Brucella* has been shown to be able to withstand NO stress. Strikingly, macrophages producing high levels of NO do not successfully eliminate *Brucella*, and instead become steadily infected with the pathogen following 24-hours of infection (Wang *et al*., 2001). One hypothesis as to what may allow for the survival of the bacteria when stressed with NO involves 4 'denitrification islands' comprised of: Nar (nitrate reductase); Nir (nitrite reductase); Nor (nitric oxide reductase); and Nos (nitrous oxide reductase).

In *B. suis*, a *norD* mutant was found to be severely attenuated in both *in vitro* (J774.1 macrophages) and *in vivo* (BALB/c mice) models of infection (Loisel-Meyer *et al*., 2006). The authors of this paper suggest that *norD* is important for the process of denitrification, by acting as a nitric oxide reductase. According to these results, NorD is critical for the ability of the bacteria to cause a chronic infection. However, a mutant of *narG*, encoding for a nitrate reductase, in both *B. suis* and *B. melitensis* did not result in either decrease survival under hypoxic conditions nor lead to attenuation in a murine model of infection (Loisel-Meyer *et al*., 2006; Haine *et al*., 2006). These data suggests that not all of the genes encoded in these 'denitrification islands' are necessary for *Brucella* persistance in the mononuclear phagocyte system.

NnrA, a transcriptional regulator apart of the NnrR family, is involved in the regulation of the Nir (*nirK*), Nor (*norC*), and Nos (*nosR*) systems in *B. melitensis* (Haine *et al*., 2006). Moreover, a mutant of *nnrA* was shown to be attenuated in BALB/c mice as well as in activated J774.1 macrophages. Importantly, this defect in survival of the *nnrA* mutant can be reversed when L-NAME, an inhibitor of NO synthase, is given to activated

macrophages, suggesting that the reason for lack of survival of the *nnrA* mutant is due to the inability to completely denitrify NO (Haine *et al*., 2006).

Acidic pH: Upon engulfment, the BCV interacts transiently with the lysosome, but ultimately escapes fusion with it. During this brief association, the BCV is rapidly acidified to a pH between 4-4.5 (Porte *et al*., 1999). Interestingly, studies have reported that inhibiting compartmental acidification leads to reduction in *Brucella* replication (Detilleux *et al*., 1991).

One of the systems that respond readily to acidic pH is the VirB type IV secretion system (T4SS). The VirB T4SS is one of the key pathogenicity system found in *Brucella* and is thought to play a variety of roles including evading fusion with the lysosome, interacting with the ER, and modulating host immune response. Phagosomal acidification, along with nutrient deprivation, has been shown to be absolutely required for *virB* expression (Rouot *et al*., 2003; Boschiroli *et al*., 2002). *Brucella virB* mutants have been shown to be defective in intracellular survival both *in vitro* and *in vivo*.

The periplasmic chaperone HdeA has been shown to play a role in acid resistance not only in *Brucella*, but also in *Escherichia coli* and *Shigella flexneri* (Gajiwala and Burley, 2000; Waterman and Small, 1996). However, although a *Brucella hdeA* mutant does show sensitivity to acidic pH, the mutant does not show attenuation in either an *in vitro* or *in vivo* model of infection (Valderas *et al*., 2005).

Antimicrobial peptides (AMPs): The generation of antimicrobial peptides (AMPs), also known as “host defense peptides”, is an oxygen-independent mechanism employed by phagocytic cells to kill invading bacteria. The AMP family includes defensins,

cathelicidins, lysozymes, and lipases/proteases, which operate in a direct mechanism to disrupt the outer membrane (OM) of the bacterial cell (Flannagan *et al*., 2009).

Although this bactericidal mechanism is an excellent host defense, *Brucella* spp. are highly resistant to AMPs. This lack of sensitivity is attributed to the properties of the OM. The OM of *Brucella* has a reduced negative change due to the absence of acidic core sugars, low proportions of phosphate groups in lipid A, and the presence of positively charged lipids (Moriyón and López-Goñi, 1998).

Major regulatory systems have been linked to the biogenesis and homeostasis of the *Brucella* OM and may indirectly contribute to this heightened resistance to AMPs. The two-component regulatory system BvrR/BvrS has been linked to altered cell-surface hydrophobicity, permeability, and sensitivity to AMPs (Guzman-Verri *et al*., 2002). Mutations in the quorum sensing transcriptional regulator VjbR have also shown changes in the OM properties of *Brucella* (Uzureau *et al*., 2007).

Nutrient deprivation: One of the major stresses *Brucella* must deal with is the lack of nutrients within the phagosomal environment. These phagocytic cells are able to remove nutrients by using the H^+-gradient generated by the V-ATPase (Flannagan *et al*., 2009). Experiments have shown *Brucella* to adapt to these conditions by making coordinated changes in proteins involved with central metabolism. During initial infection, *Brucella* reduces expression of proteins involved with the tricarboxylic acid cycle and carbon anabolism, and increases expression of proteins involved with catabolism of amino acids (Chaves-Olarte *et al*., 2012). This altered metabolism seems to reflect the limited substrate availability within the phagosomal compartment.

In addition to macronutrient availability, micronutrients are scarce within this environment as well. Some of the most crucial micronutrients *Brucella* must scavenge for are divalent cations such as Fe^{2+}, Zn^{2+}, Mg^{2+}, and Mn^{2+}. Divalent cations serve as important cofactors in a variety of proteins in both eukaryotes and prokaryotes. One of the strategies for acquiring iron is by use of the four-component FtrABCD-type transporter. This transport system has been shown to transport ferrous iron and, importantly, to play an indispensable role in *Brucella* virulence (Elhassanny *et al*., 2013). For zinc acquisition, both uptake (ZnuABC system) and export (ZntA) have been very well defined, and play a crucial part in *Brucella* pathogenesis as well (Kim *et al*., 2004; Yang *et al*., 2006; Sheehan *et al*., 2015). Since magnesium is found at such high concentrations in the bacterial cell, transport systems are presumably necessary within the macrophage environment. Two independent transporters, MgtB and MgtC, have been linked to *Brucella* survival in both *in vitro* and *in vivo* models of infection (Lestrate *et al*., 2000; Lavigne *et al*., 2005). For manganese transport, the H^+-dependent transporter MntH has been shown to be involved in both Mn^{2+} and Fe^{2+} in other bacteria (Makui *et al*., 2000). In *Brucella*, MntH is important for wild-type virulence in a mouse model of infection, and is essential to resist oxidative stress (Anderson *et al*., 2009).

Oxygen deprivation: Due to the oxygen-limiting conditions of the phagosomal compartment, *Brucella* must be able to metabolically change by altering its gene expression to successfully survive (Köhler *et al*., 2003; Roop *et al*., 2009). As mentioned previously, *Brucella* encodes two high-affinity cytochrome oxidases, which have been shown to increase in expression during limited oxygen availability. In addition to these components, a two-component system called NtrY/NtrX is also responsible for sensing

low levels of O_2 and causing transcription of denitrification genes (Carrica *et al*., 2013). As mentioned above, the denitrification genes are necessary for use of NO_3 as a terminal electron acceptor for respiration. In addition to NtrY/NtrX, another two-component system, PrrB/PrrA, is also involved in adaptation to anaerobic environments. PrrB/PrrA responds to the environmental redox state and, in turn, controls expression of regulator proteins involved in oxygen deprivation, including *ntrY* (Carrica *et al*., 2013). Without these systems in place, *Brucella* would be unable to survive these harsh conditions found in the host intracellular environment.

Importance

Although brucellosis has been termed a historical disease, the recent human brucellosis outbreaks along with the prevalence of animal brucellosis in our wildlife cannot be ignored. Instead, we must continue to study this pathogen and determine how it is able to cause disease in our animals and ourselves. Doing so will allow us to move toward developing improved therapeutics to combat the spread of brucellosis.

LysR-type transcriptional regulators (LTTRs)

General description

LysR-type transcriptional regulators (LTTRs) are the most common class of prokaryotic DNA-binding proteins, with functional orthologs present in archaea and eukaryotes (Maddocks and Oyston, 2008). The majority of LTTRs have been reported in the phylum of *Proteobacteria*, specifically found in the *Alphaproteobacteria* and *Gammaproteobacteria* (Schell, 1993). First described in 1988, this class was only comprised of 9 regulators (Henikoff *et al.*, 1988). This family was originally grouped together based on sequence similarity along with the presence of a DNA-binding domain (Maddocks and Oyston, 2008). LTTRs were first thought to be activators of a single gene that was divergently transcribed from itself. In addition, LTTRs were also found to exhibit negative autoregulation.

Since these initial reports and characterizations of LTTRs, the family has grown to over 800 members, with new regulators continually being discovered and characterized (Maddocks and Oyston, 2008). We now know that LTTRs function not only as activators of gene expression, but can also function as repressors. LTTRs can control many genes within a bacterial genome, and these regulated genes can be located proximal or distal to the LTTR gene. LTTRs are typically between 300-400 amino acids, with a conserved amino terminus helix-turn-helix (HTH) DNA-binding domain (DBD) and a variable carboxy terminus co-factor-binding domain. The genes regulated by LTTRs can vary greatly in what they encode and are involved in. Some examples of what LTTR-regulated genes are involved with include: metabolism (LysR: Stragier and Patte, 1983; ClcR: Coco *et al.*, *1993*; CatR: Chugani *et al.*, 1998; CatM: Ezezika *et al.*, 2006; PcaQ: MacLean *et al.*,

2007); nitrogen fixation (NodD: Schlaman *et al.*, 1992; LsrB: Luo *et al.*, 2005); oxidative stress (OxyR: Farr and Kogoma, 1991); virulence (OccR: Habeeb *et al.*, 1991; LrgB: Goldberg *et al.*, 1991; PhcA: Brumbley *et al.*, 1993; SpvR: Sheehan and Dorman, 1998; AphB: Kovacikova and Skorupski, 1999; MvfR: Cao *et al.*, 2001; RovM: Heroven *et al.*, 2007); and pilus/flagellan synthesis (CrgA: Deghmane *et al.*, 2000; LrhA: Lehnen *et al.*, 2002). An example of a LTTR activating the transcription of a gene in response to an environmental stimulus can be found in Figure 2.

LTTRs in prokaryotes

LysR: The family of LTTRs was named after the first characterized LTTR LysR, a transcriptional activator of the gene *lysA*. LysA is a diaminopimelate (DAP) decarboxylase involved in catalyzing the decarboxylation of DAP to lysine. In *E. coli* K12, the synthesis of DAP decarboxylase is induced by the presence of DAP (Boy *et al.*, 1979) and repressed by the presence of lysine (Patte *et al.*, 1962). Both of these amino acids are important in *E. coli*, where DAP is a component of the cell wall and lysine is a component in protein synthesis. As such, maintaining equilibrium of these two amino acids is critical for proper cellular functions.

Prior to 1983, the only mutation that was noted to lead to a Lys^- phenotype in *E. coli* was in the *lysA* gene. In 1983, Stragier and colleagues found that a mutation in the *lysR* gene encoded divergently to *lysA* lead to a Lys^- phenotype (Stragier *et al.*, 1983a). The authors concluded that the *lysR* gene encodes for a transcriptional activator of *lysA*, and LysR responds to the intracellular concentrations of DAP and lysine. Finally, LysR was also shown to autoregulate the expression of its own gene (Straiger *et al.*, *1983*).

Following the first ever description of this LTTR in *E. coli*, the identification of proteins categorized as LTTRs increased dramatically.

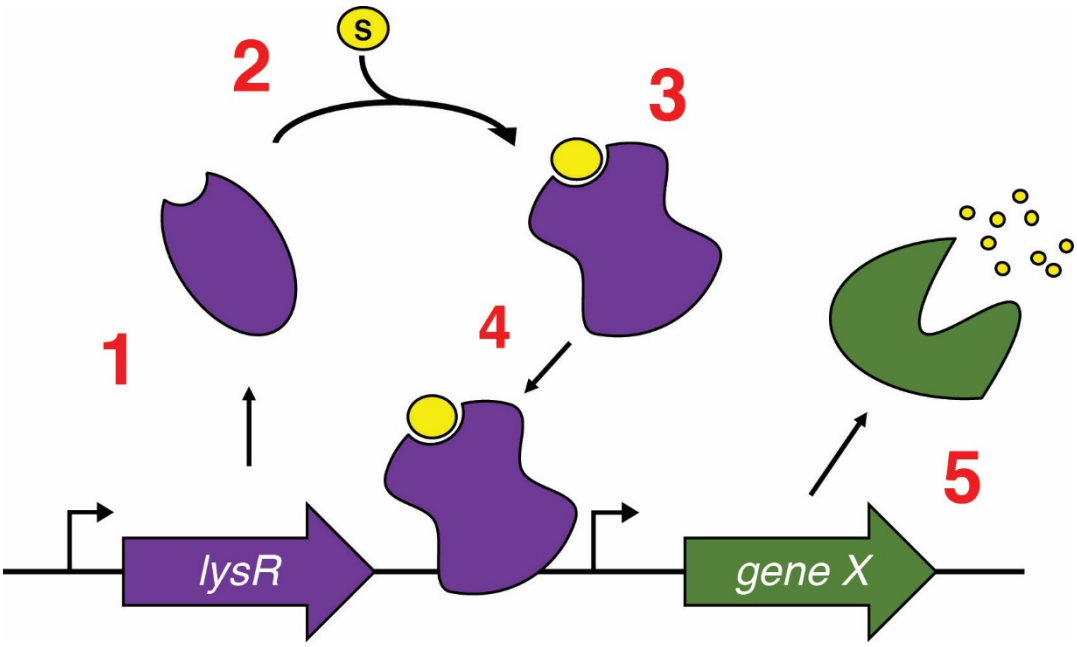

Figure 1.2. Example of the regulation by an LTTR
1. The *lysR* gene (purple) is transcribed and translated into an LTTR protein (represented in purple). Without a bound cofactor, the LTTR does not bind DNA.
2. The bacteria encounters a stress (represented by yellow circle) in its environment.
3. The LTTR binds to the cofactor produced by the stress and undergoes a conformation change.
4. The stress-induced LTTR binds to the promoter of *gene X* (represented in green) and activates its expression.
5. *gene X* encodes for a protein that is necessary for combating this certain stress.

NodD: The production of Nod-factors in rhizobia is critical to the ability of the soil bacteria to form a successful symbiotic relationship with legumes. Nod factors, encoded by *nod* genes, are responsible for initiating nodule formation to allow for uptake of the rhizobia by the host plant (Long, 2001). Induction of these *nod* genes requires the presence of the LysR transcriptional regulator, NodD. NodD has been shown to play a role in not only activating the expression of *nod* genes, but also responding to the molecules secreted by the host plant, known at flavonoids. Interestingly, although many plant-associated bacteria have this LysR regulator, NodD has evolved to control the response of the rhizobium to flavonoids in a species-specific manner (Horvath *et al.*, 1987). For example, in the plant symbiont *Sinorhizobium meliloti*, NodD1 has been shown to bind multiple types of flavonoids such as luteolin, naringenin, eriodictyol, and daidzein. However, expression of the *nod* genes only occurs when NodD1 associates with luteolin. It seems the other flavonoids act as competitive inhibitors of luteolin and bind NodD1, but are unable to initiate later steps of *nod* gene activation (Peck *et al.*, 2006). This ligand-specific nodulation activation makes sense, since each species of rhizobia establishes symbiosis with a specific set of host plants, which excrete a particular set of flavonoids to which the rhizobia can sense and respond to (Rolfe, 1988).

OxyR: The generation of toxic products during oxygen metabolism is common. As such, organisms must have systems in place to protect themselves from these potentially harmful components. As mentioned earlier, oxidative stress can be caused by exposure to ROS such as O_2^- and H_2O_2. The ability to respond to H_2O_2 is typically thought to be controlled by the LysR regulator OxyR. OxyR is responsible for regulating a plethora of

genes in response to H_2O_2 stress. Specifically, OxyR regulates catalases, alkyl hydroperoxide reductases and glutathione reductases (Storz *et al.*, 1990; Storz and Imlay, 1999). As with other LysR regulators, OxyR undergoes a conformational change when in the presence of H_2O_2. The two main forms of OxyR are its inactive, reduced form, and its active, oxidized form. Biochemical analysis revealed that OxyR is directly oxidized by H_2O_2 (Zheng *et al.*, 1998), which in turn induces gene expression. Oxidation of OxyR is by a disulfide intramolecular bond formation between cysteine residues. Following adequate response to H_2O_2, OxyR is brought back to its reduced, inactive form by glutaredoxin (one of the genes encoded in the *oxyR* regulon).

AphB: In *Vibrio cholera*, the expression of toxin-coregulated pilus (TCP) and cholera toxin (CT) is initiated at the *tcpPH* promoter located on the *Vibrio* pathogenicity island. The proteins responsible for activation of this operon are AphA, a quorum-sensing regulator, and AphB, a LysR-type transcriptional regulator (Skorupski and Taylor, 1999; Kovacikova and Skorupski, 1999). AphB was described as the transcriptional activator that functions with another protein, AphB, to co-activate *tcpPH* gene expression. To further elucidate the AphB regulon, microarray analysis revealed AphB regulating genes responsible for coping with acidic pH and oxygen tension (Kovacikova *et al.*, 2010). Crystallization of AphB revealed residues allowing AphB to sense these environmental conditions and, furthermore, turn on/off gene expression (Taylor *et al.*, 2012). Recently, AphB was shown to undergo a conformational change when *V. cholera* transitions from an aquatic, oxygen-rich environment to an intestinal, oxygen-limited environment (Liu *et al.*, 2011). In addition, AphB was found to work with the ROS resistant regulator, OhrR, to turn on/off expression of *tcpP* (Liu *et al.*, 2016).

CrgA: CrgA is a LysR family regulator found in the human pathogen *Neisseria meningitidis*. CrgA has been shown to be involved in adherence of the bacteria to host epithelial cells upon infection. A mutant of *crgA* was found to produce more capsule, which led to a decreased ability of the bacteria to adhere to cell surfaces (Deghmane *et al.*, 2002; Deghmane and Taha, 2003). In regards to regulation, there are several controversial reports of the genes CrgA activates and/or represses in *N. meningitidis*. Ieva and colleagues found CrgA to act as an activator of the gene *mdaB*, which encodes a NADPH-quinone oxidoreductase (Ieva *et al.*, 2005). In addition, contrary to earlier reports, CrgA was found to not be involved in regulating genes involved in pili and capsule synthesis. In contrast, it was shown how CrgA regulates these genes, specifically *pilE* and *lst* (lipooligosaccharide sialyltransferase) by performing real-time PCR and electrophoretic mobility shift assays (EMSAs) (Matthias and Rest, 2014). Clearly, further experiments must be done to definitively define the regulon of CrgA.

Of interest, CrgA has been reported to interacting with the protein, HPr, a component of the phosphotransferase system (PTS), in *N. meningitidis* (Derkauoi *et al.*, 2016), and identified binding of CrgA to promoter regions (P*crgA* and P*pilE*) was enhanced with addition of HPr. The authors speculated that the co-inducer domain of CrgA may be used for binding of HPr rather than a specific ligand (as is the case for many other LysR proteins). It will be interesting to see future experiments done to further characterize this novel protein-protein interaction.

LsrB: The LysR regulator LsrB found in *S. meliloti* 1021 has been shown to be necessary for the ability of the bacteria to form a symbiotic relationship with the host legume, alfalfa. A mutation in the *lsrB* gene results in the inability of *S. meliloti* to fix

nitrogen, resulting in poor alfalfa plant growth (Luo *et al.*, 2005). Recently, LsrB has been implemented in potentially regulating genes involved in protecting *S. meliloti* from ROS damage (Tang *et al.*, 2017). Moreover, *S. meliloti* LsrB contains three cysteines residues that have been shown to be involved in sensing oxidative stress, putatively by the formation of disulfide bonds (Tang *et al.*, 2017). LsrB has also been linked to regulating genes involved with lipopolysaccharide biosynthesis (Tang *et al.*, 2014).

Importance

Across prokaryotes, LTTRs are a highly distributed, functionally conserved class of transcriptional regulators that are involved in the regulation of gene expression, typically in response to an environmental signal. LTTRs serve as a critical component for bacterial adaptation, as these regulators are actively monitoring the environment and, subsequently, altering the organism's transcriptome to allow it to successfully survive and thrive. Due to their importance in bacteria, LTTRs are great systems to study, as several reports have recently pointed out that LTTRs can be targeted by therapeutics (MvfR: Starkey *et al.*, 2014; AphB: Mandal *et al.*, 2017).

Small RNAs (sRNAs)

General description

Regulation through the use of RNA has come to light as a powerful and energy-efficient way for cells to control gene expression in a timely manner. RNA-mediated regulation can act on the level of transcription, translation, and/or on the stability of a mRNA (Waters and Storz, 2009). RNA-mediated regulation in bacteria is comprised of four classes: 1) riboswitches, 2) protein-binding RNA, 3) mRNA-binding RNA, and 4) CRISPR RNA. In the following section, I will focus on and discuss in detail the class of mRNA-binding RNAs, also known as small regulatory RNAs (sRNAs).

sRNAs have been shown in myriad bacteria to play critical roles in a variety of processes, such as maintaining metal homeostasis (RhyB), coping with oxidative stress (OxyS), maintaining glucose homeostasis (Spot42, CyrA, SgrS), repressing quorum sensing (Qrr), and coping with outer membrane stress (MicF, RybB) (Storz *et al*., 2011). These regulatory transcripts are relatively small, typically between 50-300 nucleotides (Gottesman, 2005). sRNAs act by binding to target mRNAs through either perfect or imperfect complementary basepairing. Following binding, sRNAs are able to regulate the translation and/or stability of the target mRNA in a positive or negative manner. There are two types of sRNAs: cis-acting sRNAs, which are encoded on the opposite DNA strand as the target RNA they regulate; or trans-acting sRNAs, which are located elsewhere in the genome. Because cis-acting elements are encoded in the same location as the RNA they regulate, they have complete complementarity with their target. On the contrary, trans-acting sRNAs share limited complementarity to their target RNA(s). Trans-acting

sRNAs are a primary focus in the field of riboregulation, with a majority of characterized sRNAs being classified as trans-acting elements (Vanderpool *et al*., 2011).

As mentioned above, sRNAs can act through positive or negative regulation of target transcripts (Figure 3) (Waters and Storz, 2009). sRNAs can positively regulate translation by freeing the ribosome binding site (RBS) by alleviating secondary structures in the 5' untranslated region (5' UTR), or by binding to the 3' end of the transcript to stabilize it (Gottesman and Storz, 2011). Alternatively, sRNAs can negatively affect translation by binding to and blocking the RBS, targeting the mRNA for degradation by an RNase, or both. The majority of sRNAs are reported to have a negative effect on translation, and typically act by basepairing to the 5' UTR (Waters and Storz, 2009). However, several cases have been reported where sRNAs can either bind far upstream of the AUG start site, or far downstream into the coding region. sRNAs typically utilize between 10-25 nucleotides to achieve regulation of their target mRNAs, but it has been shown that only a small subset of these nucleotides is absolutely required for regulation. Because of this imperfect complementarity between sRNAs and mRNAs, a RNA chaperone named Hfq is almost always required for this interaction (De Lay *et al*., 2013).

Hfq was first described in 1968 in non-pathogenic *E. coli*, and was later found to play a critical role in bacterial physiology (Franze de Fernandez *et al.*, 1968; Tsui *et al.*, 1994; Robertson and Roop, 1999). Hfq forms a hexameric, donut-like structure that 'hugs' the sRNA-mRNA complex together. In addition to enabling this RNA-RNA interaction, Hfq is also involved in sRNA stability. Specifically, Hfq has been shown to protect sRNAs from degradation when the sRNA is not actively interacting with its target mRNA. However, Hfq has also been shown to recruit the endoribonuclease RNase E as well as other

components of the degradosome when the sRNA-mRNA complex has formed, which ultimately leads to RNA degradation. Hfq is widely conserved throughout prokaryotes and is extremely useful in identifying new sRNAs by way of co-immunoprecipitation (Vogel and Luisi, 2011).

Many bacterial sRNAs are expressed under specific growth conditions, as they become important for regulating transcripts that may or may not be needed at a certain time (Waters and Storz, 2009). For example, several sRNAs have been shown to be expressed under: low iron (RhyB: Massé and Gottesman, 2002), oxidative stress (OxyS: Altuvia *et al*., 1997), outer membrane stress (MicA: Udekwu *et al*., 2005; RybB: Papenfort *et al*., 2006), high glycine levels (GcvB: Urbanowski *et al*., 2000), changes to glucose concentrations (Spot42: Møller *et al*., 2002; CyaR: De Lay and Gottesman, 2009), and high glucose-phosphate levels (SgrS: Vanderpool and Gottesman, 2004). Another important point regarding expression of these sRNAs is their genomic locations. Many sRNAs can be found adjacent to the gene which encodes their transcriptional regulator (Gottesman and Storz, 2011). Moreover, many of these regulators are active under specific growth conditions. Some examples are: OxyR activating OxyS during oxidative stress; GcvA regulating GcvB during changes in cellular glycine levels; SgrR regulating SgrS during changes in cellular glucose-phosphate levels. Similar to how transcriptional regulators work, sRNAs are involved in the regulation of target mRNAs that in turn modulate a response to environmental changes. However, in contrast to transcriptional regulators, this regulation is post-transcriptional. So why do bacteria sometimes utilize sRNAs instead of transcriptional regulators if they both achieve the same goal?

There are several answers to this question. If a cell utilized a transcriptional regulator instead of a sRNA to regulate a target, this would be a much more energy intensive process. In contrast, if a sRNA is utilized, that sRNA must be transcribed and then directed to the correct mRNA sequence (Storz *et al*., 2011). Moreover, sRNAs are much quicker to produce (average of ~100 nucleotides) versus proteins (average of ~1000 nucleotides). The process of translating a piece of mRNA is also very energy taxing on the cell, requiring the ribosome, tRNAs, and a variety of protein factors. Finally, the regulation by sRNAs is quick, especially with cis-acting elements. For these reasons, sRNAs are considered the superior option for regulating gene expression in prokaryotes. In the following section I will switch over to discussing in detail three sRNAs, specifically focusing on ones that have multiple gene copies within a bacterial genome.

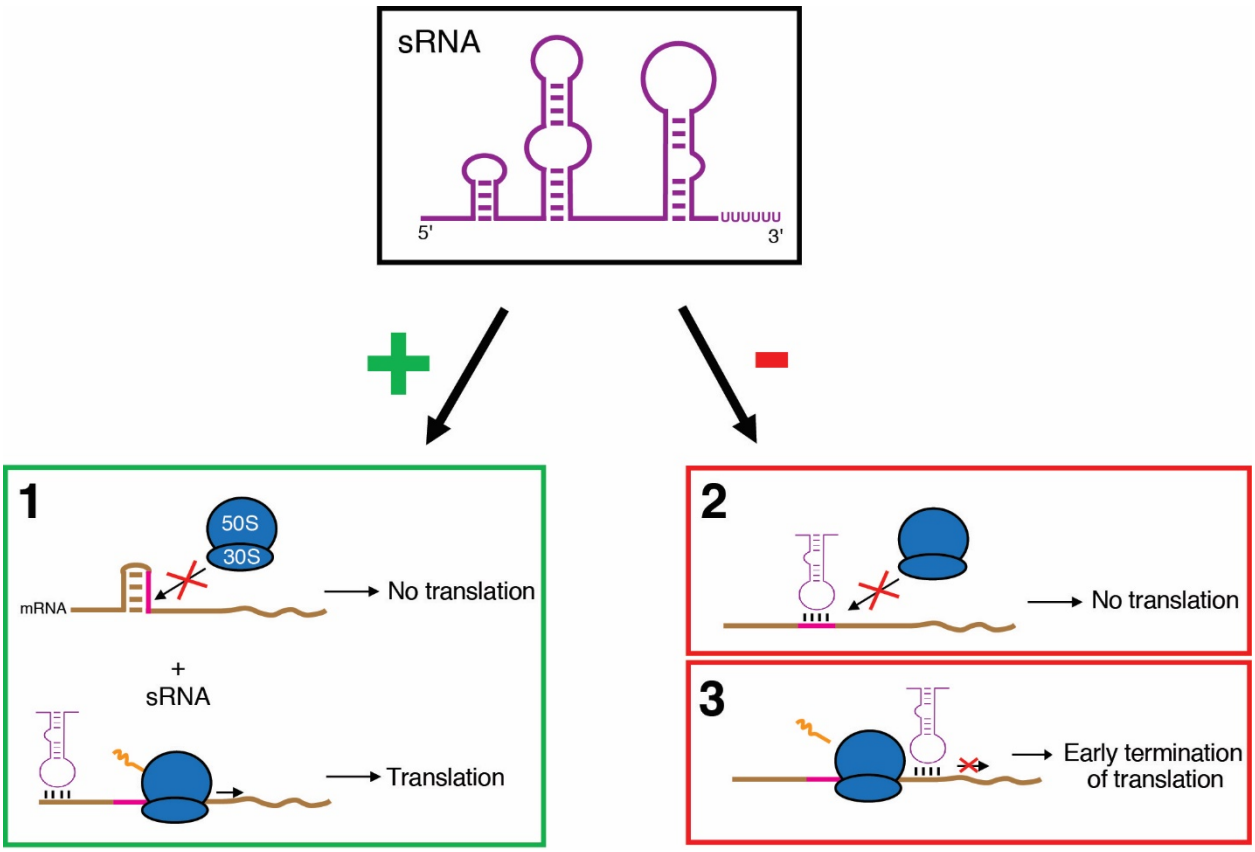

Figure 1.3. sRNA positive (left) and negative (right) regulation in prokaryotes
1. In some mRNAs (represented in brown), a secondary strucutre in the 5′ UTR, specifically occluding the Shine-Dalgarno (SD) sequence (represented in pink), can prevent ribosomal binding (represented in blue). A sRNA with complementarity to the sequence of the secondary structure can bind to and relax the mRNA, which allows for the SD sequence to be free and for the ribosome to bind and translate the mRNA.
2. For negative regulation, a sRNA can bind to the SD site in the 5′ UTR of a transcript and prevent ribosomal binding.
3. sRNAs can also result in early termination of translation by binding within the coding region of an mRNA, resulting in the ribosome coming to a halt.

Sibling sRNAs

The existence of multiple copies of the same sRNA is a phenomenon that cannot fully be explained, but there are several hypotheses as to why a bacterial cell would benefit from encoding these "sibling sRNAs" (Waters and Storz, 2009). First, these sibling sRNAs may act redundantly and act as a 'fail-safe' mechanism in the chance that one of the sRNAs become inactive, which may be the case for the AbcR sRNAs in *B. abortus* (Caswell *et al*., 2012b). Alternatively, this redundancy may be in place to increase the sensitivity of a cell to a certain response. Second, these sRNAs may act additively, as is the case with the Qrr sRNAs in *V. harveyi* (Tu and Bassler, 2007). Finally, these siblings may act independently of one another. Below are three examples of previously characterized sibling sRNAs and a description of the roles they have in their host bacterium.

NmsRs: *N. meningitidis* is a Gram-negative pathogen responsible for causing meningitis along with other forms of meningococcal disease. During infection, the bacterium is capable of moving throughout its host and, as such, must constantly adapt to new environments (e.g., changes in nutrient supplies) (Coureuil *et al*., 2013). To do this, the bacterium must constantly alter its metabolic profile and protein expression pattern to survive in specific areas within its host.

Two RNAs, named $NmsR_A$ and $NmsR_B$ (for *Neisseria* metabolic switch regulators) were recently identified in *N. meningitidis* strain H44/76 (Pannekoek *et al.*, 2017). The NmsR sRNAs share 70% sequence identity, are predicted to fold into similar secondary structures (3 hairpin structure), and are tandemly arranged in the genome. To assess what regulatory role they have in *N. meningitidis*, a double deletion of the *nmsR* sRNAs was made, and the double deletion strain along with wild type was subjected to mass

spectrophotometric analysis. This analysis found 18 proteins to be upregulated and 10 proteins to be downregulated in the mutant. Of interest, 10 of the 18 proteins that were upregulated were found to be involved in the TCA cycle (Pannekoek *et al.*, 2017). The authors went on to experimentally show through a gfp reporter system in *E. coli* that the NmsRs interact with the 5' UTR of several of the target mRNAs (Pannekoek *et al.*, 2017). Specifically, it is hypothesized that the NmsRs utilize a UC-rich region to bind targets, which is similar to how other sRNAs have been shown to interact.

In *N. meningitidis*, the TCA cycle is important when cells shift from a favorable, nutrient-rich environment to one that is unfavorable. This shift can occur as the bacterium traffics to different areas within the host such as the nasopharynx, blood, and cerebrospinal fluid (CF) (Pannekoek *et al.*, 2017). As such, being able to quickly sense and adapt nutrient availability is key to survival. The sibling NmsR sRNAs have been shown to be key regulatory modules in the ability of *N. meningitidis* to alternate between cataplerotic and anaplerotic metabolism (Pannekoek *et al.*, 2017). Further strengthening this claim was the overexpression experiments done with the NmsR sRNAs. Bacteria harboring NmsR overexpression plasmids were capable of growth in blood, but unable to grow in CF compared to wild type (Pannekoek *et al.*, 2017). This shows how the environment in which the bacteria reside can dictate the type of metabolism utilized by the organism. Overall, the *N. meningitidis* NsmRs are an example of redundant sRNAs which allow the bacteria to adapt to changes within the host environment.

LhrCs: *Listeria monocytogenes* is a Gram-positive foodborne pathogen responsible for causing the disease listeriosis. In *L. monocytogenes*, over 200 sRNAs have already been identified, which include the 7 homologous LhrC sRNAs (LhrC1-5,

Rli22, and Rli33-1) (Christiansen *et al*., 2006; Mollerup *et al.*, 2016). The LhrC family of sRNAs is the largest set of homologous sRNAs to date, and is an example of sRNAs that are hypothesized to have both redundant and independent functions. All 7 of these sRNAs are transcribed on independent promoters, have similar secondary structures, and contain between two to three CU-rich motifs.

All of the LhrC sRNAs share redundant regulatory functions. The LhrC sRNAs are responsible for negative regulating at least three mRNAs: LapB, which encodes for a virulence adhesion factor (Reis *et al.*, 2010); TcsA, which encodes for a CD4+ T-cell stimulating antigen (Sanderson *et al.*, 1995); and OppA, which encodes for an oligo-peptide binding protein (Borezee *et al.*, 2000). All three LhrC targets have been implemented in the pathogenesis of *L. monocytogenes*. In addition, 6 out of 7 of the LhrC sRNAs have been found to be involved in *L. monocytogenes* virulence, strongly suggesting that this sRNA system is essential to pathogenesis (Christiansen *et al.*, 2006; Mollerup *et al.*, 2016).

Although these sRNAs have been shown to share some redundant functions, it is likely that they also share independent activities. While 6 of the LhrC sRNAs are regulated by the two-component system LisRK in response to cell envelope stress, Rli33-1 is regulated by the general stress sigma factor σ^B (Mollerup *et al.*, 2016). Additionally, the LhrC sRNAs are differentially expressed when stressed with infection-relevant conditions. These data suggest that the LhrC sRNAs likely have some unidentified non-overlapping regulatory functions. Taken together, the family of LhrC sRNAs is an example of sRNAs sharing regulatory functions, as well as having yet to be discovered independent

regulatory profiles. In *L. monocytogenes*, the LhrCs are likely involved in avoidance of immune detection through the negative regulation of surface exposed proteins.

OmrA/OmrB: The trans-acting Omr sRNAs are conserved in almost all *Enterobacteriaceae*, and were first discovered in *E. coli* (Argaman *et al.*, 2001; Wassarman *et al.*, 2001). OmrA and OmrB, also referred to as SraE RNA, and RygA/RygB, were named following the discovery that they are activated by the response regulator OmpR (OmrA/B: OmpR-regulated sRNAs A and B) (Guillier and Gottesman, 2006). Unlike other sibling sRNAs, the Omr sRNAs share high nucleotide similarity in their 5' and 3' ends, but display unique sequences in their central regions. This could be taken as the Omr sRNAs having both redundant and unique regulatory functions, similar to what was described for the LhrC sRNAs in *L. monocytogenes*. Also in common with the LhrC sRNAs is OmrA and OmrB having differences in their expression profiles (Argaman *et al.*, 2001; Vogel *et al.*, 2003). Nevertheless, OmrA and OmrB have been shown to negatively regulate the same genes, specifically ones that encode outer membrane proteins. The Omr sRNAs bind to the 5' UTR of targets, in close proximity to the RBS (Guillier and Gottesman, 2006; Guillier and Gottesman, 2008). OmrA and OmrB have also been shown to participate in an autoregulatory feedback loop, where the sRNAs indirectly limit their own expression by regulating the OmpR mRNA. In addition to regulating components of the outer membrane, the Omr sRNAs are also involved in regulating at least one transcript, named CsgD, which is involved in bacterial adhesion and biofilm formation (Holmqvist *et al.*, 2010).

AbcR1/AbcR2: The AbcR sRNAs are another example of sibling sRNAs, and are conserved in the *Rhizobiales* (Wilms *et al*., 2011; Caswell *et al*., 2012b; Torres-Quesada

et al., 2013; Sheehan and Caswell, 2018). The AbcRs have been shown to be involved in regulation of ABC-type transport systems, and, therefore, are considered important for proper nutrient acquisition. The AbcR sRNAs and their evolutionary conservation are discussed in detail in Chapter 6.

Importance

As mentioned previously, RNA regulators provide bacteria a quick means to alter their transcriptome when faced with environmental changes that challenge their survival. The importance of sRNAs in the lifestyle of a bacterium has come to light within the last 20 years, but there is no doubt that much more remains to be discovered about this regulatory system. Regulatory RNAs are being investigated for their potential to be used as biosensors, for controlling bacterial growth, and, most importantly, as drug targets (Waters and Storz, 2009; Blount and Breaker, 2006). For example, riboswitches provide an exciting means as putative drug targets, as small synthetic molecules can be generated that fit in the binding pocket of the riboswitch, thus allowing us to control bacterial gene expression.

Concluding remarks

The regulation of and regulation by sRNAs is largely unexplored in the mammalian pathogen *B. abortus*. In the following chapters, I will describe in detail a sRNA/LysR-type regulatory system, termed the AbcR/VtlR system, which is conserved in the *Rhizobiales*. Specifically, Chapters 2 and 3 will discuss the transcriptional regulation of these sRNAs and the mechanism utilized by the AbcR sRNAs to exert their negative regulation, respectively. Chapter 4 is a comprehensive review on the AbcR system in the *Rhizobiales*. Finally, Chapter 5 details the identification of the transcriptional regulator of one of the AbcR sRNAs in the plant pathogen *A. tumefaciens*. In addition, Chapter 5 further characterizes this transcriptional regulator, named VtlR, and the importance it has in bacterial pathogenesis.

Chapter 2. A LysR-family transcriptional regulator required for virulence in *Brucella abortus* is highly conserved among the *Alphaproteobacteria*

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Abstract

Small RNAs are principal elements of bacterial gene regulation and physiology. Two small RNAs in *Brucella abortus*, AbcR1 and AbcR2, are required for wild-type virulence. Examination of the *abcR* loci revealed the presence of a gene encoding a LysR-type transcriptional regulator flanking *abcR2* on chromosome 1. Deletion of this *lysR* gene (*bab1_1517*) resulted in the complete loss of *abcR2* expression while no difference in *abcR1* expression was observed. The *B. abortus bab1_1517* mutant strain was significantly attenuated in macrophages and mice, and *bab1_1517* was subsequently named *vtlR* for virulence-associated transcriptional LysR-family regulator. Microarray analysis revealed three additional genes encoding small hypothetical proteins also under the control of VtlR. Electrophoretic mobility shift assays demonstrated that VtlR binds directly to the promoter regions of *abcR2* and the three hypothetical protein-encoding genes, and DNase I footprint analysis identified the specific nucleotide sequence in these promoters that VtlR binds to and drives gene expression. Strikingly, orthologs of VtlR are encoded in a wide range of host-associated α-proteobacteria, and it is likely that the VtlR genetic system represents a common regulatory circuit critical for host–bacterium interactions.

Introduction

Brucella spp. are Gram-negative α2-proteobacteria that are facultative intracellular pathogens capable of residing within both professional and non-professional phagocytic cells (Köhler *et al*., 2003; Roop *et al*., 2004). The brucellae can infect a variety of wild and domesticated animals, leading to abortions and sterility, and furthermore, *Brucella* infections in humans result in a debilitating condition characterized most commonly by a chronic relapsing fever (Corbel, 1997; Pappas *et al*., 2006). Owing to the zoonotic nature of *Brucella*, humans often acquire this infection by consumption of unpasteurized dairy products, direct contact with mucosal secretions from an infected animal, or by occupational exposure (i.e., veterinarians and laboratory workers) (Pappas *et al*., 2005). Currently, brucellosis remains one of the most widespread and economically devastating zoonotic diseases worldwide with an estimated 500,000 human cases per year (Pappas *et al*., 2006; Seleem *et al*., 2010).

Upon entering the host, the brucellae are engulfed by cells of the reticuloendothelial system, particularly macrophages, and following trafficking of the bacteria through the host cell, the brucellae ultimately reside in an intracellular vacuole called the replicative *Brucella*-containing vacuole, which is associated with the endoplasmic reticulum (Arenas *et al*., 2000; Celli, 2006). While trafficking through the macrophage, the bacteria encounter many potentially harmful conditions, including exposure to reactive oxygen and nitrogen species, destructive enzymes, low pH, nutrient deprivation and diminished oxygen levels (Aderem, 2003; Roop *et al*., 2009). To combat the harsh intracellular environment of the macrophage, the brucellae encode an arsenal of strategies, including the VirB type IV secretion system (O'Callaghan *et al*., 1999;

Lacerda *et al*., 2013), as well as other stress response mechanisms (Roop *et al*., 2009). Nonetheless, much remains to be elucidated about the regulatory components controlling gene expression during the intracellular life of *Brucella*.

LysR-type transcriptional regulators (LTTRs) are the most common type of prokaryotic DNA-binding protein, and these regulatory proteins can function as either activators or repressors of gene expression (Schell, 1993). As a family, LTTRs are highly conserved in protein structure, and these regulators are composed of an N-terminal helix–turn–helix DNA-binding domain and a C-terminal co-inducer binding domain (Maddocks and Oyston, 2008). Given the broad conservation of LTTRs among diverse groups of bacteria, it is not surprising that these regulators control expression of a wide variety of genes, including those involved in functions related to virulence (Russell *et al*., 2004; Doty *et al*., 1993), quorum sensing (O'Grady *et al*., 2011), motility (Heroven and Dersch, 2006) and metabolism (Hartmann *et al*., 2013). *Brucella* spp. encode several LysR proteins, few of which have been linked to virulence and, in general, very little is known about the role of LysR regulators in the brucellae (Haine *et al*., 2005).

Another prominent and widely distributed class of bacterial genetic regulators is small regulatory RNAs (sRNAs). sRNAs are comparatively short (i.e., ~50–300 nucleotides) regulatory RNA molecules that often control gene expression at the post-transcriptional level by binding directly to target mRNAs to alter their structure, stability and/or access to the ribosome-binding site. Depending on the nature of the sRNA–mRNA interaction, sRNAs may exert positive or negative effects on the expression of a given gene (Waters and Storz, 2009). Not surprisingly, bacterial sRNAs regulate myriad cellular processes, including stress response, metabolism, quorum sensing and virulence

(Bejerano-Sagie and Xavier, 2007; Hoe *et al*., 2013; Papenfort and Vogel, 2014; Murphy and Payne, 2007). The brucellae encode two highly related sRNAs called AbcR1 and AbcR2, and these sRNAs are required for wild-type virulence of *Brucella* in macrophages and mice (Caswell *et al*., 2012b). However, to date, nothing is known about the mechanisms controlling *abcR* expression in *Brucella*.

The present study describes a LTTR in *B. abortus* that activates the expression *abcR2*, but not *abcR1*, and more importantly, this regulator is essential for the capacity of the bacteria to successfully infect macrophages and mice. In addition, this LysR regulator, named VtlR for virulence-associated transcriptional LysR-family regulator, also directly activates the expression of three genes putatively encoding small hypothetical proteins of ~50 amino acids in length. Most striking is the fact that numerous host-associated α-proteobacteria appear to encode a protein orthologous to VtlR, as well as cognate orthologous small hypothetical proteins. The VtlR system may represent a common genetic regulatory mechanism required for efficient host–bacterium interactions among the α-proteobacteria.

Results

A LysR-type transcriptional regulator, VtlR, controls expression of the small RNA-encoding gene, *abcR2* in *Brucella abortus*

Two highly similar small RNAs, AbcR1 and AbcR2, are essential for the wild-type virulence of *B. abortus* (Caswell *et al*., 2012b), but to date, there is no information regarding the regulation of *abcR1* and *abcR2* expression. Flanking the *abcR2* gene in *B. abortus* 2308 is a gene designated *vtlR* (*bab1_1517*) predicted to encode a LysR-type transcriptional regulatory protein of approximately 33 kDa in size (Fig. 1A). Because LysR regulators are often encoded in close proximity to the genes they regulate (Schell, 1993), it was hypothesized that VtlR controls expression of one or both of the *abcR* genes. To test this hypothesis, an in-frame, unmarked gene deletion of *vtlR* was constructed, and the expression of the AbcR sRNAs was assessed (Fig. 1B). Northern blot analyses demonstrated that AbcR2 production was abolished by deletion of *vtlR*; however, there was no effect on the levels of AbcR1 expression in the Δ*vtlR* mutant strain compared with the parental strain 2308. As a control for RNA production in the strains tested, there were no differences observed for the production of the *Brucella* sRNA Bsr6. Taken together, these data demonstrate that VtlR is an activator of *abcR2* expression in *B. abortus* 2308.

VtlR is essential for the wild-type virulence of *B. abortus* 2308

To study the role of VtlR in the pathogenesis of *Brucella*, a series of *in vitro* and *in vivo* experiments were performed with the Δ*vtlR* mutant strain compared with the parental strain 2308 (Fig. 2). Primary murine macrophages were infected with the parental strain 2308 and the *B. abortus* Δ*vtlR* strain (Fig. 2A). The Δ*vtlR* mutant exhibited a reduced

ability to survive and replicate within macrophages compared with the parental strain 2308. The *B. abortus* Δ*vtlR* mutant strain was also assessed by its ability to establish a chronic infection in a mouse model (Fig. 2B). Female BALB/c mice were infected via the intraperitoneal route with either the Δ*vtlR* mutant strain or the parental strain 2308. The Δ*vtlR* mutant strain was significantly attenuated compared with the parental strain 2308. Furthermore, reconstruction of the Δ*vtlR* locus (*vtlR*/Comp) restored spleen colonization to levels similar to the parental strain 2308 (Fig. 2C). To assess the potential pleiotropic effect of deleting *vtlR* in *B. abortus*, *in vitro* growth of the parental strain 2308 and the Δ*vtlR* strain in both nutrient-rich (brucella broth) or nutrient-deficient Gerhardt's minimal medium (GMM) medium was assessed. No differences were observed between the strains tested in either growth conditions. Overall, these results demonstrate that VtlR is required for the full virulence of *B. abortus* 2308 *in vitro* as well as *in vivo*.

Microarray analysis reveals the VtlR regulon

Although VtlR plays a critical role in the virulence of *Brucella*, the absence of *abcR2* expression does not fully explain the attenuation of the Δ*vtlR* mutant strain. Caswell *et al*., demonstrated that AbcR1 and AbcR2 are functionally redundant sRNAs in *Brucella*, as attenuation of the brucellae was only observed when both *abcR1* and *abcR2* were deleted, but not when either *abcR1* or *abcR2* were individually deleted (Caswell *et al.*, 2012b). Therefore, we hypothesized that VtlR must regulate the expression of other genes required for survival and replication of the bacteria in the host. To determine the full genetic regulon of VtlR in *B. abortus*, microarray analysis was employed using RNA isolated from the parental strain 2308 and the *vtlR* mutant strain grown in rich medium

(Table 1; Geo Accession GSE69564). Eight genes, including *bab1_0310*, *bab1_0313*, *bab1_0314* and *bab1_1794*, which are known targets of the AbcR sRNAs, were shown to be significantly elevated in the Δ*vtlR* mutant strain. Moreover, three genes encoding putative small hypothetical proteins, *bab1_0914*, *bab2_0512* and *bab2_0574*, exhibited diminished levels in the Δ*vtlR* mutant strain compared with the parental strain 2308.

To confirm the regulation of these hypothetical protein-encoding genes by VtlR, northern blot analyses were performed with RNA isolated from the parental strain 2308, the Δ*vtlR* mutant strain and the reconstructed *vtlR* mutant strain (*vtlR*/Comp) (Fig. 3). The *vtlR* mutant exhibited no production of the BAB1_0914, BAB2_0512, BAB2_0574 or AbcR2 RNAs. Importantly, AbcR1 production was maintained in the Δ*vtlR* mutant strain compared with strain 2308. Complementation of *vtlR* expression in the Δ*vtlR* mutant strain restored production of AbcR2, BAB1_0914, BAB2_0512 and BAB2_0574 RNAs. These data confirm that VtlR activates the expression of these genes in *B. abortus* 2308.

VtlR binds directly to the promoter regions of *abcR2*, *bab1_0914*, *bab2_0512* and *bab2_0574*

Electrophoretic mobility shift assays (EMSAs) were employed to assess the direct regulation of *abcR2*, *bab1_0914*, *bab2_0512* and *bab2_0574* by VtlR in *B. abortus* 2308 (Fig. 4). First, recombinantly purified *B. abortus* VtlR (rVtlR) was incubated with and subsequently bound to radiolabeled DNA corresponding to the promoter region of *abcR2* in a concentration-dependent manner (Fig. 4A). Importantly, specific, unlabeled DNA (i.e., *abcR2* promoter region DNA) abolished binding between rVtlR and the labeled *abcR2* promoter DNA, but non-specific, unlabeled DNA (i.e., *abcR1* promoter region DNA) did

not affect interactions between rVtlR for the labeled *abcR2* promoter. Subsequent EMSA experiments demonstrated that, in addition to the *abcR2* promoter, VtlR also binds directly to the promoter regions of *bab1_0914*, *bab2_0512* and *bab2_0574*, but not to the *abcR1* promoter (Fig. 4B). Overall, these experiments confirm the *B. abortus* VtlR protein interacts directly with the promoters of *abcR2*, *bab1_0914*, *bab2_0512* and *bab2_0574* to activate gene transcription.

DNase I footprinting was employed to determine the specific DNA motif within the *abcR2* promoter region that is bound by VtlR in *B. abortus* (Fig. 5). An approximately 200-bp 6-Fluorescein amidite (6FAM)-labeled DNA fragment corresponding to the promoter region of *abcR2* was incubated with or without rVtlR, and the complexes were treated with DNase. Following DNA fragment analysis by capillary electrophoresis, it was determined that a 35-bp region in the *abcR2* promoter was protected from DNase digestion by VtlR (Fig. 5A). DNA sequencing analyses determined the sequence of the protected region to be CAATGCAGCCATGCATTTTACGCATGACTGCATTG. This sequence is composed of an inverted repeat of CAATGCAGCCATGCA separated by an AT-rich region, suggesting that VtlR may bind as a dimer to the *abcR2* promoter. Moreover, the CAATGCAGCCATGCA motif is only found in the promoter regions of *abcR2*, *bab1_0914*, *bab2_0512* and *bab2_0574* and not in any other location of the *B. abortus* 2308 chromosomes (Fig. 5B). Taken together, these data demonstrate the specific nucleotide sequence utilized by VtlR to activate gene expression in *B. abortus* 2308.

Deletion of four VtlR-regulated genes does not lead to attenuation of *B. abortus* in macrophages

As demonstrated in Fig. 2, a *B. abortus vtlR* mutant exhibits a decreased ability to survive and replicate within macrophages compared with the parental strain 2308 (Fig. 2A). To begin to elucidate the role of the VtlR-regulated genes (i.e., *abcR2*, *bab1_0914*, *bab2_0512*, *bab2_0574*) in *B. abortus* virulence, isogenic mutant strains, as well as a strain with all four VtlR-regulated genes deleted, were constructed. Primary naïve macrophages were isolated from BALB/c mice and infected with either *B. abortus* 2308, Δ*vtlR*, Δ*vltR* with a reconstructed locus (*vtlR*/Comp), Δ*abcR2*, Δ*bab1_0914*, Δ*bab2_0512*, Δ*bab2_0574* or Δ*abcR2*/*bab1_0914*/*bab2_0512*/*bab2_0574* (Δ*quad*). As expected, the Δ*vtlR* mutant strain was attenuated compared with strain 2308, and reconstruction of the *vtlR* locus in the mutant restored intracellular levels of brucellae to those of the parental strain (Fig. 6). However, *B. abortus* strains with individual deletions of *abcR2*, *bab1_0914*, *bab2_0512* or *bab2_0574* did not exhibit decreased capacities to survive and replicate in macrophages, and likewise, a strain with all four of the genes deleted was not attenuated in this model.

Extensive conservation of VtlR in the α-proteobacteria

Putative orthologs of VtlR are present in multiple α-proteobacteria, including the soil-dwelling bacterium *Ochrobactrium anthropi*, the human pathogen *Bartonella henselae*, the plant symbiont *Sinorhizobium meliloti*, and the plant pathogen *Agrobacterium tumefaciens*. Although LTTRs are the most common prokaryotic transcriptional regulators (Schell, 1993; Maddocks and Oyston, 2008), VtlR orthologs found in α-proteobacteria

exhibit a strikingly high degree of amino acid identity and similarity, suggesting a common function across this group of bacteria. Furthermore, orthologs of VtlR have been demonstrated to play an important role in host–bacterium interactions in other α-proteobacteria. For instance, in *S. meliloti* 1021, the ortholog of VtlR, designated LsrB, is critical for the successful symbiosis between *S. meliloti* and alfalfa plants (Luo *et al.*, 2005). An *S. meliloti* Δ*lsrB* mutant is deficient in both establishing an effective symbiosis and fixing nitrogen compared to the parental strain 1021. Altogether, these data suggest that the VtlR regulatory system is paramount for the host–bacterium relationship of α-proteobacteria and their respective hosts.

Discussion

The VtlR protein (BAB1_1517) is required for *B. abortus* virulence (Fig. 2), and the link between *Brucella* virulence and VtlR has been observed previously in *B. suis* (BR1498; Foulongne *et al.*, 2000) and in *B. melitensis* (BMEI0513; Wu *et al.*, 2006). However, although these previous studies reported observations of attenuation of strains generated by transposon mutagenesis, the present work defines the role of VtlR in *Brucella* pathogenesis. Moreover, this study sheds light on the molecular basis for the role of VtlR in *Brucella* virulence. Specifically, VtlR directly activates the expression of *abcR2*, encoding an important small regulatory RNA, as well as three genes encoding novel small hypothetical proteins (Figs 1, 3–5, and Table 1; Geo Accession GSE69564). Although VtlR was also linked to the expression of other genes (Table 1; Geo Accession GSE69564), the promoter regions of these genes do not contain the putative VtlR-binding motif CAATGCAGCCATGCA and thus, we conclude that these genes are indirectly regulated by VtlR. For example, several of the genes exhibiting increased expression in the *B. abortus* Δ*vtlR* mutant strain (i.e., *bab1_0310*, *bab1_0313*, *bab1_0314* and *bab1_1794*) are AbcR sRNA target genes, and expression of these genes is known to be elevated when the AbcR sRNAs are deleted (Caswell *et al.*, 2012b). In the same vein, the genes *bab1_0355*, *bab1_0356*, *bab2_0348* and *bab2_0961* are likely indirectly regulated by VtlR, but presently, the link between VtlR and the expression of these genes remains to be elucidated.

The attenuation of the *B. abortus* Δ*vtlR* mutant is striking (Fig. 2), but given the fact that both *abcR1* and *abcR2* are required for wild-type virulence of *B. abortus* 2308 (Caswell *et al.*, 2012b), the absence of *abcR2* in this mutant strain cannot fully account

for the virulence defect exhibited by the *B. abortus vtlR* mutant. Interestingly, the VtlR-regulated genes *bab1_0914*, *bab2_0512* and *bab2_0574* also do not appear to play a role in the wild-type survival and replication of *B. abortus* in murine macrophages (Fig. 6). Additional work aimed at dissecting the full VtlR regulon (i.e., both directly and indirectly regulated genes), as well as animal model experiments, are needed to fully elucidate the biological role(s) of *bab1_0914*, *bab2_0512* and *bab2_0574* in *B. abortus*. Interestingly, genes encoding other putative small proteins in *Brucella* have been linked to the virulence of *Brucella* strains (Köhler *et al.*, 2002; Lavigne *et al.*, 2005; Sun *et al.*, 2012).

To date, bioinformatics approaches have failed to provide insight into the biological function(s) of BAB1_0914, BAB2_0512 or BAB2_0574 in *Brucella*, but ongoing work in our laboratory is aimed at defining the role of these hypothetical proteins in the biology of *Brucella* (Budnick, unpublished data). Nonetheless, the putative proteins encoded by *bab1_0914*, *bab2_0512* and *bab2_0574*, as well as the regulator VtlR, are all highly conserved among the α-proteobacteria. Although it is currently unknown if these genes encode authentic small proteins in *Brucella*, we have shown that the RNAs encoded by *bab1_0914*, *bab2_0512* and *bab2_0574* are stably produced in a *B. abortus hfq* mutant, indicating that these RNAs are not prototypical small regulatory RNAs and likely encode proteins. Our laboratory is currently interrogating *B. abortus* 2308 to determine if BAB1_0914, BAB2_0512 and/or BAB2_0574 are bona fide proteins produced in this strain.

VtlR orthologs are encoded flanking the *abcR* genes of *A. tumefaciens* (Wilms *et al.*, 2011) and *S. meliloti* (Torres-Quesada *et al.*, 2013). Unlike the situation in the brucellae, the *abcR* genes in *A. tumefaciens* and *S. meliloti* are encoded in tandem, and

thus, it is likely that the VtlR orthologs in these bacteria control the expression of one or both of the *abcR* genes in these bacteria. In fact, a sequence highly similar to the CAATGCAGCCATGCA VtlR-binding motif of the *Brucella abcR2* promoter is present in the promoter region of the *abcR1* in *S. meliloti*. Orthologs of the other three genes directly regulated by VtlR in *B. abortus* (i.e., *bab1_0914*, *bab2_0512* and *bab2_0574*) are also highly conserved in the α-proteobacteria, and the promoter regions of these orthologs also contain a putative VtlR-like binding site. Therefore, it is likely that the VtlR system activates the expression of the *abcR* genes, as well as three hypothetical protein-encoding genes in other members of the α-proteobacteria.

The conservation of VtlR orthologs is high in the α-proteobacteria. While we propose that the VtlR systems are important in each of their respective bacteria for interactions in their specific environment, there is evidence of evolutionary adaptation of each unique protein to each niche. The VtlR ortholog in *S. meliloti* has been identified and characterized, and this protein was designated LsrB for LysR-type symbiosis regulator (Luo *et al.*, 2005). Interestingly, *lsrB* is required for efficient symbiosis of *S. meliloti* with alfalfa plants, and it was one of two LTTRs (from a total of 90 screened) necessary for symbiosis and nitrogen fixation. Heterologous complementation of the *B. abortus* Δ*vtlR* mutant strain with the *lsrB* gene from *S. meliloti* was unable to restore the capacity of the Δ*vtlR* mutant to colonize mice at wild-type levels (data not shown), which alludes to the fact that these LTTRs having evolved over time to suit their specific environment and host, more likely by altering the C-terminal co-inducer binding domain. More recently, LsrB was shown to play a role in the LPS biosynthesis in *S. meliloti* (Tang *et al.*, 2014). To date, there is no evidence linking VtlR to LPS biosynthesis in *Brucella*.

To summarize, the findings in this paper reveal VtlR as the LTTR activator of the sRNA-encoding gene *abcR2*, as well as three additional genes, *bab1_0914*, *bab2_0512* and *bab2_0574*, encoding small hypothetical proteins. VtlR was also found to play a critical role in the survival of *B. abortus* in both macrophage and mouse models of infection. The conservation of VtlR, along with the three hypothetical proteins, in α-proteobacteria seems to be a common genetic system necessary for the interaction of the bacteria with its host, regardless of whether this interaction is pathogenic or symbiotic, and thus, the VtlR system is likely a common genetic determinant of host–bacterium interactions in the α-proteobacteria.

Materials and Methods

Bacterial strains and growth conditions

Brucella abortus 2308 and derivative strains were routinely grown on Schaedler agar (BD, Franklin Lakes, NJ, USA) containing 5% defibrinated bovine blood (Quad Five, Ryegate, MT, USA) (SBA), or in brucella broth (BD). For cloning and recombinant protein expression, *Escherichia coli* strains (DH5a and BL21) were grown routinely on tryptic soy agar (BD) or in Luria–Bertani (LB) broth. When appropriate, growth media were supplemented with ampicillin (100 μg ml^{-1}) or kanamycin (45 μg ml^{-1}). All cultures of and experiments using *Brucella* strains were carried out in a Biosafety Level-3 (BSL-3) laboratory.

Construction of *B. abortus* mutants and genetic complementation

The *vtlR* gene (*bab1_1517*) was mutated using an unmarked gene excision strategy described previously (Caswell *et al.*, 2012a). An approximately 1-kb fragment of the upstream region of the gene was amplified by PCR using the primers BAB1_1517-Up-For and BAB1_1517-Up-Rev, genomic DNA from *B. abortus* 2308 as a template, and Platinum® Pfx DNA Polymerase (Invitrogen, Carlsbad, CA, USA). Likewise, an approximately 1-kb fragment of the downstream region of the gene was amplified by PCR using the primers BAB1_1517-Down-For and BAB1_1517-Down-Rev. The upstream fragment was digested with the restriction enzyme BamHI, whereas the downstream fragment was digested with PstI, and both fragments were treated with polynucleotide kinase (Monserate Biotechnology Group, San Diego, CA, USA). The fragments were combined in a single ligation mix with BamHI/PstI-digested pNPTS138 (Spratt *et al.*,

1986), which has a kanamycin resistance marker and *sacB* gene for counter-selection with sucrose, and T4 DNA ligase (Monserate Biotechnology Group). The resulting plasmid, pC^{3}038, was introduced into *B. abortus* 2308, and a merodiploid clone was obtained by selection on SBA+kanamycin. Kanamycin-resistant transformants were grown for 6–8 hours in brucella broth and then plated onto SBA plates containing 10% sucrose. Genomic DNA from sucrose-resistant, kanamycin-sensitive colonies was isolated and screened by PCR for loss of the *vtlR* gene. The isogenic *vtlR* mutant derived from *B. abortus* 2308 was named CC126.

Plasmids for isogenic mutagenesis of Δ*bab1_0914*, Δ*bab2_0512* and Δ*bab2_0574* were constructed as described above. The quadruple mutant of *abcR2*, *bab1_0914*, *bab2_0512* and *bab2_0574* was constructed by sequential mutagenesis of *B. abortus* 2308 using the constructed plasmids used for generation of the isogenic mutant strains. Reconstruction of the *vtlR* loci was done using the *sacB* counter-selection strategy described above but with modification. The wild-type *vtlR* locus was amplified using primers BAB1_1517-Up-For and BAB1_1517-Down-Rev, genomic DNA from *B. abortus* 2308 as a template, and Taq polymerase (Monserate Biotechnology Group). The approximately 3-kb fragment was digested with BamHI and PstI and ligated into BamHI/PstI-digested pNPTS138. The resulting plasmid, pLS003, was introduced into *B. abortus* CC126 (*vtlR* mutant), and selection and screening were carried out as described above. The strain carrying the reconstructed *vtlR* locus was named LS002.

Northern blot analysis

RNA was isolated from *Brucella* cultures as described previously (Caswell *et al.*, 2012a). Ten micrograms of RNA was separated on a denaturing 10% polyacrylamide gel containing 7 M urea and 1 × TBE (89 Tris-base, 89 mM boric acid and 2 mM EDTA). A low-molecular-weight DNA ladder (New England BioLabs, Ipswich, MA, USA) was labeled with [γ-^{32}P]-ATP (Perkin-Elmer, San Jose, CA, USA) and polynucleotide kinase. This radiolabeled ladder was also separated on the polyacrylamide gel. Following electrophoresis in 1 × TBE buffer, the ladder and RNA samples were transferred to an Amersham Hybond^{TM}-N^{+} membrane (GE Healthcare, Piscataway, NJ, USA) by electroblotting in 1 × TBE buffer. The samples were UV-cross-linked to the membrane and the membrane was pre-hybridized in ULTRAhyb®-Oligo Buffer (Ambion, Austin, TX, USA) for 2 hours at ~45°C in a rotating hybridization oven. The oligonucleotide probes [AbcR-Northern (for detection of both AbcR1 and AbcR2), 0914-Northern, 0512-Northern, 0574-Northern, and Bsr6-Northern] were end-labeled with [γ-^{32}P]-ATP and polynucleotide kinase. The radiolabeled probes were incubated with the prehybridized membranes at ~45°C in a rotating hybridization oven overnight (~12 hours). The next day, membranes were washed four times for 30 minutes each with 2 × SSC (300 mM sodium chloride and 30 mM sodium citrate), 1 × SSC, 0.5 × SSC and 0.025 × SSC at ~45°C in a rotating hybridization oven. Each SSC wash buffers contained 0.1% sodium dodecyl sulphate (SDS). The membranes were then exposed to X-ray film and visualized by autoradiography.

Microarray analysis

RNA was isolated from *Brucella* cultures grown to late exponential phase in brucella broth (Caswell *et al.*, 2012a), and contaminating genomic DNA was removed by treatment with RNase-free DNase I. Ten micrograms of each RNA sample, *B. abortus* 2308 and *B. abortus vtlR* mutant, were reverse transcribed, fragmented and 3′ biotinylated 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, USA). Signal intensities were normalized to the median signal intensity value for each GeneChip, averaging and analyzed with GeneSpring Software X. RNA species exhibiting ≥ 2-fold change in expression, as determined by Affymetrix algorithms to be statistically differentially expressed (*t*-test; $P < 0.05$), between *B. abortus* 2308 and the *vtlR* mutant strain were stated. The microarrays used in this study were developed based on *B. melitensis* biovar *abortus* 2308 and all *B. abortus* GenBank entries that were available at the time of design. In total, predicted open reading frames and intergenic regions were represented on PMD2308a520698F. The GenBank accession number for the microarray data is GSE69564.

Production and purification of recombinant proteins

For EMSAs in Fig. 4A, the *Strep*-tag II system (IBA, Göttingen, Germany) was used to produce a recombinant version of the *Brucella* VtlR protein in *E. coli* strain BL21. The coding region of the *vtlR* gene (*bab1_1517*) was amplified using the primers rVtlR-For and rVtlR-Rev, *B. abortus* 2308 chromosomal DNA as a template, and Taq polymerase. The DNA fragment was digested with BbsI and ligated into BsaI-digested pASK-IBA6,

which encodes an amino-terminal *Strep*-tag on the protein of interest that is localized to the periplasmic space of the host cell. The resulting plasmid, pLS014 (rVtlR-pIBA6), was transformed into *E. coli* strain BL21, and the strains harboring the recombinant protein expression plasmid were grown to an optical density at 600 nm of approximately 0.6 in LB broth before the production of the recombinant protein was induced by adding anhydrotetracycline (200 µg ml^{-1} final concentration). Following incubation for 2 hours at 37°C with shaking, the cells were collected by centrifugation (4,200 × *g* for 10 minutes at 4°C) and lysed by treatment with CelLyticB (Sigma, St Louis, MO, USA) in the presence of the protease inhibitor phenylmethanesulfonyl fluoride. The supernatant from the suspension of lysed cells was cleared by centrifugation (14 000 × *g* for 10 minutes at 4°C) and passed through an affinity column packed with Strep-Tactin Sepharose. The column was washed extensively with Buffer W (100 mM Tris-HCl, 300 mM NaCl, pH 8.0) and the recombinant protein was eluted with 2.5 mM desthiobiotin in Buffer W. The degree of purity of the recombinant VtlR was high as judged by visualization of a single, major band on SDS-PAGE.

For EMSAs in Fig. 4B and DNase I footprinting in Fig. 5, the *Strep*-tag II system (IBA, Göttingen, Germany) was used as above to generate a recombinant *Brucella* VtlR, but with modification. The *vtlR* DNA fragment was digested with BbsI and ligated into BsaI-digested pASK-IBA7, which incorporates an amino-terminal *Strep*-tag on the protein of interest. The protein is then localized in the cytosol of the cell. The resulting plasmid, rVtlR-pIBA7, was transformed into *E. coli* strain BL21, and the strains harboring the recombinant protein expression plasmid were grown and induced as described above. Following incubation for 2 hours at 37°C with shaking, the cells were collected by

centrifugation (4,200 × *g* for 10 minutes at 4°C) and lysed by treatment with CelLyticB (Sigma) and DNase I in the presence of the protease inhibitor phenylmethanesulfonyl fluoride. Subsequent purification steps of rVtlR was followed as described above.

Electrophoretic mobility shift assays (EMSAs)

All rVtlR EMSA experiments were carried out in a final volume of 20 µl containing binding buffer composed of 10 mM Tris-HCl (pH 7.4), 50 mM KCl, 1 mM dithiothreitol, 6% glycerol, 0.5 mM EDTA, 50 µg ml^{-1} bovine serum albumin and 50 µg ml^{-1} salmon sperm DNA. Approximately 200-bp DNA fragments of the *abcR1*, *abcR2*, *bab1_0914*, *bab2_0512* and *bab2_0574* promoter regions were amplified by PCR using primers: abcR1-EMSA-For, abcR1-EMSA-Rev; abcR2-EMSA-For, abcR2-EMSA-Rev; 0914-EMSA-For3; 0914-EMSA-Rev3; 0512-EMSA-For3; 0512-EMSA-Rev3; 0574-EMSA-For3; 0574-EMSA-Rev3. Chromosomal DNA from *B. abortus* 2308 was used as a template in all PCR reactions. The amplified fragments were purified by agarose gel electrophoresis, and the fragments were end-labeled with [γ-^{32}P]ATP and polynucleotide kinase. In Fig. 4A, increasing amounts of rVtlR were mixed with the radiolabeled *abcR2* fragment in binding buffer. As controls, 50 × molar concentrations of non-radiolabeled *abcR2* promoter region DNA (specific competitor) or nonradiolabeled *abcR1* promoter region DNA (non-specific competitor) were added to some reactions. In Fig. 4B, 160 nM of rVtlR was either added (+) or not added (−) to binding reactions with the promoter fragments of *abcR1*, *abcR2*, *bab1_0914*, *bab2_0512* and *bab2_0574*. All binding reactions were incubated at room temperature for 20 minutes and then subjected to electrophoresis on 6% native polyacrylamide gels in 0.5 × TB (Tris-borate) running buffer for approximately 1 hour at

4°C. Following electrophoresis, gels were dried onto 3MM Whatman® paper using a vacuum gel dryer system and visualized by autoradiography.

DNase I footprinting

DNase I footprinting was performed as previously described by Zianni *et al.* (2006). The approximately 200-bp *abcR2* promoter fragment was labeled with a 5′-6FAM modification and amplified by PCR using primers abcR2-6FAM-For and abcR2-EMSA-Rev, Taq polymerase, and *B. abortus* 2308 chromosomal DNA. Following gel purification, the 6FAM-labeled *abcR2* probe was mixed in EMSA binding buffer with or without 160 nM of rVtlR, to a total volume of 20 µl, and incubated at room temperature for 20 minutes. All reactions were then treated with 0.50 U DNase I (Ambion) for 4 minutes at room temperature. The DNase I reactions were terminated by addition of 0.5 M EDTA and incubation at 90°C for 5 minutes. The DNA fragments were purified using phenol-chloroform extraction and ethanol precipitation. Fragment analysis was performed with a GenescanTM-500-Liz® size standard on an Applied Biosystems 3130 (Applied Biosystems, Grand Island, NY, USA) at the Virginia Bioinformatics Institute (Blacksburg, VA, USA). To determine the sequence of the VtlR-binding site, sequencing reactions with the 5′-FAM-labeled *abcR2* oligonucleotide and Thermo Sequenase Dye Primer Manual Cycle Sequencing Kit (Affymetrix) were used according to the manufacturer's directions.

Virulence of *Brucella* mutant strains in murine macrophages and mice

Experiments to test the virulence of *Brucella* mutant strains in primary, peritoneal murine macrophages were carried out as described previously (Gee *et al.*, 2005). 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 50:1. After 2 hours, the infected macrophages were treated with gentamicin (50 μg 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 hour time points, macrophages were washed with PBS following gentamicin treatment and fresh cell culture medium containing gentamicin (20 μg 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 and colonization of mice by *Brucella vtlR* mutant was performed as described previously (Gee *et al.*, 2005). Briefly, 6-week-old female BABL/c mice (five mice per *Brucella* strain) were infected intraperitoneally with ~5×10^4 CFU of each *Brucella* strain in sterile PBS. The mice were sacrificed at 1, 4 and 8 weeks post-infection and serial dilutions of spleen homogenates were plated on SBA. All data was analyzed using the statistical software JMP 11.0.0 (SAS Institute, Cary, NC, USA).

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Figures/Figure Legends

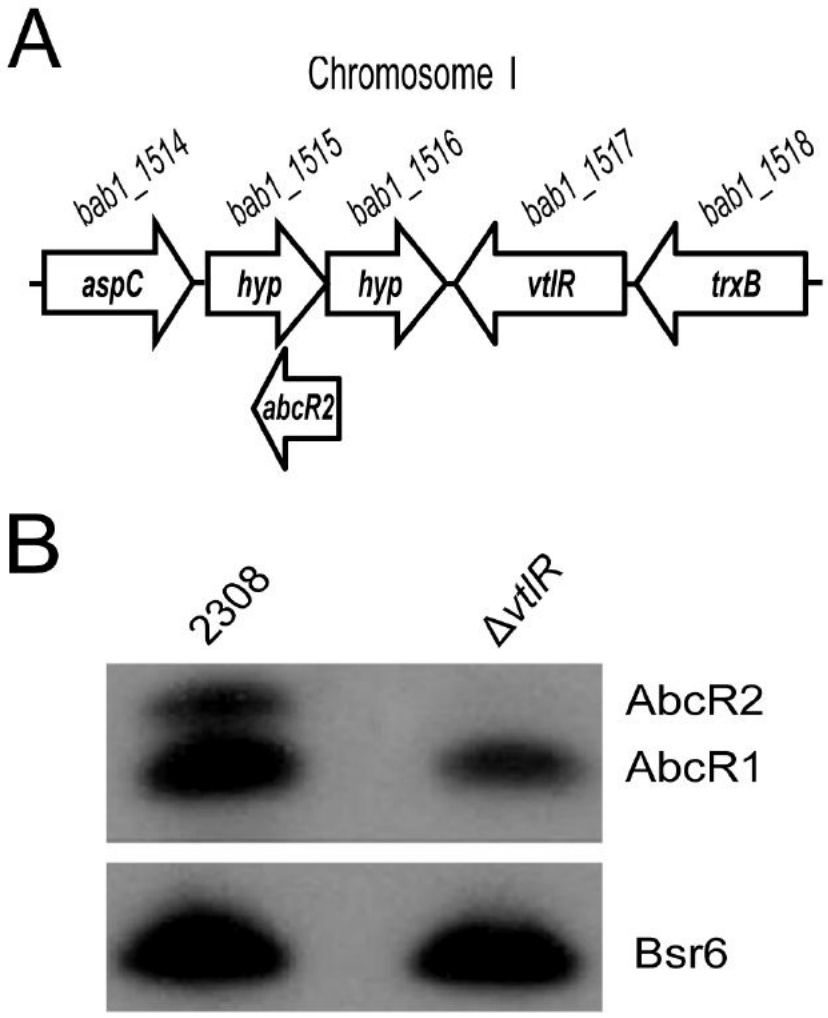

Figure 2.1. VtlR, a LysR-type transcriptional regulator, is an activator of the small RNA-encoding gene *abcR2*

A. Diagram of the *abcR2* locus on chromosome 1 of *Brucella abortus* 2308. In this study, the gene *vtlR* (*bab1_1517*) encoding a LysR-type regulator was hypothesized to control the transcription of the small RNA, *abcR2*.

B. Northern blot analysis assessing the levels of the AbcR sRNAs in the parental strain *B. abortus* 2308 and in the *B. abortus* Δ*vtlR* mutant strain. The *Brucella* sRNA, Bsr6, served as a loading control for this experiment.

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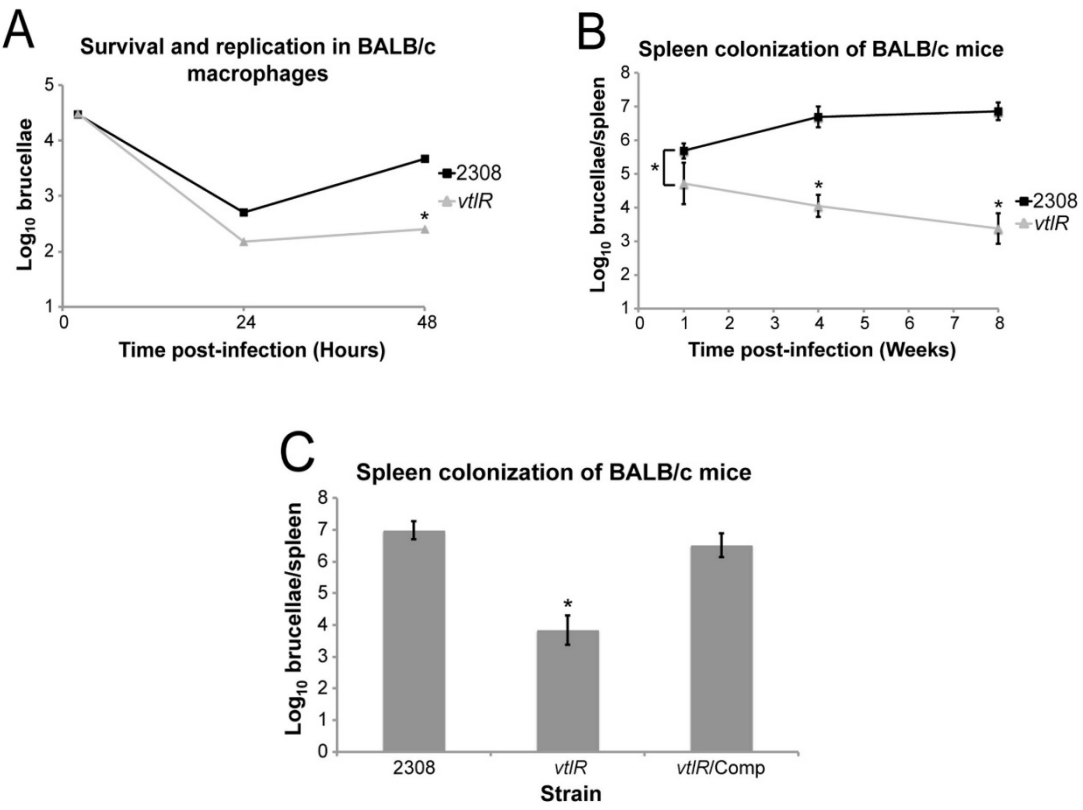

Figure 2.2. VtlR is essential for virulence of *Brucella abortus* 2308
A. Survival and replication of the *B. abortus* Δ*vtlR* mutant in primary murine macrophages. Primary macrophages were collected from the peritoneal cavity of female BALB/c mice and infected with the parental *B. abortus* strain 2308 or the Δ*vtlR* mutant strain. At 2, 24 and 48 hours post-infection, macrophages were lysed, and serial dilutions of the lysates were plated on blood agar to determine the number of intracellular brucellae. Statistically significant differences (one-way ANOVA; $P < 0.005$) are indicated by an asterisk (*).
B. Spleen colonization of BALB/c mice infected with the *B. abortus* Δ*vtlR* mutant strain. Six week old female BALB/c mice were infected intraperitoneally with *B. abortus* 2308 or the Δ*vtlR* mutant strain. Five mice were used for each *Brucella* strain at each time point. At 1, 4 and 8 weeks post-infection, mice were sacrificed, and the spleens were removed and homogenized. Serial dilutions of the spleen homogenates were plated onto blood agar to enumerate the number of brucellae per spleen. Data are presented as average brucellae per spleen +/− the standard deviation from the five mice infected with each *Brucella* strain. Statistically significant differences (*t*-test; $P < 0.05$) are indicated by an asterisk (*).
C. Spleen colonization of BALB/c mice infection with the reconstructed Δ*vtlR* mutant strain (Δ*vtlR*/Comp). This experiment was performed as described above in Fig. 2B except spleen colonization was evaluated after a single time-point of 4 weeks post-infection. Data is presented as average brucellae per spleen +/− the standard deviation from the five mice infected with each *Brucella* strain. Statistically significant differences (*t*-test; $P < 0.05$) are indicated by an asterisk (*).

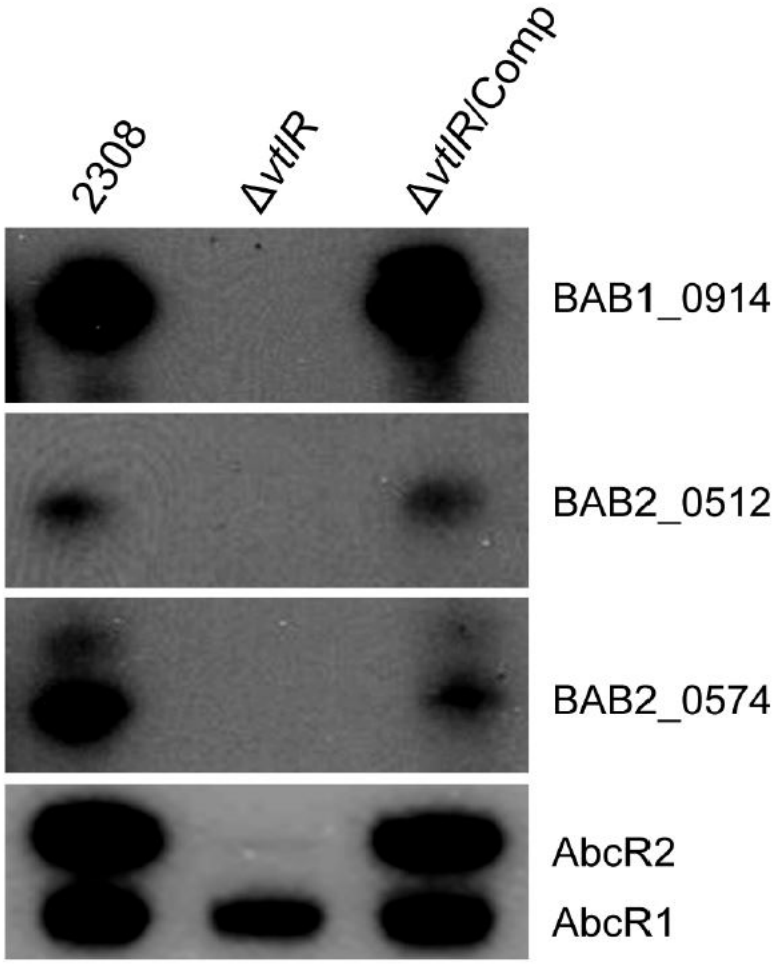

Figure 2.3. VtlR activates the expression of *abcR2* and three hypothetical protein-encoding genes

Northern blot analyses evaluating expression of the genes encoding the AbcR sRNA, and the genes encoding BAB1_0914, BAB2_0512 and BAB2_0574. RNA isolated from the parental strain *B. abortus* 2308, the *vtlR* mutant strain, and the genetically complemented *vtlR* mutant strain (*vtlR*/Comp) were separated by denaturing polyacrylamide gel electrophoresis, and following transfer to a positively-charged membrane, the membranes were probed for RNAs corresponding to the AbcR sRNAs (a common probe that recognizes AbcR1 and AbcR2), BAB1_0914, BAB2_0512 or BAB2_0574.

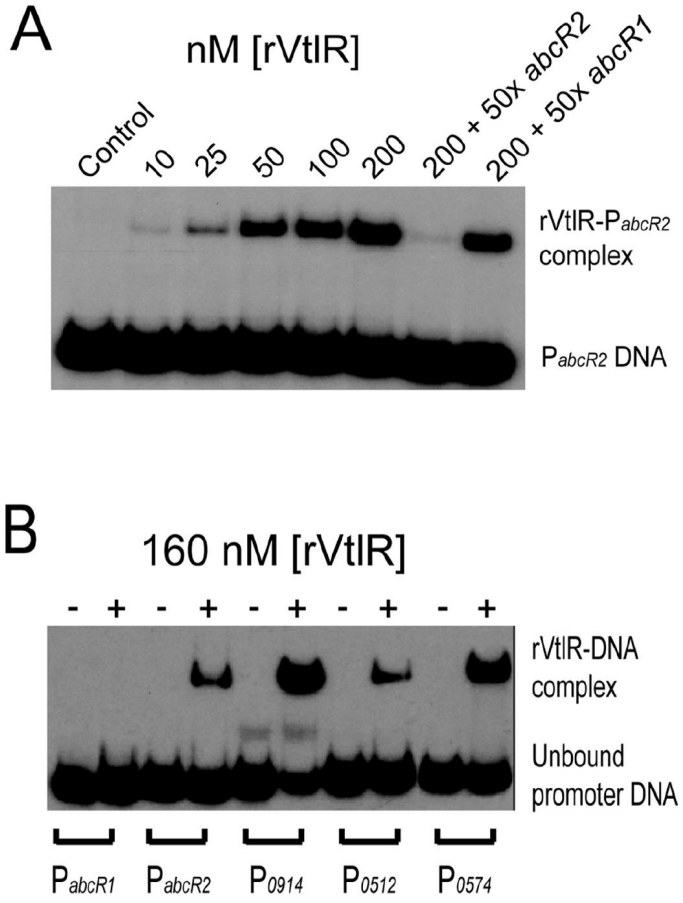

Figure 2.4. VtlR binds to the promoter regions of the small RNA *abcR2*, as well as three genes *bab1_0914*, *bab2_0512* and *bab2_0574* encoding hypothetical proteins
A. Recombinant VtlR (rVtlR) protein was evaluated for binding to the *abcR2* promoter region using electrophoretic mobility shift assays (EMSAs). Increasing concentrations of rVtlR were incubated with radiolabeled *abcR2* promoter region DNA. In some binding reactions, either unlabeled specific (*abcR2* promoter region DNA) or unlabeled non-specific (*abcR1* promoter region DNA) competitor DNA fragments were added to the binding reactions.
B. EMSAs of rVtlR protein binding to the promoter regions of *abcR1*, *abcR2*, *bab1_0914*, *bab2_0512* and *bab2_0574*. Radiolabeled promoter DNA fragments (approximately 200 bp) were either incubated with (+) or without (−) 160 nM rVtlR.

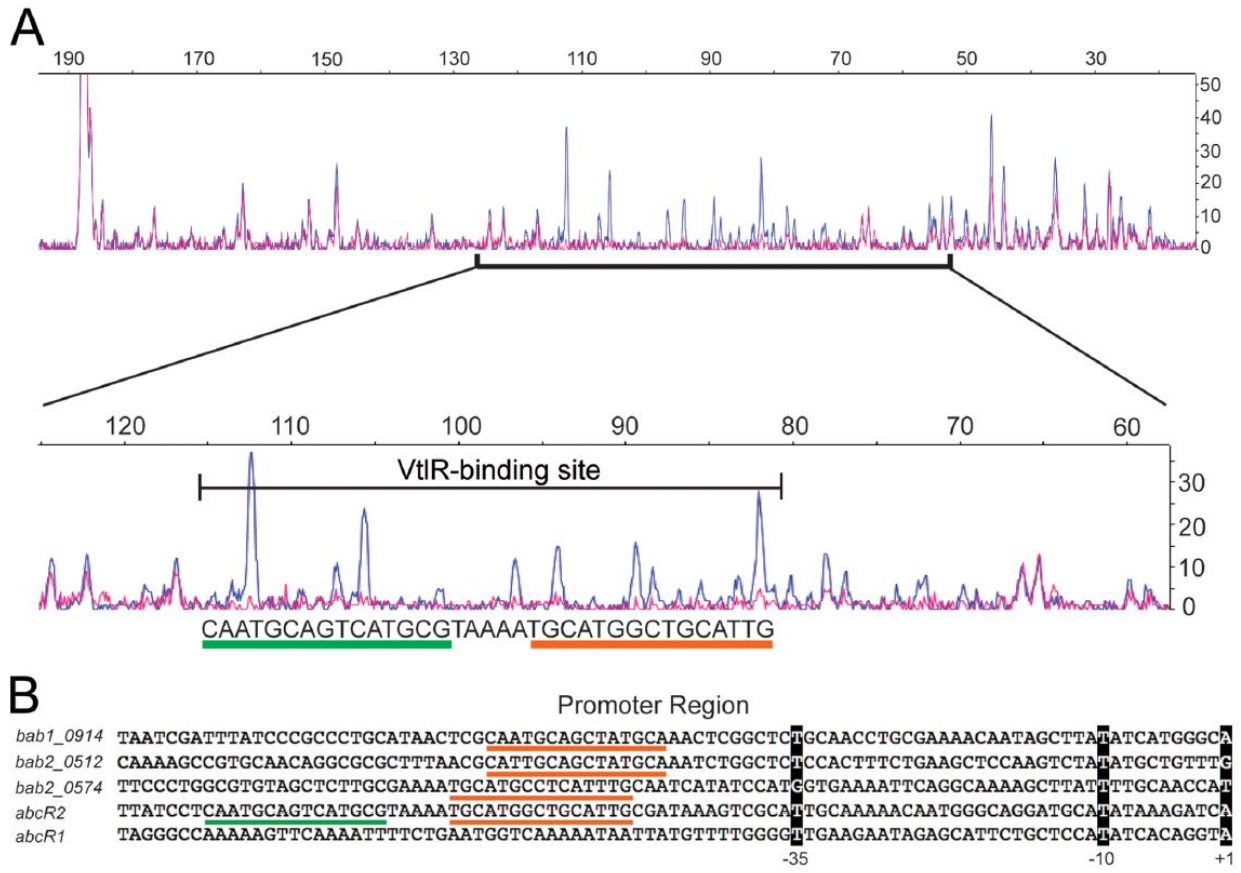

Figure 2.5. DNase I footprinting analysis of VtlR binding site in the *abcR2* promoter
A. Overlaid DNase digestion pattern of the *abcR2* 200 bp promoter region with (pink line) or without (blue line) 160 nM rVtlR. The protected region of *abcR2* is indicated by the black horizontal line labeled 'VtlR-binding site', and the nucleotide sequence is indicated underneath of the digestion pattern. Orange underlining beneath nucleotides indicates the conserved sequence, whereas the green underlining beneath nucleotides indicates the inverted repeat within the 35 bp VtlR-binding site of P*abcR2*.
B. The promoter regions (P*gene*) of the VtlR-regulated genes, *bab1_0914*, *bab2_0512*, *bab2_0574*, and *abcR2*, and *abcR1* as a negative control. The transcriptional start site (+1), as well as the −10 and −35 regions are indicated by nucleotides highlighted in black. The conserved nucleotide sequence is indicated by the orange underline. As in panel B, the inverted repeat found in P*abcR2* is indicated by the green underline.

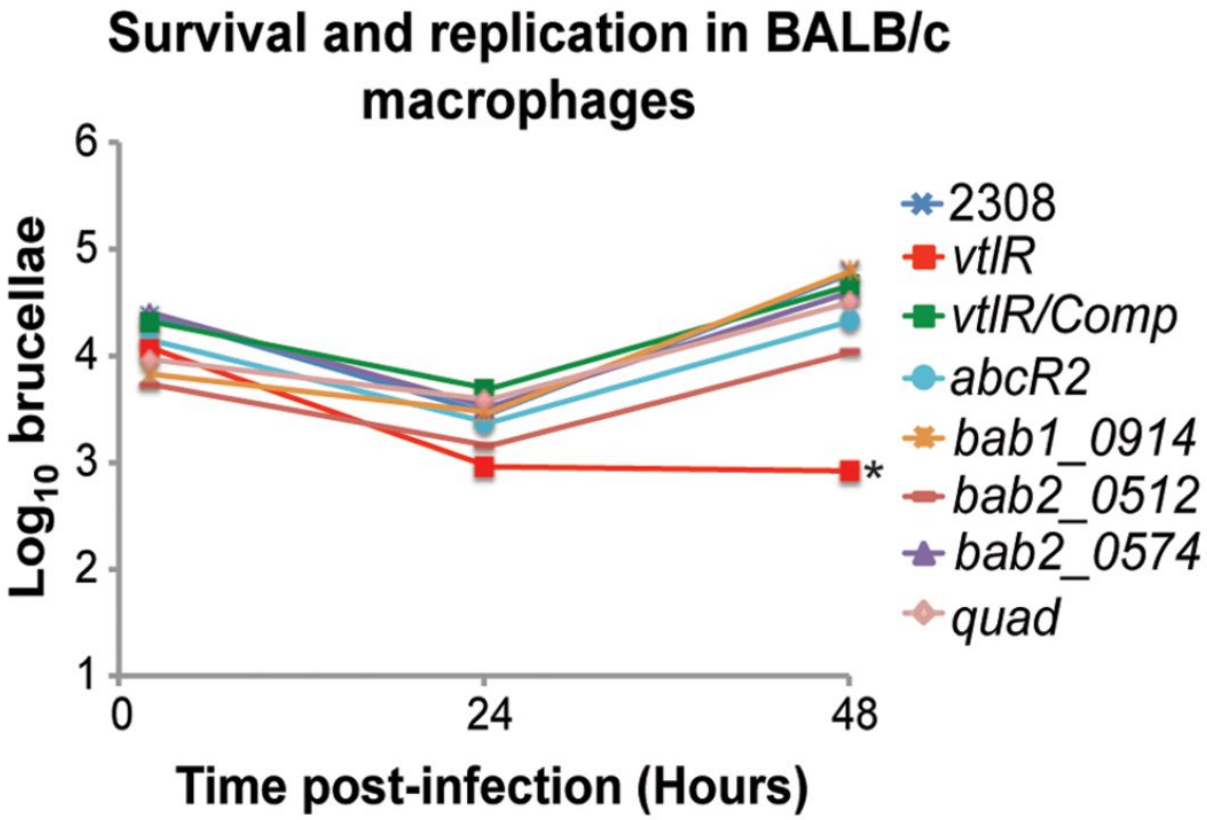

Figure 2.6. Assessment of the role of VtlR-regulated genes in *Brucella abortus* virulence in macrophages

Survival and replication of *B. abortus* 2308, Δ*vtlR*, reconstructed *ΔvtlR* locus (*vtlR*/Comp), *ΔabcR2*, *Δbab1_0914*, *Δbab2_0512*, *Δbab2_0574*, and a quadruple mutant of *abcR2*, *bab1_0914*, *bab2_0512* and *bab2_0574* (*quad*) in primary murine macrophages. Macrophages were collected from the peritoneal cavity of female BALB/c mice and infected with the *Brucella abortus* strains. At 2, 24 and 48 h post-infection, macrophages were lysed, and serial dilutions of the lysates were plated on blood agar to determine the number of intracellular brucellae. Statistically significant differences (one-way ANOVA; $P < 0.005$) compared with the wild-type are indicated by an asterisk (*).

Tables

Microarray Analysis

BAB #	Fold decrease in Δ*vtlR* strain	Description of gene product
bab1_0914	10.1	Hypothetical protein
bab1_1517	14.5	Transcriptional regulator (VtlR)
bab2_0512	8.4	Hypothetical protein
bab2_0574	15.7	Hypothetical protein
bab2_0801	2.0	Hypothetical protein
	Fold increase in Δ*vtlR* strain	
bab1_0310	2.3	Allantoate amidohydrolase
bab1_0313	3.0	Dihydropyrimidine dehydrogenase
bab1_0314	3.5	Putative oxidoreductase
bab1_0355	2.2	LrgA family protein
bab1_0356	2.2	LrgB family protein
bab1_1794	2.2	Leu/Ile/Val-binding family protein
bab2_0348	2.7	Protein kinase
bab2_0961	2.0	Dihydrodipicolinate synthetase

Table 2.1: Identification of the VtlR-regulated genes in *Brucella abortus* 2308
List of genes differentially expressed in the *B. abortus* Δ*vtlR* mutant strain compared to the parental strain 2308. Microarray analysis was performed with total cellular RNA from *Brucella* strains grown to late exponential phase in nutrient-replete brucella broth. The genes listed are more than two-fold decreased in the Δ*vltR* mutant strain, or more than two-fold increased in the Δ*vltR* mutant strain.

Chapter 3. A 6-nucleotide regulatory motif within the AbcR small RNAs of *Brucella abortus* mediates host-pathogen interactions

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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**.

Abstract

In *Brucella abortus*, two small RNAs (sRNAs), AbcR1 and AbcR2, are responsible for regulating transcripts encoding ABC-type transport systems. AbcR1 and AbcR2 are required for *Brucella* virulence, as a double chromosomal deletion of both sRNAs results in attenuation in mice. Although these sRNAs are responsible for targeting transcripts for degradation, the mechanism utilized by the AbcR sRNAs to regulate mRNA in *Brucella* has not been described. Here, two motifs (M1 and M2) were identified in AbcR1 and AbcR2, and complementary motif sequences were defined in AbcR-regulated transcripts. Site-directed mutagenesis of M1 or M2 or of both M1 and M2 in the sRNAs revealed transcripts to be targeted by one or both motifs. Electrophoretic mobility shift assays revealed direct, concentration-dependent binding of both AbcR sRNAs to a target mRNA sequence. These experiments genetically and biochemically characterized two indispensable motifs within the AbcR sRNAs that bind to and regulate transcripts. Additionally, cellular and animal models of infection demonstrated that only M2 in the AbcR sRNAs is required for *Brucella* virulence. Furthermore, one of the M2-regulated targets, BAB2_0612, was found to be critical for the virulence of *B. abortus* in a mouse model of infection. Although these sRNAs are highly conserved among *Alphaproteobacteria*, the present report displays how gene regulation mediated by the AbcR sRNAs has diverged to meet the intricate regulatory requirements of each particular organism and its unique biological niche.

Introduction

Regulatory small RNAs (sRNAs) are important components of bacterial gene regulation, allowing organisms to quickly shift gene expression in response to changes in environmental conditions. sRNAs are typically less than 500 nucleotides in length and are capable of post-transcriptionally regulating mRNA targets by complementary, imperfect base pairing (Waters and Storz, 2009; Gottesman and Storz, 2011), and these sRNA-mRNA interactions are commonly facilitated by the RNA chaperone Hfq (Møller *et al.*, 2002; Sauter *et al.*, 2003; Vogel and Luisi, 2011). Once bound, mRNAs can experience one or more of several fates: sRNA can relieve hairpin structures in the untranslated region (UTR) of transcripts, allowing the ribosomal binding site (RBS) to be relaxed and translation to occur; sRNAs can block the RBS and inhibit binding of the ribosome, thus impeding the start of translation; and/or sRNAs can bind to either the UTR or coding region (CDR) of the transcript and target the mRNA or the entire sRNA-mRNA complex for degradation by an RNase (Fröhlich and Vogel, 2009; Gottesman, 2011; De Lay *et al.*, 2013).

AbcR1 and AbcR2 are two highly conserved sRNAs found throughout species in the class of *Alphaproteobacteria* (del Val *et al.*, 2007; Vercruysse *et al.*, 2010; Wilms *et al.*, 2011). Although the AbcR sRNAs are similar in nucleotide composition and secondary structure among bacteria, they have diverged greatly in their regulatory capacity. In the plant pathogen *Agrobacterium tumefaciens*, AbcR1, but not AbcR2, has been shown to regulate gene expression (Wilms *et al.*, 2011). *A. tumefaciens* AbcR1 regulates expression of ABC-type transporter genes, the majority of which encode amino acid and sugar uptake systems. In contrast, AbcR1 and AbcR2 in the plant symbiont *Sinorhizobium*

meliloti are both responsible for regulating specific and shared transcripts (Torres-Quesada *et al.*, 2013; Torres-Quesada *et al.*, 2014).

In plant-associated *Alphaproteobacteria* such as *A. tumefaciens* and *S. meliloti*, AbcR1 and AbcR2 are chromosomally encoded in tandem, directly downstream of a gene encoding a LysR-type transcriptional regulator. In the human pathogen *Brucella abortus*, *abcR1* is located on chromosome II, while *abcR2* is positioned on chromosome I. In *B. abortus*, expression of *abcR2*, but not *abcR1*, is controlled by VtlR, the orthologous LysR-type transcriptional regulator found upstream of the genes encoding the *A. tumefaciens* and *S. meliloti* AbcR sRNAs (Sheehan *et al.*, 2015). These differences in regulatory roles, genetic organization, and transcriptional regulation highlight the evolutionary differentiation of the AbcR regulatory system, which may have helped drive the host-bacterium relationships of *Alphaproteobacteria*.

In *B. abortus*, AbcR1 and AbcR2 negatively regulate several mRNA transcripts and are critical for the ability of the bacteria to cause a chronic infection in mice (Caswell *et al.*, 2012a). Although *abcR1* and *abcR2* are located on different chromosomes, the sRNAs exhibit a high degree of nucleotide identify and very similar secondary structures, suggesting regulatory redundancy. Importantly, these two sRNAs seem to be functionally redundant, as isogenic chromosomal mutations in *abcR1* or *abcR2* do not affect the virulence of *B. abortus* but a double deletion of both *abcR1* and *abcR2* (Δ*abcR1/2*) results in attenuation *in vitro* and *in vivo*. Microarray analysis of *B. abortus* Δ*abcR1/2* revealed over 20 transcripts with elevated levels, demonstrating negative regulation by the sRNAs. Importantly, this regulation is posttranscriptional, as the results from the microarray analyses closely mirrored the results of the quantitative proteomic analyses.

For this study, we chose to focus on four AbcR targets that were identified as being upregulated over 3-fold in both microarray analysis and quantitative proteomics: BAB1_0314, BAB1_1794, BAB2_0612, and BAB2_0879. Importantly, although over 20 transcripts were found to be differently expressed in the *B. abortus abcR1/2* deletion strain, these four targets appear to be part of four independent AbcR-regulated systems. *bab1_0314* is predicted to encode subunit A of a dihydropyrimidine dehydrogenase along with *bab1_0313*, *bab1_1794* is predicted to encode a periplasmic binding protein of a putative ABC-type transport system encoded by *bab1_1794* to *bab1_1799*, *bab2_0612* is a putative glutamate-binding protein predicted to be a part of an ABC-type transport system, and *bab2_0879* is a putative polyamine-binding protein predicted to be a part of an ABC-type transport system encoded by *bab2_0874* to *bab2_0879*. Although AbcR1 and AbcR2 are known to play a critical role in promoting degradation of these transcripts in *B. abortus*, the regulatory redundancy and the mechanism by which the sRNAs target mRNAs for degradation have not been characterized.

As discussed above, AbcR1 and AbcR2 are important regulatory components in *B. abortus* and are critical for the ability of the brucellae to cause infection. Although the targets of AbcR-mediated gene regulation have been defined, the mechanism of regulatory redundancy remains uncharacterized. Here, we determined that AbcR1 and AbcR2 exhibit redundant mechanisms to control gene expression, and we have defined two nucleotide sequence motifs, called M1 and M2, that are required for AbcR-mRNA interactions. Most importantly, M2, but not M1, is critical for the wild-type virulence of *Brucella abortus*. To date, this is the first demonstration of a molecular mechanism of sRNA-mediated gene expression in *Brucella*.

Results

AbcR1 and AbcR2 share redundant regulatory functions in B. abortus 2308

Caswell *et al*., demonstrated that chromosomal isogenic deletion of either *abcR1* or *abcR2* in *B. abortus* resulted in a wild-type virulence phenotype but that a double *abcR1 abcR2* mutant led to significant attenuation in a mouse model of infection (Caswell *et al.*, 2012a). Although this suggested functional redundancy, it was unknown if one or both of the sRNAs regulate the identified mRNA targets, which were identified using the *abcR1 abcR2* double mutant strain. To determine this, RNA from *B. abortus* 2308, Δ*abcR1*, Δ*abcR2*, and Δ*abcR1/2* was reverse transcribed to cDNA, and quantitative reverse transcriptase PCR (qRT-PCR) was performed on the four most highly regulated transcripts: *B. abortus bab1_0314*, encoding a putative oxidoreductase; *bab1_1794*, encoding a putative amino acid-binding protein; *bab2_0612*, encoding a putative glutamate-binding protein; and *bab2_0879*, encoding a putative polyamine-binding protein (Fig. 1). Individual deletions of either *abcR1* or *abcR2* did not lead to altered target mRNA levels; however, a strain in which both *abcR1* and *abcR2* were deleted exhibited statistically significantly increased levels of the mRNA targets compared to wild-type strain 2308, clearly demonstrating that AbcR1 and AbcR2 possess redundant regulatory functions in *B. abortus*.

AbcR1 and AbcR2 directly bind to mRNA targets

Electrophoretic mobility shift assays (EMSAs) were employed to assess the ability of the *B. abortus* AbcR sRNAs to directly bind to target mRNAs, specifically, to the 5' UTR of the BAB2_0879 mRNA (Fig. 2). BAB2_0879 RNA was radiolabeled using *in vitro*

transcription that employed the T7 promoter, and the labeled BAB2_0879 RNA was incubated with increasing concentrations of *in vitro* transcribed AbcR1 or AbcR2. Both AbcR1 and AbcR2 exhibited concentration-dependent binding to BAB2_0879 mRNA. To ensure that sRNA-mRNA binding was specific to the AbcR-regulated target RNA, a negative-control binding reaction was performed with RNA corresponding to the 5' UTR of BabR, a *Brucella* LuxR-type transcriptional regulatory protein. No binding between the AbcR sRNAs and BabR RNA was observed in this experiment (Fig. 2, lower panel). Overall, these data demonstrate the direct interaction of the AbcR sRNAs with a target mRNA in *B. abortus*.

Identification of two regulatory sequences in the AbcR sRNAs

In *A. tumefaciens*, two putative regulatory motifs were computationally predicted in AbcR1, and EMSAs revealed that AbcR1 employs both motifs to bind the 5' UTRs or CDRs in mRNA transcripts (Overlöper *et al.*, 2014). Analysis of the *B. abortus* AbcR sRNA secondary structures identified two conserved sequences comprised of 6-nucleotides each in both AbcR1 and AbcR2 (Fig. 3A). The first motif (M1), CUCCCA, is located in the first hairpin of the sRNAs, while the second motif (M2), GUUCCC, is found between the first and second hairpins. AbcR-regulated mRNA transcripts were subsequently examined to identify putative complementary binding sequences with respect to the AbcR sRNAs. Several transcripts were found to contain complementary M1 and/or M2 sequences in either their 5' UTR or within the CDR. This variability in the location of binding sites is not uncommon for negatively regulated transcripts, as sRNAs can bind and block ribosome at the Shine-Dalgarno sequence in the 5' UTR or can cause early

termination of translation and targeted degradation by binding to the CDR of the mRNA (Fröhlich and Vogel, 2009; Gottesman, 2011; De Lay *et al.*, 2013).

The AbcR sRNAs regulate expression of mRNAs using the M1 and M2 motifs

Following identification of the M1 and M2 motifs in the AbcR sRNAs, we sought to determine the involvement, if any, of the motifs in AbcR-mediated gene regulation (Fig. 3). Site-directed mutagenesis was carried out on both AbcR sRNAs in *B. abortus* to mutate the M1 CUCCCA sequence to a nonsense sequence, UGAUAC (denoted as *abcR-M1*^{mut}). Likewise, the M2 GUUCCC sequence was mutated to ACGTAT (denoted as *abcR-M2*^{mut}). Finally, a double-motif mutant was constructed (denoted as *abcR-M1/2*^{mut}) (Fig. 3B). Northern blot analyses were performed on all AbcR motif mutant strains to ensure that sequence changes did not negatively affect sRNA transcription and stability (Fig. 3C). Indeed, all *B. abortus* strains produced the corresponding AbcR sRNA.

Following sRNA mutagenesis, qRT-PCR was employed to determine if gene expression of the mRNA targets was affected by changes made to the M1 and M2 putative binding motifs (Fig. 4; Table 1). These data showed that the AbcR sRNAs regulated transcripts by utilizing M1 or M2 or a combination of M1 and M2. Expression of *bab1_1799*, encoding a putative permease of an amino acid ABC transport system, was the sole mRNA target found to be regulated by M1 (Table 1). Expression levels of *bab2_0506*, *bab2_0612*, *bab2_0879*, and *bab2_1062* were significantly upregulated in the *abcR-M2*^{mut} strain compared to wild-type strain 2308, suggesting that these mRNA targets are regulated solely by M2. Finally, expression levels of *bab1_0313*, *bab1_0314*, *bab1_1794*, and *bab2_0491* were significantly upregulated in both the *abcR-M1*^{mut} and

abcR-M2^{mut} strains, suggesting that both motifs are utilized by the AbcR sRNAs to regulate expression of these targets.

To further validate these findings, a *B. abortus* double M2 motif mutant of the AbcR sRNAs and BAB2_0879 mRNA was constructed to assess if negative regulation could be restored (Fig. 5). For mRNA mutagenesis, the wild-type *B. abortus* BAB2_0879 M2 motif, UAAGGG, was mutated to UGCAUA (Fig. 5A). The BAB2_0879 motif was mutated to be complementary to the M2 motif in the *B. abortus abcR-M2*^{mut} strain, in the hope that negative regulation by the AbcR sRNAs could be reestablished (Fig. 5B). Following mutagenesis, qRT-PCR was performed to analyze expression of *bab2_0879* in the *B. abortus* double *abcR mRNA-M2* mutant. As expected, downregulation of BAB2_0879 was restored. Taken together, these experiments identified and confirmed two 6-nucleotide motifs, M1 and M2, within the AbcR sRNAs that are required for regulation of mRNA targets in *B. abortus*.

Deletion of M2, but not M1, in the AbcR sRNAs results in attenuation of *Brucella abortus* in mice

As demonstrated previously, AbcR1 and AbcR2 are essential for the wild-type virulence of *Brucella abortus* (Caswell *et al.*, 2012a). The potential reason for the attenuation seen with a *B. abortus abcR1 abcR2* double deletion may be the upregulation of a numerous and rather diverse set of ABC-type transport systems. Since the M1 and M2 motifs in the sRNAs were demonstrated to be involved in regulation of transcripts (Fig. 4), it was hypothesized that mutagenesis of the M1 and M2 motifs in the AbcR sRNAs would affect the virulence of *B. abortus* (Fig. 6). To test this hypothesis, BALB/c mouse macrophages

were infected with the AbcR sRNA motif mutants (Fig. 6A). Surprisingly, while the *B. abortus abcR-M1*^{mut} trended with the wild-type strain, the *B. abortus abcR-M2*^{mut} strain was unable to successfully infect and survive in macrophages. Furthermore, similar results were observed in a mouse model of infection, where the *abcR-M2*^{mut} strain exhibited a decreased ability to chronically infect mice (Fig. 6B). These data suggest that the 6-nucleotide M2 motif in AbcR sRNAs is required for the full virulence of *B. abortus*.

An AbcR M2-regulated target, BAB2_0612, is critical for colonization of *Brucella abortus* in mice

Following the demonstration that M2 in the AbcR sRNAs is critical for *Brucella* pathogenesis, we sought to determine if deletion of any of the M2-regulated targets would result in attenuation in a mouse model of infection (Fig. 7). Isogenic, unmarked, in-frame gene deletions were made of two M2-regulated targets: *bab2_0612*, encoding a putative glutamate-binding protein, and *bab2_0879*, encoding a putative polyamine-binding protein. Subsequently, the strains were assessed for their ability to cause a chronic infection in mice. *B. abortus* Δ*bab2_0612* was significantly attenuated in BALB/c mice compared to wild-type strain 2308 (Fig. 7A), whereas *B. abortus* Δ*bab2_0879* showed no difference from the wild-type strain in colonization (Fig. 7B). These data suggest that BAB2_0612 is a critical component of *Brucella* virulence.

Discussion

The AbcR small RNAs were previously shown to be vital components of the virulence of *B. abortus* and, moreover, to be involved in negatively regulating transcripts largely encoding ABC-type transport systems (Caswell *et al.*, 2012a). Importantly, and as further defined in this study, the AbcR sRNAs are a prime example of sibling sRNAs, as they share identical regulatory repertoires in *B. abortus* 2308 (Caswell *et al.*, 2012a; Caswell *et al.*, 2014) (Fig. 1). While the link between the AbcR sRNAs and *Brucella* pathogenesis is evident, it was unknown how the AbcR sRNAs target and bind transcripts, ultimately leading to mRNA degradation.

In the present study, we identified and experimentally confirmed that two motifs, M1 and M2, which are comprised of six nucleotides each, are the primary sequences used by the AbcR sRNAs to bind transcripts in *B. abortus* (Fig. 3 to 5; Table 1). In the plant pathogen *A. tumefaciens*, AbcR1 is the main regulatory AbcR sRNA, as AbcR1 regulates transcripts encoding a diverse set of nutrient uptake systems (Wilms *et al.*, 2011; Overlöper *et al.*, 2014). In contrast, AbcR2 has not been reported to have any regulatory function in *A. tumefaciens*. Although AbcR2 is speculated to have arisen from gene duplication, it is important to note a major difference in the *A. tumefaciens* AbcR2 that may explain its lack of regulatory capability: the M1 sequence is absent. In contrast, both M1 and M2 are present in *A. tumefaciens* AbcR1. Importantly, the M1 and M2 sequences in *A. tumefaciens* AbcR1 are identical to the M1 and M2 sequences in the *B. abortus* AbcR sRNAs. However, *A. tumefaciens* AbcR2 contains only the M2 site. This absence of M1 may contribute to the lack of a regulatory function by AbcR2 in *A. tumefaciens*. Furthermore, because of the importance that the M2 site has in *Brucella*

virulence (Fig. 6), it will be interesting to learn what role, if any, the AbcR M2 motif plays in *Agrobacterium* biology and pathogenesis.

In the plant symbiont *S. meliloti*, AbcR1 has been hypothesized to utilize M1 to bind transcripts (Torres-Quesada *et al.*, 2013). However, there is currently no experimental evidence to confirm this interaction. As is the case in *A. tumefaciens*, AbcR1 is defined as having the dominant regulatory role in *S. meliloti*. However, AbcR2 was also shown to have some regulatory capacity as well, although not to the same degree as AbcR1. This difference in AbcR regulation levels may also be linked to the two motifs. M1 and M2 are present in *S. meliloti* AbcR1 and are identical to the AbcR motifs in both *Agrobacterium* and *Brucella*. Conversely, the M1 sequence in AbcR2 is mutated from CUCCCA to CUCCCC. Though this difference is in one nucleotide, it may compromise the regulatory ability of AbcR2, but this remains to be experimentally determined.

In *B. abortus*, the AbcR sRNAs are interesting examples of sibling sRNAs (Caswell *et al.*, 2014), as the two *Brucella* AbcR sRNAs have identical regulatory roles and utilize two conserved motifs for binding transcripts. Although the regulatory profiles of M1 and M2 are not identical (Fig. 4; Table 1), only M2 was found to be critical for the virulence of *B. abortus* in both macrophages and mice (Fig. 6). The attenuation seen in an *abcR-M2* mutant may be due to the overproduction of potentially immunogenic proteins such as BAB2_0506, BAB2_0612, BAB2_0879, and/or BAB2_1062. To start to investigate if any of these M2-regulated targets have a role in pathogenesis, isogenic gene deletions were made in two of the M2 targets: *bab2_0612* and *bab2_0879*. *bab2_0612* is predicted to encode a periplasmic binding protein of an ABC-type glutamate transport system. Aside from this prediction, nothing is currently known about BAB2_0612 in *Brucella*.

Interestingly, in *A. tumefaciens*, Atu1879, the orthologue of BAB2_0612, is regulated by AbcR1, but the regulation is performed specifically by the M1 motif (Overlöper *et al.*, 2014). However, the results of our present study show that BAB2_0612 in *Brucella* is controlled entirely by the M2 motif (Fig. 4). In mice, the Δ*bab2_0612* mutant is attenuated compared to wild-type strain (Fig. 7A), highlighting the importance of this M2-regulated target in infection. These seemingly small but strikingly important differences in M1-mediated versus M2-mediated gene regulation will need to be better defined in the *Alphaproteobacteria* in order to understand the evolutionary consequences of AbcR-linked gene expression for host specificity of the bacteria. In terms of *Brucella*, current work is aimed at further characterization of BAB2_0612, particularly by developing a *bab2_0612* overexpression strain and determining the function of this protein in *B. abortus*.

bab2_0879 is predicted to encode a putative periplasmic protein in an ABC-type polyamine transport system. In bacteria, polyamines can be viewed a double-edged sword (Shah and Swiatlo, 2008). Polyamines provide protection against free radicals (Khan *et al.*, 1992, Ha *et al.*, 1998) and promote the expression of acid resistance genes during stress (Jung and Kim, 2003); both activities could be beneficial to intracellular organisms such as *Brucella*. The increase in the level of BAB2_0879 in the *abcR-M2* mutant could be linked to polyamine toxicity. However, this is strictly speculation as no studies have linked polyamine synthesis or transport to *Brucella* pathogenesis. Deletion of *bab2_0879* in *B. abortus* did not affect colonization of the bacteria in a mouse model of infection, but previous studies have shown that deletion of this gene in *B. melitensis* results in attenuation (Delrue *et al.*, 2004). However, because an *abcR-M2* mutant is

hypothesized to overproduce BAB2_0879, deletion of these targets may not recapitulate what is happening in a *B. abortus abcR-M2* mutant strain. Current work is targeted at identifying and subsequently mutating complementary M2 motifs in all M2-regulated targets (i.e., BAB2_0506, BAB2_0612, BAB2_0879, and/or BAB2_1062) in *B. abortus* 2308 and at assessing the ability of these overexpressing *Brucella* strains to infect both macrophages and mice.

sRNAs typically regulate transcripts through the direct or indirect sensing of specific environmental stimuli (Waters and Storz, 2009; Gottesman and Storz, 2011). It is by this mechanism that sRNAs can quickly alter the bacterial transcriptome to respond to and adapt to stress. In regard to the *Brucella* AbcR sRNAs, no specific stimuli have been identified that are responsible for altering their regulatory activities. Current research in our laboratory is aimed at identifying the stimuli responsible for changing the expression levels of the sRNAs. Importantly, we previously identified the transcriptional activator of *abcR2*, VtlR (Sheehan *et al.*, 2015). VtlR belongs to the class of LysR-type transcriptional regulators, whose members typically have an N-terminal DNA-binding domain and a C-terminal coinducer binding domain. Current research is aimed at identifying the putative coinducer with which VtlR is interacting, which may in turn result in the alteration of expression of *abcR2*. Moreover, since external stimuli added to culture can never fully recapitulate intracellular environmental conditions, our laboratory is aiming to perform transcriptome sequencing (RNA-seq) from brucellae extracted from macrophages during various phases of infection. This experiment will give us insight into the transcriptome of *Brucella in vitro* and, more specifically, will give us a clear picture of the AbcR system during intracellular infection.

The AbcR sRNAs are involved in the negative regulation of mRNA transcripts, ultimately targeting them for degradation. It has been suggested that RNase E may be involved in AbcR sRNA degradation, as a *S. meliloti rnaseE* gene mutant was reported to show increased stability of AbcR1 (Becker *et al.*, 2014). RNase E is a highly conserved bacterial endoribonuclease involved in the processing and decay of RNA (reviewed in Mackie, 2013). Moreover, pertaining to its association with sRNAs, the C-terminus of RNase E has been reported to bind the sRNA chaperone Hfq and assist in sRNA-mRNA decay (Valentin-Hansen *et al.*, 2004; Aiba, 2007; Brennan and Link, 2007; Ikeda *et al.*, 2011). Empirical evidence suggests that RNase E is essential in *B. abortus*, but, importantly, the data suggested that a nonlethal C-terminal truncation of RNase E could be constructed in *B. abortus* (Xavier DeBolle, personal communication). Therefore, to determine if RNase E (BAB1_0930) is responsible for AbcR sRNA-mRNA degradation in *B. abortus*, a chromosomal *B. abortus rnaseE* mutant was constructed which lacked the C-terminal domain. Northern blot analysis of the AbcR sRNAs, as well as qRT-PCR analysis for determination of mRNA levels of AbcR-regulated targets, found no association between RNase E and AbcR-mRNA degradation (data not shown). It is possible that the region associated with sRNA degradation was not deleted in this *B. abortus* strain, but we have not experimentally tested this hypothesis. In addition to RNase E, several other *B. abortus* RNases were also tested for their potential role in AbcR-mRNA degradation: YbeY, a putative single-strand and double-strand endoribonuclease (Davies and Walker, 2008; Davies *et al.*, 2010; Jacob *et al.*, 2013; Saramago *et al.*, 2017); RNase J, a 5'–3' exoribonuclease (reviewed in Condon, 2010); and RNase R, a 3'–5' hydrolytic exoribonuclease (reviewed in Domingues *et al*., 2015).

None of those RNases were found to be associated with the AbcR sRNAs or with their targets (data not shown). Current work is focused on identifying the RNase involved in AbcR sRNA degradation in *B. abortus*.

In summary, this work experimentally confirmed the presence of two regulatory motifs, comprised of only 6-nucleotides each, in the highly conserved *B. abortus* AbcR sRNAs. The data demonstrate how the AbcR M1 and M2 motifs are essential for proper regulation of mRNA targets in the pathogenic bacterium *B. abortus* (Fig. 8). Upon transcription of *abcR2* and *abcR1* by VtlR and a yet-to-be-described protein, respectively, the two sRNAs adopt similar secondary structures, exposing two single-stranded RNA motifs, CUCCCA (M1) and GUUCCC (M2). To date, only one mRNA target has been identified as being regulated by the M1 motif alone. In contrast, the majority of transcripts are regulated by M2 or by both M1 and M2. We hypothesize that, upon interaction, the sRNA-mRNA complex is targeted for degradation by an RNase, leading to inhibition of a diverse set of ABC-type transport systems. To our knowledge, this is the first report demonstrating the precise mechanism in *Brucella* by which sRNAs interact with and bind to mRNAs. Most importantly, only the M2 sequence in the AbcR sRNAs is critical for the survival of the brucellae within macrophages and mice, highlighting the importance of relatively short sequence elements in mediating the host-bacterium relationship.

Materials and Methods

Bacterial strains and growth conditions

Brucella abortus strains were grown on Schaedler agar (BD, Franklin Lakes, NJ) supplemented with 5% defibrinated bovine blood (Quad Five, Ryegate, MT) (SBA) or in brucella broth (BD). For cloning, *Escherichia coli* strains (DH5α) were grown on tryptic soy agar (BD) or in Luria-Bertani broth. When necessary, the medium was supplemented with kanamycin (45 μg ml^{-1}) or ampicillin (100 μg ml^{-1}). All cultures of and experiments utilizing *Brucella* strains were performed in a biosafety level 3 (BSL-3) facility.

Brucella abortus strain constructions

(i) Wild-type plasmid constructs

The *B. abortus abcR1*, *abcR2*, and double *abcR1 abcR2* reconstruction and deletion plasmids were previously constructed (Caswell *et al.*, 2012a). To construct a *B. abortus bab2_0879* reconstruction plasmid, oligonucleotides were designed to amplify the wild-type locus, plus 1-kb upstream and 1-kb downstream, using *Brucella abortus* 2308 genomic DNA as a template and platinum Pfx polymerase (Invitrogen, Carlsbad, CA). Following amplification, the fragment was digested with BamHI and PstI, ligated into pNPTS138 (Spratt *et al.*, 1986), and transformed into *E. coli* DH5a. The resulting plasmid, pLS012, was sequenced by the Biocomplexity Institute (BI) at Virginia Tech.

(ii) Gene deletion plasmid constructs and *B. abortus* strains

The *B. abortus bab2_0612* and *bab2_0879* genes were mutated by an unmarked gene excision strategy as previously described (Caswell *et al.*, 2012). Briefly, 1-kb fragments

of the upstream and downstream regions of the genes were amplified by PCR using primers, genomic *B. abortus* 2308 DNA as a template, and platinum Pfx polymerase (Invitrogen). The upstream fragments were digested with the BamHI restriction enzyme, while the downstream fragments were digested with the PstI restriction enzyme. All fragments were phosphorylated with polynucleotide kinase (Monserate Biotechnology Group, San Diego, CA). Fragments were then combined in a single ligation mix with BamHI/PstI-digested pNPTS138 and T4 DNA ligase (Monserate Biotechnology Group). The resulting plasmids, pC^{3}041 (Δ*bab2_0612*) and pLS001 (Δ*bab2_0879*) were transformed into *B. abortus* 2308 by electroporation, and a merodiploid clone for each was obtained by selection on SBA plus kanamycin (as previously described in references Sheehan *et al.*, 2015). Kanamycin-resistant colonies were grown in brucella broth for 6 to 8 hours and then plated on plates with SBA plus 10% sucrose. Sucrose-resistant, kanamycin-sensitive colonies were screened by PCR for loss of either *bab2_0612* or *bab2_0879*. The isogenic *bab2_0612* mutant derived from *B. abortus* 2308 was named CC069, while the isogenic *bab2_0879* mutant was named LS006.

(iii) Site-directed mutagenesis of motifs in AbcR sRNAs

For all motif mutants, a protocol for site-directed mutagenesis was followed (QuikChange II XL site-directed mutagenesis kit; Agilent Technologies, Santa Clara, CA) with slight modifications. The *abcR1* and *abcR2* plasmids generated by Caswell *et al*., 2012a, (pC^{3}032 [RC-abcR1] and pC^{3}033 [RC-abcR2]) were used individually as templates for site-directed mutagenesis. For mutagenesis of the motifs in either *abcR1* or *abcR2*, overlapping oligonucleotides were designed which included either a mutated version of

the motif 1 (M1) nucleotide sequence (CUCCCA to UGAUAC) or a mutated version of the motif 2 (M2) nucleotide sequence (GUUCCC to ACGUAU). pC^{3}032, for *abcR1* mutagenesis, and pC^{3}033, for *abcR2* mutagenesis, were used as templates, and Pfurious DNA polymerase (Monserate Biotechnology Group) was utilized in subsequent PCRs. All plasmid products were then subjected to DpnI (Thermo Fisher Scientific, Waltham, MA) digestion to remove any remaining template DNA and were transformed into *E. coli* DH5a cells. The resulting plasmids were named pLS013 (*abcR1-M1^{mut}*), pLS014 (*abcR1-M2^{mut}*), pLS015 (*abcR2-M1^{mut}*), and pLS016 (*abcR2-M2^{mut}*). For generation of double-motif mutants, either pLS013 (*abcR1-M1^{mut}*) or pLS015 (*abcR2-M1^{mut}*) was used as a template, and primers for M2 mutagenesis were utilized. The resulting plasmid constructs were named pLS017 (*abcR1-M1/2^{mut}*) and pLS018 (*abcR2-M1/2^{mut}*).

Motif mutagenesis of *B. abortus abcR1* or *abcR2* was carried out using a *sacB* counterselection strategy, as described above. Plasmids pLS015 (*abcR2-M1^{mut}*), pLS016 (*abcR2-M2^{mut}*), and pLS017 (*abcR1-M1/2^{mut}*) were introduced individually into the *B. abortus abcR1 abcR2* double deletion strain (previously generated as described in reference 15 [CC097]) via electroporation. The pNPTS138 backbone plasmid contains a kanamycin resistance marker gene and a *sacB* gene for counterselection on sucrose. Following electroporation, merodiploid clones were obtained by selection on SBA-kanamycin. Single kanamycin-resistant colonies were picked, grown for 6 to 8 hours in brucella broth, and plated on SBA containing 10% sucrose. Colony PCR, utilizing confirmation oligonucleotides, was employed with sucrose-resistant, kanamycin-sensitive colonies for reconstruction of either the *abcR1* locus or the *abcR2* locus, and the resulting *B. abortus* strains were named LS030 (*abcR2-M1^{mut}*), LS038 (*abcR2-M2^{mut}*), and LS039

(*abcR1-M1/2*^{mut}). For generation of double *abcR1 abcR2* motif mutants, *B. abortus* strains LS030, LS038, and LS039 were subjected to electroporation with pLS013 (*abcR1-M1*^{mut}), pLS014 (*abcR1-M2*^{mut}), and pLS018 (*abcR2-M1/2*^{mut}), respectively. Following resolution of sucrose-resistant, kanamycin-sensitive colonies, PCR was employed to identify reconstruction of either the *abcR1* locus or the *abcR2* locus. The resulting *B. abortus* strains were named LS032 (*abcR1/2-M1*^{mut}; referred to here as *abcR-M1*^{mut}), LS040 (*abcR1/2-M2*^{mut}; referred to here as *abcR-M2*^{mut}), and LS041 (*abcR1/2-M1/2*^{mut}; referred to here as *abcR-M1/2*^{mut}). A schematic of the strains constructed can be found in Fig. 3B.

Site-directed mutagenesis of M2 in *bab2_0879*

Similar to the *abcR* site-directed mutagenesis, plasmid pLS012 (*bab2_0879*) was used as the template and Pfurious DNA polymerase was utilized for subsequent PCRs. Following plasmid mutagenesis, DpnI treatment was carried out and the plasmid was transformed into *E. coli* DH5α. For *bab2_0879*, the putative M2 site located in the 5' UTR was mutated from UAAGGG to UGCAUA. The resulting plasmid was named pLS023 (*bab2_0879-M2*^{mut}). Construction of a *B. abortus bab2_0879* M2 mutant strain was carried out with the *sacB* counterselection strategy as described above. For plasmid pLS023 (*bab2_0879-M2*^{mut}), electroporation was performed with *B. abortus* Δ*bab2_0879*. The resulting strain was named LS054 (*bab2_0879-M2*^{mut}). A schematic of the strains constructed can be found in Fig. 5A.

Northern blot analysis

RNA was isolated from *Brucella* strains grown in brucella broth as previously described (Caswell *et al.*, 2012). Briefly, 10 micrograms of RNA was 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). For identification of sizes, a low-molecular-weight ladder (New England Biolabs, Ipswich, MA) was radiolabeled with (γ-^{32}P)ATP (PerkinElmer, San Jose, CA, USA) and polynucleotide kinase (Monserate Biotechnology Group). Following electrophoresis, the radiolabeled ladder and RNA samples were transferred to an Amersham™ Hybond-N^{+} membrane (GE Healthcare, Piscataway, NJ) by electroblotting in 1 × TBE buffer for 2 hours. Following transfer, membranes were subjected to UV cross-linking to the membrane and then prehybridized in ULTRAhyb®-Oligo buffer (Thermo Fisher Scientific) for 2 hours at ~45°C in a rotating hybridization oven. The oligonucleotide probes (AbcR1/AbcR2 [for detection of both wild-type AbcR1 and wild-type AbcR2], AbcR1- M1mut [for detection of AbcR1 with M1 mutated], AbcR2-M1mut [for detection of AbcR2 with M1 mutated], AbcR1/AbcR2-M2mut [for detection of AbcR1 and AbcR2 with M2 mutated], and 5S-Northern [for detection of 5S ribosomal RNA]) were end labeled with [γ-^{32}P]ATP and polynucleotide kinase. The radiolabeled probes were incubated with the prehybridized membranes overnight (~12 hours). The following day, all membranes were washed four times for 30 minutes each time with 2 × SSC (1 × SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 1 × SSC, 0.5 × SSC, and 0.025 × SSC, each containing 0.1% sodium dodecyl sulfate (SDS), at ~45°C in a rotating hybridizing oven. All membranes were exposed to X-ray film and subsequently visualized by autoradiography.

Quantitative reverse transcriptase PCR (qRT-PCR)

RNA was isolated from *Brucella abortus* strains as previously described (Caswell *et al.*, 2012b). Briefly, *B. abortus* strains were grown to an appropriate phase of growth (late

exponential to early stationary phase) in brucella broth with constant shaking at 37°C. For storage, an equal volume of cold 1:1 ethanol-acetone was added to cultures and stored at 80°C for up to 1 month. For RNA isolation, the cell/ethanol-acetone mixtures were thawed for ~10 minutes at room temperature, and cells were pelleted at 16,000 × *g* for 3 minutes. RNA was isolated from cells by the use of TRIzol reagent (Invitrogen, Carlsbad, CA) and subsequent ethanol precipitation.

Following RNA isolation, genomic DNA was removed by use of DNase I (Thermo Fisher Scientific, Waltham, MA) as previously described (Caswell *et al.*, 2012). Briefly, 30 µg of RNA was incubated with DNase I at 37°C for 1 hour. Following incubation, samples were cleaned up by phenol-chloroform extraction and subsequent ethanol precipitation. All RNA samples were resuspended in nuclease-free H_2O, and their concentrations and purity were checked for by the use of a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific). All samples had an A_{260}/A_{280} ratio of ~2.0 with a yield of ~1 g μl^{-1}. Following isolation, cDNA was synthesized. For this, ~1 µg of RNA was added to 4 µl of 5 × qScript cDNA SuperMix (QuantaBio, Beverly, MA) and brought to a final volume of 20 µl with nuclease-free H_2O. The qScript cDNA SuperMix contained the following ingredients: optimized concentrations of $MgCl_2$, dATP, dCTP, dGTP, and dTTP; recombinant RNase inhibitor protein (RIP); qScript reverse transcriptase; and titrated concentrations of a random hexamer and oligo(dT) primer. The following PCR was carried out for RT of all samples: 5 minutes at 25°C; 30 minutes at 42°C; and 5 minutes at 85°C.Following RT-PCR, cDNA was utilized for quantitative PCR. As a control for all experiments, 16S rRNA primers were used. cDNA samples were diluted 1:50 in nuclease-free H_2O, and 2µl of diluted cDNA was added to 12.5 µl of 2 × iTaq Universal SYBR green

SuperMix (Bio-Rad, Hercules, CA) and 1.5 µl of 5 µM primer mixture (i.e., forward and reverse), and the reaction mixture was brought to a final volume of 25 µl with nuclease-free H_2O. The SuperMix contained iTaq DNA polymerase, deoxynucleoside triphosphates (dNTPs), $MgCl_2$, SYBR green I dye, enhancers, stabilizers, and a blend of dyes. Gene-specific primers were generated to assess the relative expression levels of *B. abortus* AbcR-specific targets (i.e., *bab1_0313*, *bab1_0314*, *bab1_1794*, *bab1_1799*, *bab2_0491*, *bab2_0612*, *bab2_0879*, and *bab2_1062*). All amplicon lengths were ~150 bp, and all samples were run in triplicate on quantitative PCR (qPCR) plates. qRT-PCR parameters included a single denaturing step for 3 minutes at 95°C, followed by 40 cycles (denaturing for 15 seconds at 95°C, annealing for 15 seconds at 51°C, and extension for 20 seconds at 72°C) of amplification. Fluorescence representing SYBR green incorporation was analyzed by the use of an iCycler machine (Bio-Rad, Hercules, CA), and the relative levels of mRNA abundance were subsequently assessed using the Pfaffl equation (Pfaffl, 2001).

Electrophoretic mobility shift assays (EMSAs)

All RNA was generated by *in vitro* transcription with the T7 RNA polymerase promoter and a MAXIscript T7 kit (Ambion, Austin, TX) as previously described (Caswell *et al.*, 2012b). Briefly, primers bab2_0879-T7-For and bab2_0879-T7-Rev were used to amplify 215-nucleotides corresponding to the 5' UTR and the beginning CDR of the *bab2_0879* mRNA. Similarly, primers abcR1-T7-For and abcR1-T7-Rev were used to amplify *abcR1* (100-nucleotides), and primers abcR2-T7-For and abcR2-T7-Rev were used to amplify *abcR2* (110-nucleotides). All fragments were amplified with Taq polymerase and cloned

into pGEM-T Easy vector (Promega, Madison, WI). Plasmid DNA was purified from putative clones and subjected to sequencing at the BI at Virginia Tech. For control experiments, the previously constructed plasmid with the 5' UTR of *babR* was utilized (Caswell *et al.*, 2012).

Single-stranded RNA was then generated according to the manufacturer's instructions. Briefly, the cloned fragments were excised from the pGEM-T Easy backbone by use of the restriction enzymes NcoI and NheI. Following purification of the fragments, *in vitro* transcription was carried out. For generation of radiolabeled probes, BAB2_0879 mRNA and BabR mRNA were uniformly incorporated with [γ-^{32}P]UTP (PerkinElmer).

For RNA-RNA binding assays, increasing amounts of either AbcR1 or AbcR2 were mixed with either radiolabeled BAB2_0879 mRNA or BabR mRNA. Binding reactions were performed in a 1 × structure buffer (Ambion) and tRNA (1 μg μl^{-1}) and were brought to a final volume of 20 μl with nuclease-free H_2O. All binding reactions were heated to 90°C for 2 minutes and then incubated at room temperature for 30 minutes. Binding reaction products were then subjected to electrophoresis on a native 6% polyacrylamide gel in 0.5 × TBE running buffer on ice for ~1 hour at room temperature. Following electrophoresis, gels were dried on 3MM Whatman paper for 1 hour at 80°C using a vacuum gel dryer system. Gels were then visualized by autoradiography.

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

To determine the virulence of the *Brucella abcR-M1^{mut}*, *abcR-M2mut*, and *abcR-M1/2^{mut}* strains, experiments using murine peritoneal macrophages were carried out as previously

described (Gee *et al.*, 2005). Murine macrophages were harvested from BALB/c mice and seeded into 96-well plates with Dulbecco's modified Eagle's medium (3 wells per *Brucella* strain) supplemented with 5% fetal bovine serum. Macrophages were then infected with opsonized brucellae at a multiplicity of infection (MOI) of 50:1 and were incubated at 37°C for 2 hours. Following incubation, infected macrophages were treated with gentamicin (50 μg ml^{-1}) for 1 hour to eliminate extracellular brucellae. Macrophages were then lysed with 0.1% deoxycholate, and serial dilutions were then plated on SBA to count CFUs. For the 24 hour and 48 hour time points, macrophages were washed once with PBS following gentamicin treatment, and fresh cell culture medium with gentamicin (20 μg ml^{-1}) was added to all macrophages. At the indicated time points, macrophages were lysed with 0.1% deoxycholate, and serial dilutions were plated on SBA in triplicate to count CFUs.

Determination of the ability of *Brucella* mutant strains to infect and colonize BALB/c mice was achieved as previously described (Gee *et al.*, 2005). Six-week-old female BALB/c mice were infected intraperitoneally with ~5 × 10^4 CFU of each *Brucella* strain suspended in sterile PBS. Following infection, mice were sacrificed at 1, 4, and 8 weeks post-infection, and spleen homogenates were serially diluted and plated on SBA. For the strains listed in Fig. 6 and 7B, five mice were sacrificed per strain per time point. For the strains listed in Fig. 7A, five mice were sacrificed per strain at the 4 week time point, and four mice were sacrificed per strain at the 8 week time point. All data presented were analyzed utilizing JMP 12.0.0 statistical software (SAS Institute, Cary, NC).

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Figures/Figure Legends

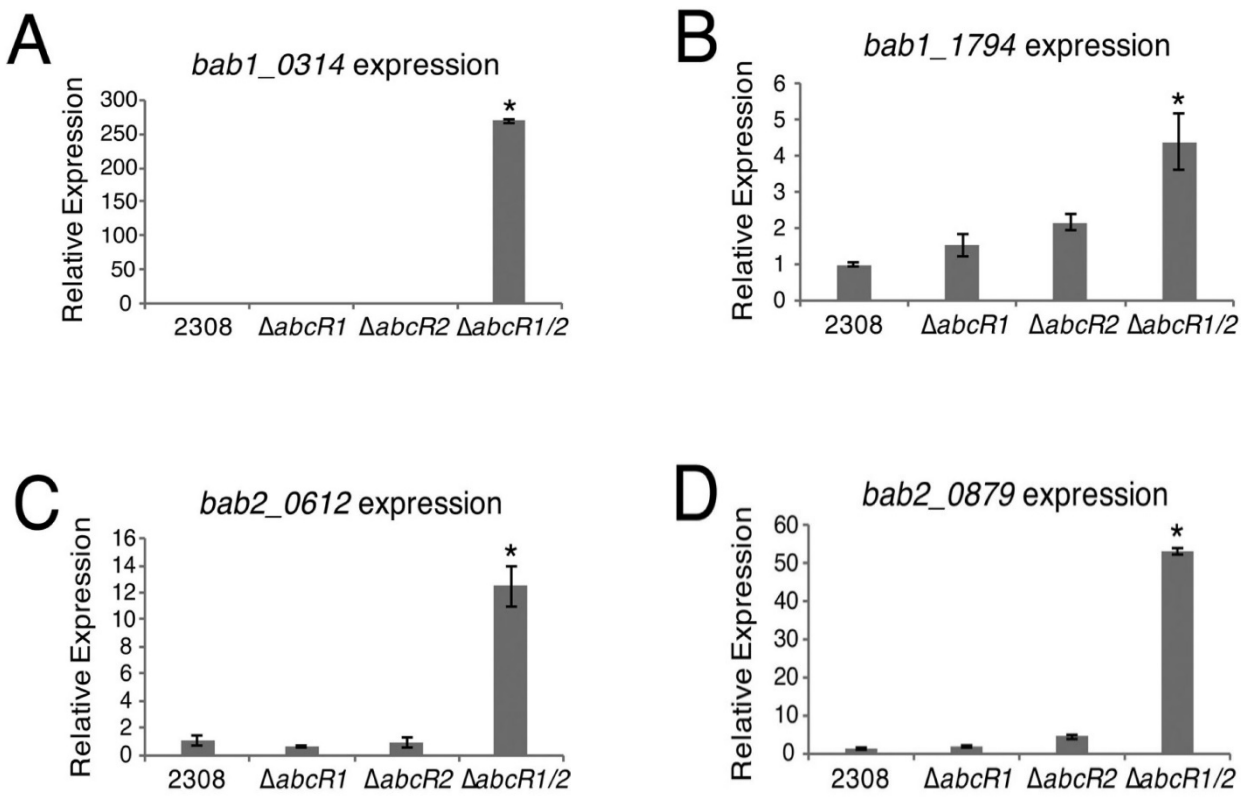

Figure 3.1. The AbcR sRNAs serve redundant regulatory functions in *Brucella abortus*

Brucella abortus 2308, Δ*abcR1*, Δ*abcR2*, and Δ*abcR1*/*2* strains were grown in brucella broth to an appropriate phase of growth, and RNA was extracted and reverse transcribed to cDNA. Quantitative reverse transcriptase PCR (qRTPCR) was carried out with gene-specific primers for amplification of **(A)** *bab1_0314*, **(B)** *bab1_1794*, **(C)** *bab2_0612*, and **(D)** *bab2_0879*. Amplification was achieved utilizing incorporation of SYBR green. Relative expression levels of the AbcR targets were normalized to that of *Brucella abortus* 2308, and 16S rRNA was used as a control. Data represent the average relative levels of expression from each *B. abortus* strain ± the standard deviations of results from triplicate samples. Statistical significance was evaluated using one-way analysis of variance (ANOVA) followed by the Tukey-Kramer posttest. Asterisks denote significance ($P < 0.0001$).

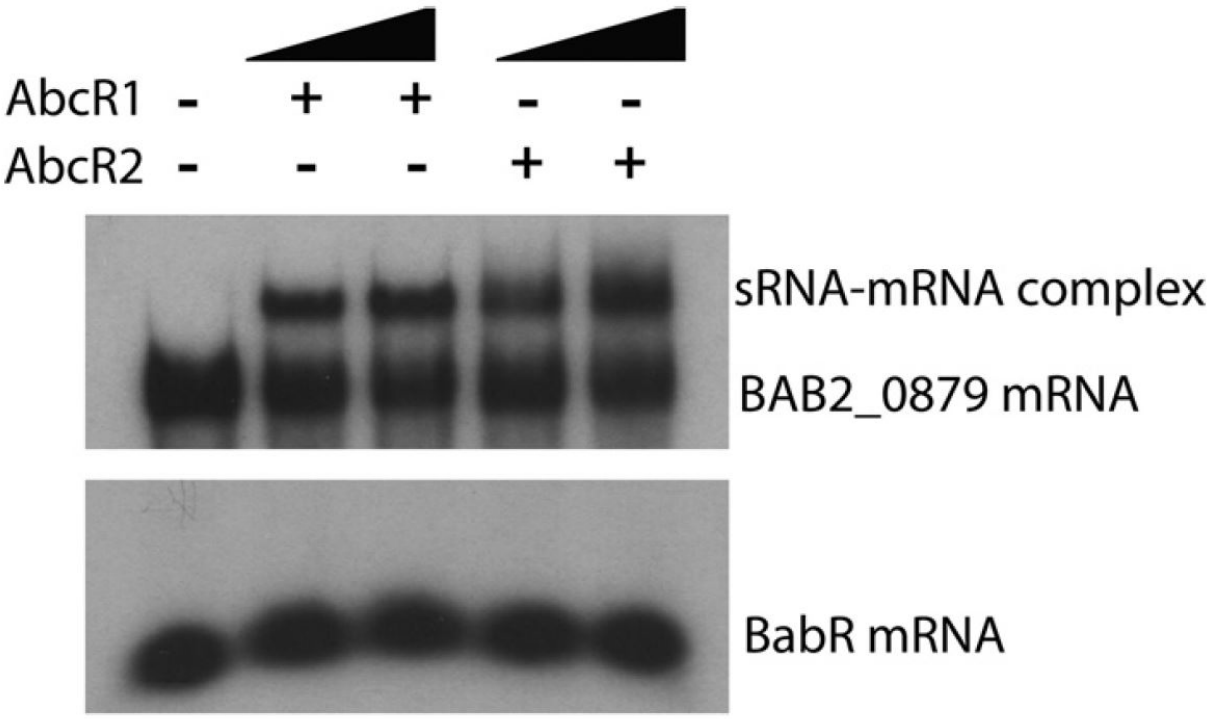

Figure 3.2. AbcR1 and AbcR2 bind to BAB2_0879 mRNA in a concentration-dependent manner

A. *In vitro* transcribed AbcR1 and AbcR2 were assessed for their ability to individually bind radiolabeled BAB2_0879 mRNA (~1 ng). Increasing amounts (500-fold to 1,000-fold excess) of either AbcR1 or AbcR2 were added to BAB2_0879 mRNA, and the reaction mixture was heated to 90°C for 2 minutes and then incubated at room temperature for 30 minutes.

B. Radiolabeled BabR mRNA (~1 ng) was used as a control.

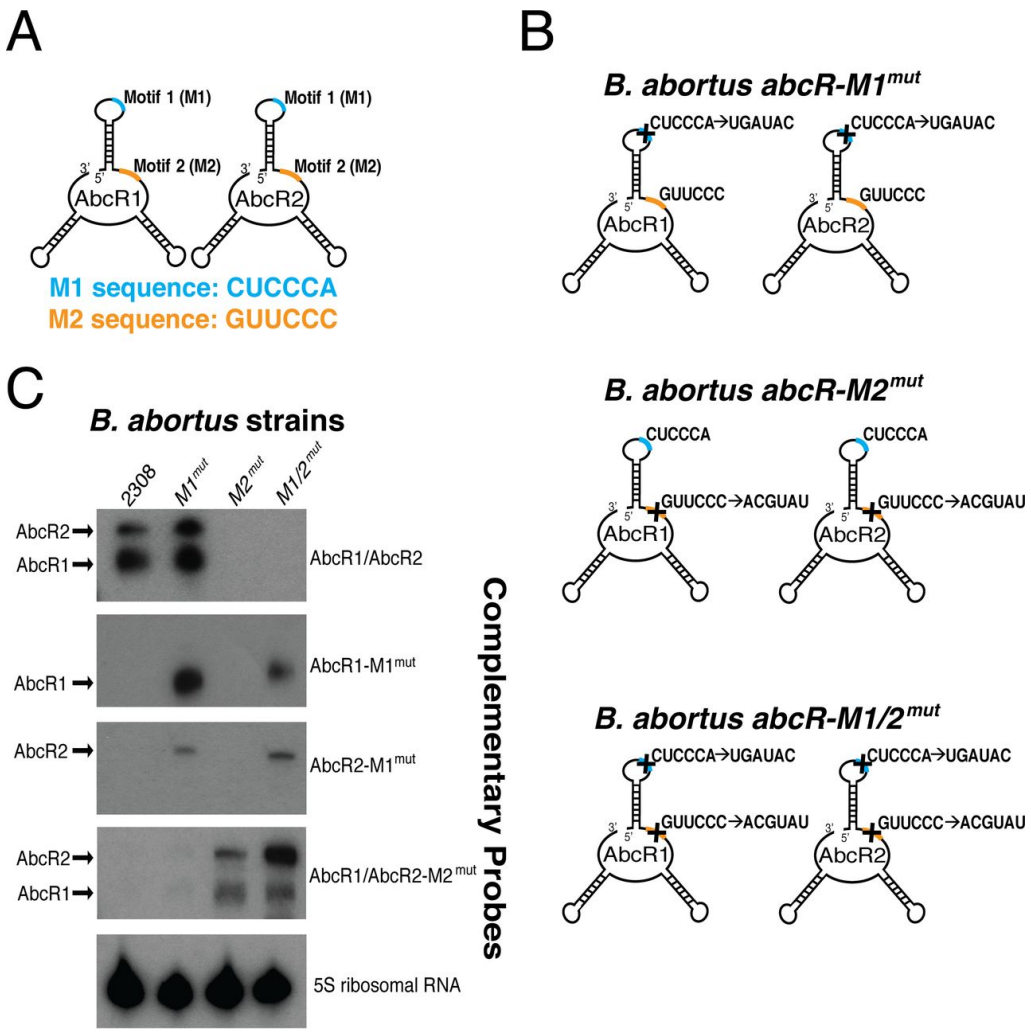

Figure 3.3. The AbcR sRNAs contain two putative 6-nucleotide binding motifs
A. Schematic of AbcR sRNAs encoding two conserved motifs: motif 1 (M1: CUCCCA) (shown in cyan) and motif 2 (M2: GUUCCC) (shown in orange). M1 is a 6-nucleotide single-strand RNA sequence, located in the first hairpin on the sRNAs. M2 is also a 6-nucleotide single-strand RNA sequence, located between the first and second hairpins.
B. To determine the regulatory capacity of M1 and M2 in the AbcR sRNAs, site-directed mutagenesis was performed. All *B. abortus* strain names are listed above a schematic of the AbcR mutagenesis strategy. A "**+**" denotes the location where mutagenesis was performed.
C. Northern blot analysis was employed to ensure that mutagenesis of AbcR sRNAs did not result in degradation. RNA from *B. abortus* 2308, *abcR-M1*^{mut}, *abcR-M2*^{mut}, and *abcR-M1/2*^{mut} was separated on a denaturing polyacrylamide gel and transferred to a nitrocellulose membrane. Here, the specific motif mutations were probed for. The wild-type AbcR1/AbcR2 probe was specific to AbcR1 and AbcR2 in *B. abortus* 2308 (first panel). It should be noted that this probe sequence contains wild-type M2 (i.e., GUUCCC) but not wild-type M1. Thus, *abcR-M1*^{mut} was detected.

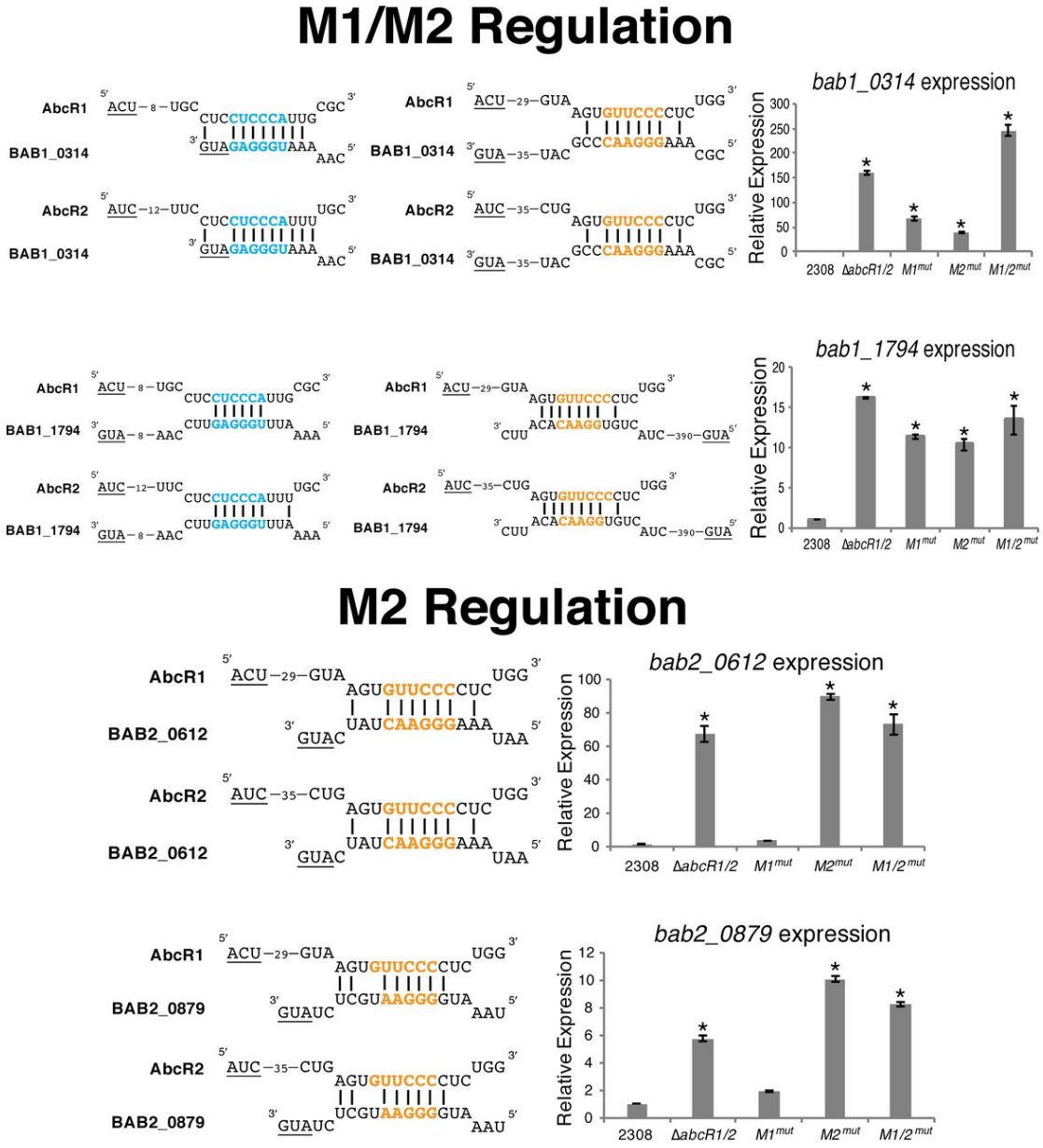

Figure 3.4. The AbcR sRNAs utilize M1 and/or M2 to regulate mRNA targets
To evaluate M1 and M2 in the regulation of AbcR-specific mRNA targets, qRT-PCR was employed on *B. abortus* 2308, Δ*abcR1*/*2*, *abcR-M1*^{mut}, *abcR-M2*^{mut}, and *abcR-M1*/*2*^{mut} for amplification of *bab1_0314*, *bab1_1794*, *bab2_0612*, and *bab2_0879*. Underlined nucleotides denote the start codon. M1 motifs are denoted by cyan nucleotides; M2 motifs are denoted by orange nucleotides. Relative expression levels of all genes were normalized to that of *B. abortus* 2308, and 16S rRNA was used as a control. Data represent the average relative levels of expression from each *B. abortus* strain ± the standard deviations of results from triplicate samples. Statistical significance was evaluated using one-way analysis of variance (ANOVA) followed by the Tukey-Kramer posttest. Asterisks denote significance ($P < 0.0001$).

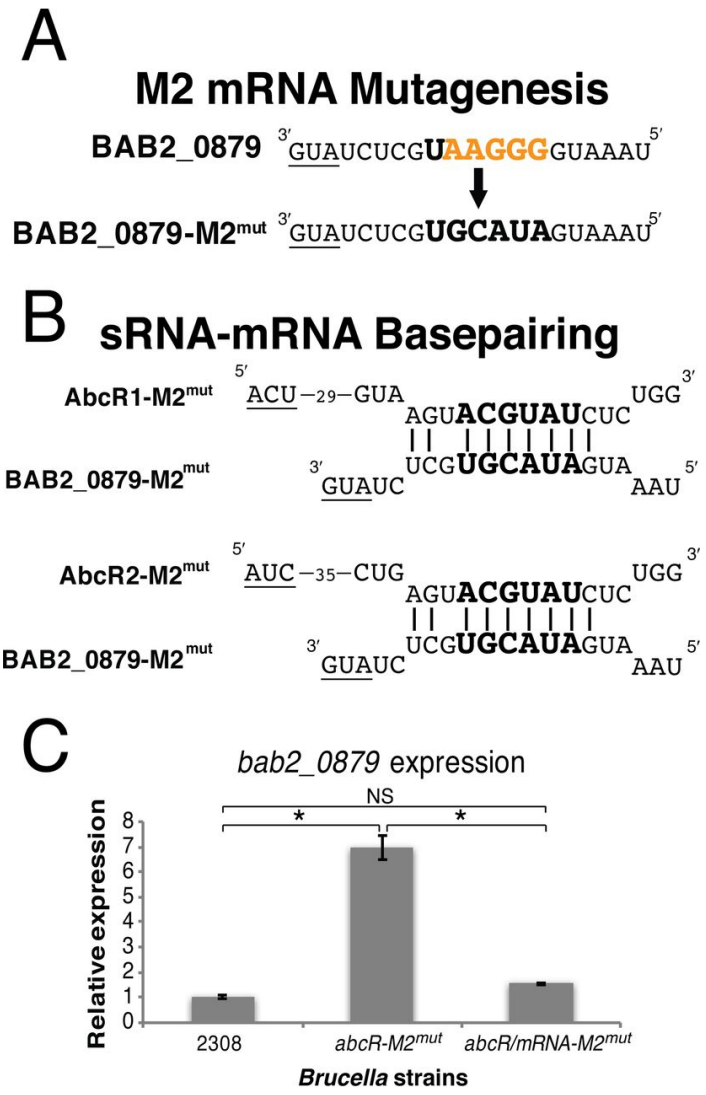

Figure 3.5. Mutagenesis of M2 in *bab2_0879* results in reestablishment of negative regulation in the *B. abortus abcR-M2^{mut}* strain

A. Illustration of site-directed mutagenesis carried out on the putative M2 site in *bab2_0879*. For this, the putative M2 site, located in the 5′ UTR of *bab2_0879*, was mutated from UAAGGG to UGCAUA.

B. This mutated sequence, which is complementary to M2 in the *abcR-M2^{mut}* strain, was predicted to result in the reestablishment of regulation by the AbcR sRNAs.

C. qRT-PCR was employed on *B. abortus* 2308, *abcR-M2^{mut}*, and *abcR*/*bab2_0879-M2^{mut}* for amplification of *bab2_0879*. Relative expression levels were normalized to that of *B. abortus* 2308, and 16S rRNA was used as a control. Data represent the average relative levels of expression from each *B. abortus* strain ± the standard deviations of results from triplicate samples. Statistical significance was evaluated using one-way analysis of variance (ANOVA) followed by the Tukey-Kramer posttest. Asterisks denote significance ($P < 0.0001$). NS, not significant.

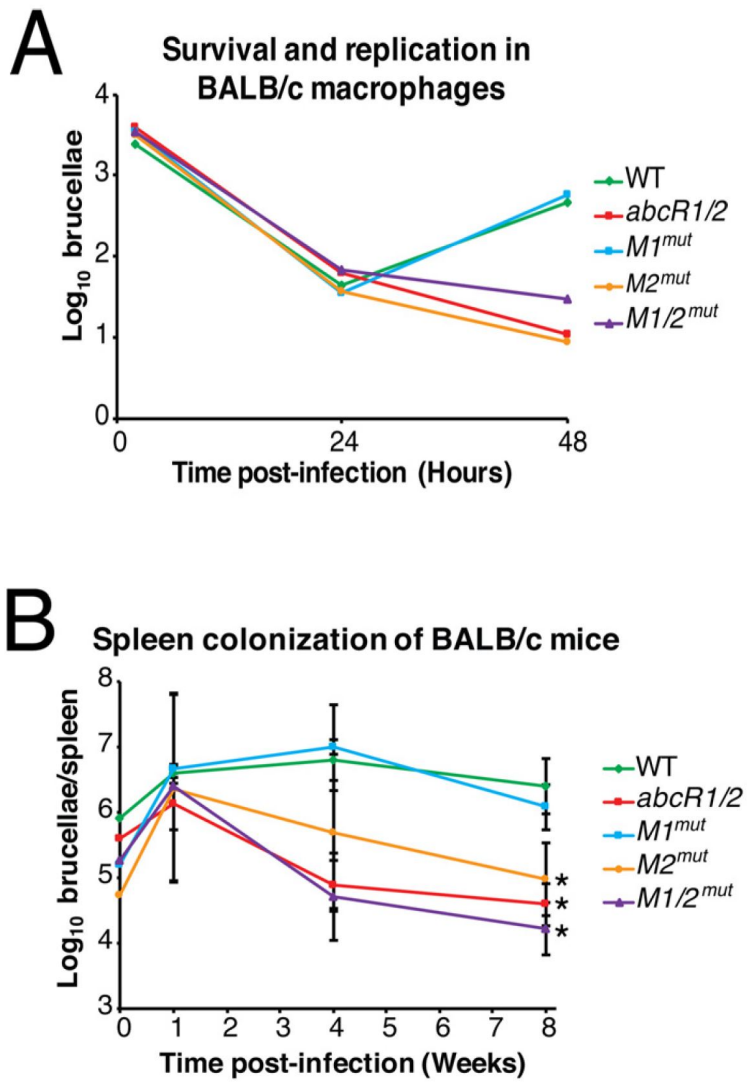

Figure 3.6. M2, but not M1, in the AbcR sRNAs is involved in *Brucella* pathogenesis
A. Naive, peritoneal BALB/c macrophages were infected with *B. abortus* 2308, Δ*abcR1/2*, *abcR-M1*^{mut}, *abcR-M2*^{mut}, or *abcR-M1/2*^{mut} to evaluate survival and colonization of the brucellae. At 2, 24, and 48 h postinfection, macrophages were lysed with deoxycholate, and serial dilutions were made on SBA to determine the number of intracellular brucellae. WT, wild type.
B. Spleen colonization of BALB/c mice by *Brucella abortus abcR* motif mutants. Six-week-old BALB/c mice were infected with *B. abortus* strains via intraperitoneal infections. At 1, 4, and 8 week postinfection, mice were sacrificed and spleen homogenates were serially diluted to determine the number of brucellae per spleen. Data represent average CFU counts per spleen ± the standard deviations of results from five mice infected with each *Brucella* strain. Statistical significance was evaluated using one-way analysis of variance (ANOVA) followed by the Tukey-Kramer posttest. Asterisks denote significance ($P < 0.05$).

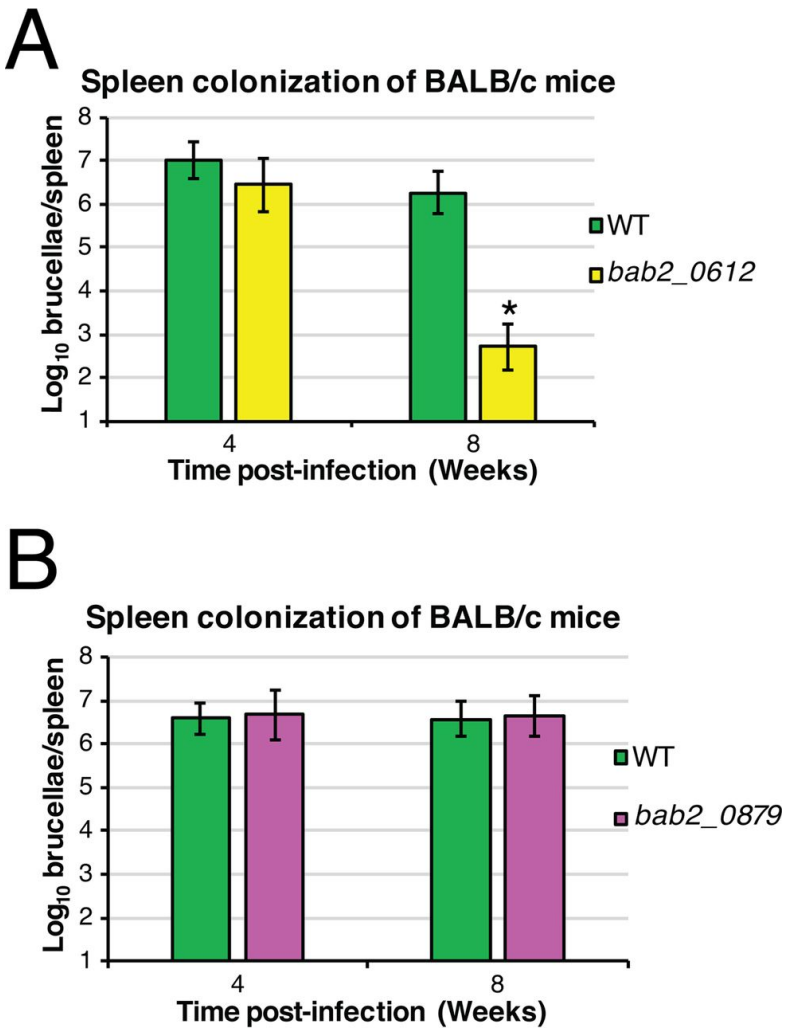

Figure 3.7. *Brucella abortus* Δ*bab2_0612* is unable to cause a wild-type chronic infection in BALB/c mice

A. Spleen colonization of BALB/c mice by *Brucella abortus* Δ*bab2_0612*. Six-week-old BALB/c mice were infected with *B. abortus* strains via intraperitoneal infections. At 4 and 8 weeks postinfection, mice were sacrificed and spleen homogenates were serially diluted to determine the number of brucellae per spleen. Data represent average CFU counts per spleen ± the standard deviations of results from five mice infected with each *Brucella* strain for the 4-week time point and from four mice infected with each *Brucella* strain for the 8-week time point. Statistical significance was evaluated using one-way analysis of variance (ANOVA) followed by the Tukey-Kramer posttest. Asterisks denote significance ($P < 0.05$).

B. Spleen colonization of BALB/c mice by *Brucella abortus* Δ*bab2_0879*. Six-week-old BALB/c mice were infected with *B. abortus* strains via intraperitoneal infections. At 4 and 8 weeks postinfection, mice were sacrificed and spleen homogenates were serially diluted to determine the number of brucellae per spleen. Data represent average CFU counts per spleen ± the standard deviations of results from five mice infected with each *Brucella* strain.

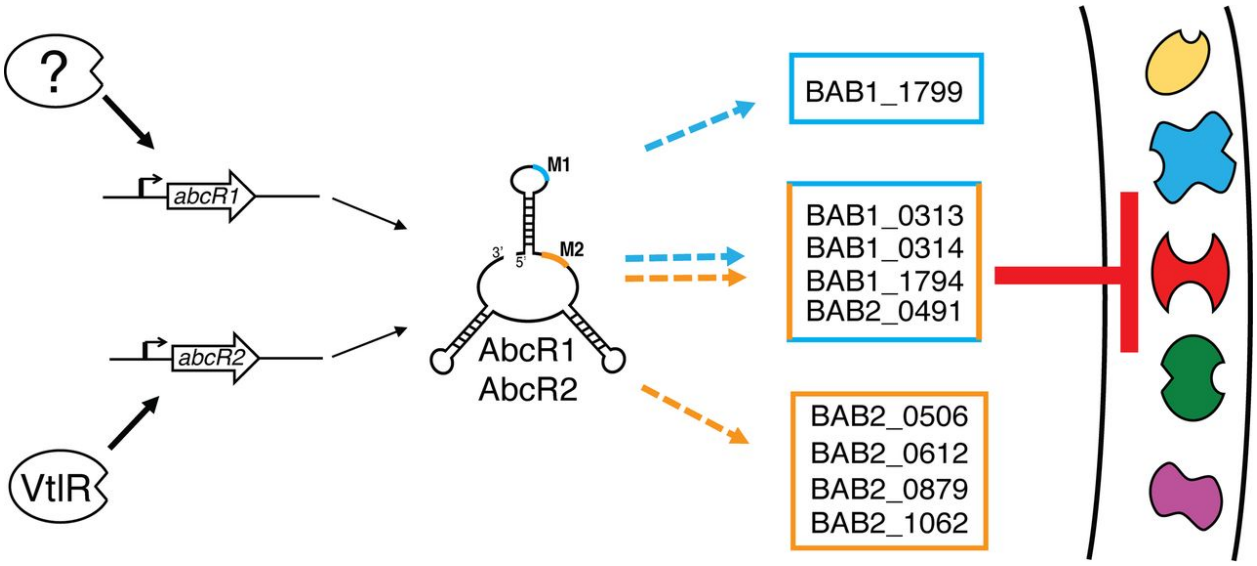

Figure 3.8. AbcR-mediated regulation in *Brucella abortus*
A working model illustrating AbcR sRNA regulation of mRNAs in *Brucella abortus* 2308 is shown. Following transcription of *abcR2* by VtlR and of *abcR1* by an unknown protein, the AbcR sRNAs utilize two conserved motifs, M1 (cyan) and M2 (orange), to bind and regulate target mRNAs, many of which encode components of ABC-type transport systems.

Tables

	***Brucella abortus* strains**				
	2308	**Δ*abcR1/2***	***M1*^{mut}**	***M2*^{mut}**	***M1/2*^{mut}**
bab1_0313	1.000	143.694	39.720	13.053	181.008
bab1_1799	1.000	1.737	2.291	0.756	2.474
bab2_0491	1.000	3.474	2.686	4.134	3.685
bab2_0506	1.000	2.164	0.990	2.562	2.116
bab2_1062	1.000	3.533	0.667	4.318	3.975
16S rRNA	1.000	0.989	0.983	0.978	0.999

Table 3.1. **qRT-PCR for additional targets regulated by the AbcR sRNAs**
qRT-PCR was carried out with *B. abortus* 2308, Δ*abcR1/2*, *abcR-M1*^{mut}, *abcR-M2*^{mut}, *abcR-M1/2*^{mut} to determine expression of the remaining mRNA targets previously identified by microarray analysis. Expression of all genes were normalized to *B. abortus* 2308, and 16S rRNA was used as a control.

Chapter 4. An account of evolutionary specialization: the AbcR small RNAs in the *Rhizobiales*

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Abstract

The AbcR small RNAs (sRNAs) are a fascinating example of two highly conserved sRNAs that differ tremendously at the functional level among organisms. From their transcriptional activation to their regulatory capabilities, the AbcR sRNAs exhibit varying characteristics in three well-studied bacteria belonging to the *Rhizobiales* order: the plant symbiont *Sinorhizobium meliloti*, the plant pathogen *Agrobacterium tumefaciens*, and the animal pathogen *Brucella abortus*. This review outlines the similarities and differences of the AbcR sRNAs between each of these organisms, and discusses reasons as to why this group of sRNAs has diverged in their genetic organization and regulatory functions across species. In the end, this review will shed light on how regulatory systems, although seemingly conserved among bacteria, can vary based on the environmental niche and lifestyle of an organism.

Introduction

Small RNAs (sRNAs) are regulatory components in prokaryotes that allow organisms to respond to stimuli through rapid alteration of gene expression. Importantly, sRNAs often exert their regulatory functions by interacting imperfectly with target mRNAs, either by binding to the untranslated region (UTR) or the coding region (CDR) of the target mRNA (Storz *et al*., 2006; Waters and Storz, 2009; Gottesman and Storz, 2011; Storz *et al*., 2011). The presence of regulatory sRNAs has been well documented in both Gram-positive (Johansson *et al*., 2002; Mann *et al.*, 2012; Soutourina *et al.*, 2013; Guillet *et al.*, 2013; Miller *et al.*, 2014) and Gram-negative (Hershberg *et al.*, 2003; Vogel, 2009; Postic *et al.*, 2010; Bardill and Hammer, 2012; Gómez-Lozano *et al.*, 2012; Schiano *et al.*, 2014; McClure *et al.*, 2014; Baddal *et al.*, 2015) bacteria. However, only a handful of sRNAs have been identified and characterized in *Alphaproteobacteria*, and in this class of bacteria, one predominant group of sRNAs is the αr15 family (del Val *et al.*, 2012).

The first members of the ar15 family, Smr15C2 and Smr15C1, were identified in 2007 in the plant symbiont *Sinorhizobium meliloti* (del Val *et al.*, 2007). ar15 sRNAs are around 100 nucleotides in length, and are commonly located within intergenic regions. The secondary structures are well conserved, generally folding into three stem loops. ar15 sRNAs are commonly trans-acting, regulating transcripts throughout the bacterial transcriptome (del Val *et al.*, 2012). Interestingly, the ar15 family has partial homology to the SuhB non-coding RNA, which is commonly maintained in several copies on the bacterial chromosome and has a secondary structure with at least one hairpin that displays a CU-rich anti-Shine Delgarno (anti-SD) sequence (Corbino *et al.*, 2005; del Val *et al.*, 2012).

The AbcR (ATP-binding cassette regulator) sRNAs, AbcR1 and AbcR2, are members of the ar15 family and have been identified throughout the *Rhizobiales* (del Val *et al.*, 2007; Ulvé *et al.*, 2007; Valverde *et al.*, 2008; Voss *et al.*, 2009; Schlüter *et al.*, 2010; Vercruysse *et al.*, 2010; Wilms *et al.*, 2011; Caswell *et al.*, 2012; Torres-Quesada *et al.*, 2013). *S. meliloti* has been predicted to have a third AbcR sRNA on the pSymA megaplasmid (del Val *et al.*, 2012), but no experimental evidence has validated this hypothesis. The AbcR sRNAs are interesting examples of extremely conserved and structurally similar sRNAs that have minor differences between organisms, which allow them to have vastly different regulatory roles. In this MicroReview, we will focus on discussing the AbcRs in three rhizobiales: *Sinorhizobium meliloti*, *Agrobacterium tumefaciens* and *Brucella abortus*.

Sinorhizobium meliloti

Lifestyle

Sinorhizobium meliloti is a plant symbiont, capable of forming mutualistic associations with leguminous plants, including those from the genera *Medicago*, *Melilotus* and *Trigonella* (Roumiantseva *et al.*, 2002). This bacterium lives either in soil, where it competes with organisms for essential nutrients, colonizes plants as an endophyte or resides within nodules in plant roots, where it relies on intracellular nutrients to survive and, in turn, fixes atmospheric nitrogen for the plant (Jones *et al.*, 2007; Pini *et al.*, 2012). *S. meliloti*, along with other rhizobial species, encodes around 200 ABC transport systems, allowing for the bacterium to take up nutrients, metals and other molecules that are necessary for its survival (Kaneko *et al.*, 2000; Cheng *et al.*, 2011). As these transport systems are critical to the longevity of *S. meliloti* both extra- and intracellularly, it is of interest to understand the regulatory circuitry that governs the expression of these transporters.

Expression of the AbcR small RNAs in *S. meliloti*

The AbcR sRNAs were among several sRNAs first identified by del Val *et al.*, in *S. meliloti* (del Val, 2007). Formally referred to as Smr15C2 and Smr15C1, the sRNAs are located in tandem between *smc01225*, encoding the LysR-type transcriptional regulator LsrB, and *smc01226*, encoding an ArsR-type transcriptional regulator (Fig. 1A). Smr15C2, named AbcR1, and Smr15C1, named AbcR2, are expressed independently and in response to various stimuli (Torres-Quesada *et al.*, 2013). Specifically, AbcR1 is predominantly expressed in early phases of growth and can be detected in high abundance in mature

M. sativa nodules. Conversely, AbcR2 reaches its highest expression at late stationary phase and is stress-induced (i.e., osmotic stress (NaCl), membrane stress (EtOH), low pH (5.6) and microaerobiosis). Although expression of each sRNA differs, it is interesting that the AbcR sRNAs are strikingly similar in both nucleotide sequence (85% identity) and predicted secondary structure. *S. meliloti* AbcR1 and AbcR2, in line with the ar15 sRNA family, are predicted to fold into 3 hairpins, where one hairpin exposes the ar15-conserved anti-SD UCCUCCC motif (Torres-Quesada *et al.*, 2013).

AbcR1 and AbcR2 possess both independent and redundant regulatory functions in *S. meliloti*

The first target experimentally confirmed to be negatively regulated by *S. meliloti* AbcR1, but not AbcR2, was LivK (Torres-Quesada *et al.*, 2013). LivK encodes for a periplasmic binding protein of an ABC transporter system, which is predicted to regulate the uptake of branched-chain amino acids. Although AbcR1 and AbcR2 possess almost identical anti-SD sequences, computational predictions of the sRNA-mRNA interactions found AbcR1 to have three additional complementary nucleotide sequences to the LivK mRNA sequence as compared to AbcR2 (Torres-Quesada *et al.*, 2013). Although additional AbcR targets were predicted in *S. meliloti*, the full regulatory profile of the AbcR sRNAs remains to be elucidated.

Many sRNAs depend on the global RNA chaperone Hfq for both their own stability and successful interactions with target transcripts (reviewed in Vogel and Luisi 2011). As such, it is important to note that the AbcR sRNAs are dependent on Hfq in *S. meliloti* (Torres-Quesada *et al.*, 2013). A deletion of *hfq* results in instability and subsequent

degradation of the sRNAs, thus resulting in absence of AbcR regulation. In *S. meliloti*, a study utilizing RNA-seq to analyze RNA populations following the co-immunoprecipitation (Co-IP) with Hfq reveled new targets of the AbcR sRNAs (Torres-Quesada *et al.*, 2014). In addition to the enrichment of LivK, the previously identified target of AbcR1, two additional targets were identified: SMa0495, encoding an ABC transporter amino acid binding protein; and PrbA, encoding an ABC transporter substrate-binding protein. SMa0495 and PrbA are down-regulated by both AbcR1 and AbcR2 (Torres-Quesada *et al.*, 2014). Computational bioinformatics predicted that the sRNAs bind the 5' UTRs, specifically in the ribosome-binding site. For interactions with PrbA, AbcR1 and AbcR2 are predicted to utilize an extended version of the previously mentioned anti-SD region, which contains a 6-nucleotide motif (CUCCCA) referred to as M1, located in the first hairpin. Conversely, the sRNAs are predicted to employ a 32-nucleotide motif, which contains a 6-nucleotide motif (GUUCCC) referred to as M2, for interaction with SMa0495 (Torres-Quesada *et al.*, 2014). Overall, these findings shed light on how AbcR1 and AbcR2, although strikingly similar in appearance, have significant differences in their regulatory profiles. It will be interesting to further define the *S. meliloti* AbcR regulatory profile, and determine the extent of regulatory redundancy and/or non-redundancy that exists between AbcR1 and AbcR2 in *S. meliloti.*

Agrobacterium tumefaciens

Lifestyle

Agrobacterium tumefaciens, a close relative of *S. meliloti*, is a plant pathogen that causes tumors, known as crown galls, in dicotyledonous plants. Following association with the plant, *A. tumefaciens* takes advantage of the plant by injecting a short piece of its DNA, known as T-DNA, into the host cells (Moore *et al.*,1997). This ultimately leads to tumor formation and the production/secretion of opines, which serve as energy sources for the bacterium. As a defense mechanism, wounded plants secrete a molecule called g-aminobutyric acid (GABA). GABA is taken up by an ABC transport system in *A. tumefaciens*, and can lead to disruption of bacterial cell-to-cell communication (Chevrot *et al.*, 2006; Haudecoeur *et al.*, 2009). Importantly, *A. tumefaciens* has been demonstrated to control the expression of this GABA transport system via the AbcR1 sRNA (Wilms *et al.*, 2011).

AbcR1 regulates the ABC transport system responsible for importing GABA

Wilms *et al*., was the first to begin mechanistic characterization of the AbcR sRNAs in *A. tumefaciens*. Identical to the organization of the AbcRs in *S. meliloti*, the *abcR1* and *abcR2* genes are in tandem on the circular chromosome, located in the intergenic region of *atu2186*, encoding a LysR-type transcriptional regulator, and *atu2187*, encoding a ArsR-type transcriptional regulator (Fig. 1B). In contrast to *S. meliloti*, AbcR1 and AbcR2 are expressed at similar levels in *A. tumefaciens* during different growth phases in rich medium (Wilms *et al.*, 2011).

Deletion of *abcR1* in *A. tumefaciens* leads to an abundance of two proteins: Atu1879 and Atu2422. Similar to the regulatory capacity of *S. meliloti* AbcR1, *A. tumefaciens* AbcR1 plays the predominant role in regulating these two targets (Wilms *et al.*, 2011). Both Atu1879 and Atu2422 putatively encode periplasmic components of ABC transport systems. Importantly, *A. tumefaciens* Atu2422 is the ortholog of *S. meliloti* LivK, a confirmed AbcR1 target. Moreover, Atu2422 was previously shown to be a GABA and proline transporter.

To investigate the link between GABA transport and AbcR1 regulation, uptake experiments utilizing radiolabeled GABA were conducted with *A. tumefaciens* strains. As expected, a deletion of *abcR1* resulted in increased import of GABA, due to the overproduction of the Atu2422 (Wilms *et al.*, 2011). Additionally, Atu2422 was previously demonstrated to function in the import of GABA and proline, and thus, excess amounts of unlabeled proline abolished uptake of radiolabeled GABA. Given this, it is not surprising that AbcR1 is critical to the process of GABA uptake as the sRNA downregulates the expression of Atu2422, reducing the uptake of GABA.

AbcR1 is involved in the complex regulation of myriad transcripts in *A. tumefaciens*

Subsequent proteomic and bioinformatic analyses demonstrates over 60 additional targets of AbcR1, the majority of which encode components of ABC transport systems (Overlöper *et al.*, 2014). Overall, AbcR1 regulation in *A. tumefaciens* is dynamic, where the sRNA is involved in both increased and decreased levels of transcripts. In some cases, AbcR regulation is governed by the growth phase of *A. tumefaciens*. For example, ChvE is activated by AbcR1 in exponential phase and repressed in stationary phase

(Overlöper *et al.*, 2014). ChvE is of particular interest, as this protein encodes a sugar-binding periplasmic protein a part of an ABC transport system. Importantly, ChvE is involved in *A. tumefaciens* pathogenesis, where it activates critical virulence cascades and transport systems (Winans *et al.*, 1986; Cangelosi *et al.*, 1990; Huang *et al.*, 1990; Shimoda *et al.*, 1990). These findings, in conjunction with AbcR1-mediated regulation of Atu2422, support the hypothesis that the AbcR system is involved in the pathogenesis of *A. tumefaciens*.

Through the use of CopraRNA, two binding motifs were identified in *A. tumefaciens* AbcR1 (Wright *et al.*, 2013). The first motif, called M1 (CUCCCA), is located in the first hairpin, and the second motif, called M2 (GUUCCC), is located in a single-stranded region between the first and second hairpins. M1 was previously shown to bind the anti-SD region of Atu2422 (Wilms *et al.*, 2011). For the additional targets identified, AbcR1 utilized M1 and/or M2 to bind the 5' untranslated region (5' UTR) or coding region (CDR) of transcripts (Overlöper *et al.*, 2014). Overall, AbcR1 is a predominant regulatory AbcR sRNA, and is involved in the regulation of both ABC transport system and virulence factors.

Brucella abortus

Lifestyle

Brucella spp. are pathogenic bacteria capable of causing serious infections in a wide range of animals, such as cattle, swine, goats, sheep and humans. In cattle, *Brucella abortus* results in spontaneous abortions, which can lead to substantial economic losses (Boschiroli *et al.*, 2001; Pappas *et al.*, 2005, 2006). In humans, brucellosis, the disease caused by *Brucella* spp., presents as an undulating fever with common flu-like symptoms (Franco *et al.*, 2007). On infection, the brucellae reside and replicate within host phagocytic cells, such as macrophages and dendritic cells. As such, eliminating the bacteria can prove to be difficult. Following entry into the host cell environment, the brucellae quickly and efficiently altering gene expression to adapt to the new environment by (Köhler *et al.*, 2003; Roop *et al.*, 2004). A common mechanism to shift their transcriptome to one that is favorable for their intracellular survival is through regulatory sRNAs.

AbcR1 and AbcR2 are functionally redundant in *B. abortus*

Unlike the chromosomal organization in *S. meliloti* and *A. tumefaciens*, the *B. abortus* AbcR sRNAs are encoded separately, with *abcR1* on chromosome II and *abcR2* on chromosome I. *abcR1* is located between *bab2_0515*, encoding a glycine decarboxylase and *bab2_0516*, encoding a small hypothetical protein. *abcR2* is positioned on the opposite strand from *bab1_1515* and *bab1_1516*, both of which encode small hypothetical proteins. *abcR2* is flanked by *bab1_1514*, encoding an aspartate aminotransferase, and *bab1_1517*, encoding the LysR-type transcriptional regulator VtlR

(Fig. 1C). VtlR, for virulence-associated transcriptional LysR-family regulator, is the bona fide transcriptional activator of *abcR2*, but not *abcR1* (Sheehan *et al.*, 2015). Although the genetic organization of the *B. abortus* sRNAs differs from other bacteria, a homologue of VtlR is found upstream of *abcR1* and *abcR2* in both *A. tumefaciens* and *S. meliloti*.

Similar to *S. meliloti* and *A. tumefaciens*, AbcR1 and AbcR2 are dependent on the RNA chaperone Hfq in *B. abortus*. In contrast to *A. tumefaciens*, *B. abortus* AbcR1 and AbcR2 are redundant in function, where only a double deletion of *abcR1* and *abcR2* results in reduced virulence (Caswell *et al.*, 2012). Given that AbcR1 and AbcR2 are functionally redundant, much emphasis has been directed to defining the molecular mechanism(s) of the redundancy between AbcR1 and AbcR2 in *B. abortus*.

B. abortus AbcR1 and AbcR2 share the same regulatory roles

Microarray and quantitative proteomic analyses of a *B. abortus abcR1 abcR2* double mutant found that the AbcR sRNAs negatively regulate over 20 transcripts, the majority of which encode components of ABC transport systems (Caswell *et al.*, 2012). Therefore, the AbcR regulatory profiles in *S. meliloti*, *A. tumefaciens* and *B. abortus* overlap, where the sRNAs largely regulate periplasmic proteins of ABC transport systems. In fact, one target, BAB1_1794 in *B. abortus*, is orthologous to LivK and Atu2422 in *S. meliloti* and *A. tumefaciens* respectively.

Quantitative reverse-transcriptase PCR with the *B. abortus* single and double *abcR* mutants found that the sRNAs are truly redundant, with the isogenic *abcR* mutants resulting in gene expression levels comparable to the wild-type strain, and a double *abcR* mutant leading to over-expression of target genes (Sheehan and Caswell, 2017).

Furthermore, AbcR1 and AbcR2 both physically bind to the 5' UTR of one of the targets, BAB2_0879, confirming the redundant roles these sRNAs have in *Brucella*. Thus far, it appears this redundancy in both regulation and function is restricted to the *Brucella* AbcR sRNAs.

AbcR1 and AbcR2 utilize two motifs, M1 and M2, to regulate transcripts

After determining the redundancy of the *B. abortus* AbcR sRNAs; two motifs, named M1 and M2, were identified in both AbcR1 and AbcR2 (Sheehan and Caswell, 2017). Similar to the organization in *A. tumefaciens* AbcR1, M1 (CUUCCC) is located in the first hairpin, and M2 (GUUCCC) is located in a single-stranded region between the first and second hairpin (Fig. 1C). Chromosomal mutations of either one or both motifs in the AbcR sRNAs found that the AbcRs utilize both motifs for their regulatory activity. Interestingly, the majority of transcripts tested were either regulated solely by M2, or by both M1 and M2. Only one target transcript, BAB1_1799, encoding a putative ABC transporter permease, was regulated by M1 alone. Conversely, four targets were regulated exclusively by M2, three of which encode putative ABC transporter periplasmic binding proteins (Sheehan and Caswell, 2017). Altogether, it seems that the AbcR sRNAs have retained almost identical motifs to carry out their regulatory roles in the *Rhizobiales* examined to date.

A *B. abortus abcR1 abcR2* double mutant has a severe virulence defect in macrophages and experimentally infected mice (Caswell *et al.*, 2012). Due to the role M1 and M2 play in regulation of transcripts, the single and double abcR motif mutants were tested to assess the role of these regulatory motifs in *Brucella* virulence. Surprisingly, while the *abcR-M1* mutant exhibited wildtype infection levels, the *abcR-M2* and *abcR-*

M1/2 mutant strains were attenuated in cells and mice (Sheehan and Caswell, 2017). This result was interesting, as both motifs in the sRNAs are necessary for the proper regulation of transcripts in *Brucella*. However, it stands to reason that one motif, M2, may be responsible for regulating targets that are directly or indirectly involved in pathogenesis. One of these M2-regulated targets, BAB2_0612, was found to be involved in *Brucella* pathogenesis, strengthening this hypothesis (Sheehan and Caswell, 2017), and will be interesting to determine if any other M2 targets are linked to *Brucella* pathogenesis.

The AbcR sRNA system in other members of the *Rhizobiales*

Although the AbcRs have been characterized extensively in three organisms, it is plausible that this sRNA family is conserved and functional in other bacteria in the *Rhizobiales*. A bioinformatics approach revealed the AbcR sRNA system in several other members of the *Rhizobiales* (del Val *et al.*, 2012; Fig. 2). In addition to the presence of one or both sRNAs, several of the AbcR targets and complementary binding motifs are found in several organisms including *Ochrobacterium anthropi*, *Bartonella henselae* and *Mesorhizobium loti*. Altogether, the AbcR system, from the LysR-type transcriptional regulator to the ABC-type transport systems, is very well conserved in this order of bacteria. The majority of ABC-type transporters regulated by the AbcR sRNAs are involved in nutrient acquisition, specifically by uptake of amino acids and polyamines (Wilms *et al.*, 2011; Caswell *et al.*, 2012; Torres-Quesada *et al.*, 2013; Overlöper *et al.*, 2014; Torres-Quesada *et al.*, 2014). It is likely this is a necessary mechanism for rhizobiales to quickly and efficiently turn-on or turn-off transporters when in certain environments (i.e., nutrient-rich vs. nutrient-limited).

ABC transport systems have been shown to play important roles in the formation of symbiotic relationships, specifically between *R. leguminosarum bv. viciae* and its pea plant host (Lodwig *et al.*, 2003). Mutations in two ABC-type broad range amino acid transporters result in defective symbiosis and the inability of the bacteriod to fix nitrogen. However, mutations of these two ABC transporters in *S. meliloti* do not have any effect on nitrogen fixation or symbiosis with alfalfa plants (Prell *et al.*, 2010). This brings up an interesting point regarding the AbcR-regulated transport systems in rhizobiales. Although deletion of the *abcR* sRNAs, and subsequent alternation of the expression of ABC

transport systems, in *S. meliloti* does not result in a symbiotic defect (Torres-Quesada *et al.*, 2013), this may not hold true in other symbionts such as *R. leguminosarum bv. viciae*. In this case, dysregulation of these ABC transport systems, which may play roles in proper nitrogen fixation, may lead to the inability of the bacterium to form a symbiotic relationship with its host. It will be interesting to see how the AbcR sRNAs effect nitrogen-fixation and subsequent symbiosis in various rhizobiales since there are obvious differences in the role ABC transporters play in their respective organism.

Another widely conserved sRNA that has been well studied and is involved in regulating nutrient acquisition systems is GcvB. First identified in *E. coli*, GcvB is a posttranscriptional sRNA regulator responsible for regulating genes involved in amino acid transport and metabolism (Urbanowski *et al.*, 2000). GcvB is an important regulatory component in many Gram-negative bacteria, tightly-controlling energy expenditure linked to the transport and biosynthesis of amino acids (McArthur *et al.*, 2006; Sharma *et al.*, 2007; Sharma *et al.*, 2011; Miyakoshi *et al.*, 2015). It seems likely that the AbcR sRNAs and GcvB are functional homologues, since both sRNAs are involved in regulating nutrient uptake in several bacteria. Moreover, at least one of the AbcR-targets in *B. abortus*, BAB1_1587, is shared with GcvB in *Salmonella* (Sharma *et al.*, 2011; Caswell *et al.*, 2012). Altogether, this conservation of sRNAs involved in the precise regulation of nutrient acquisition in Gram-negative bacteria is important for organisms to successfully adapt and survive in a variety of environment conditions.

The genetic organization of *abcR1* and *abcR2* in the *Rhizobiales*

The main difference in the genetic organization of the *abcRs* revolves around the location of the genes: are they in tandem or separated? Evolutionarily, this split is defined where *abcR1* and *abcR2* can be found in tandem in the *Rhizobiaceae* family (Sinorhizobium spp. and *Agrobacterium* spp.). In contrast, the *Brucellaceae* (*Brucella* spp. and *Ochrobacterum* spp.), *Bartonellaceae* (*Bartonella* spp.) and *Phyllobacteriaceae* (*Mesorhizobium* spp.) families have the *abcR* genes separated on their chromosomes (Fig. 2). This divergence of the sRNAs may prove to be beneficial for some organisms, such as *Brucella*, since the sRNAs are redundant in function and regulatory capacity (Caswell *et al.*, 2012; Sheehan and Caswell, 2017). If one AbcR sRNA is incapacitated (i.e., spontaneous mutation, loss of DNA, degradation of RNA, etc.), the second copy located in a difference genomic locus acts as a fail-safe. In contrast, full redundancy of the AbcRs is not observed in *S. meliloti* or *A. tumefaciens* (Wilms *et al.*, 2011; Torres-Quesada *et al.*, 2013; Torres-Quesada *et al.*, 2014; Overlöper *et al.*, 2014). Nonetheless, the AbcR sRNAs are critical regulatory components of gene expression in *Rhizobiales*, allowing organisms to adapt to and survive in specific environmental niches.

A LysR regulator is involved in the regulation of the AbcR sRNAs

Taken together, the AbcR sRNAs have been retained by multiple members of the *Rhizobiales*, but have diverged in their genomic location and regulation. VtlR, the activator of *abcR2* in *Brucella abortus*, is highly conserved among the *Alphaproteobacteria* (Sheehan *et al.*, 2015), and in the bacteria examined in this review, VtlR is always found upstream of either one or both sRNAs (Figs. 1 and 2). It is likely that this LysR-type regulator is involved in the regulation of either *abcR1* or *abcR2* across the *Rhizobiales*. In *S. meliloti*, LsrB (for LysR-type symbiosis regulator), the ortholog of VtlR, is critical for proper symbiosis between the bacterium and its plant host (Luo *et al.*, 2005). LsrB has been shown to be the transcriptional regulator of several genes involved in LPS biosynthesis, which is required for proper nitrogen fixation in alfalfa plants (Tang *et al.*, 2014). Analysis of the *S. meliloti abcR* promoter region revealed a putative LsrB-binding site, specifically upstream of *abcR1*. Along with preliminary evidence from our laboratory, it is likely LsrB is the transcriptional activator of *abcR1* in *S. meliloti*. However, since both sRNAs are transcribed individually and, in some cases, have differential expression in their respective organisms, what is the other abcR transcriptional regulator?

Unfortunately, this question remains to be elucidated. Analysis of the *abcR2* promoter regions in *S. meliloti* and *A. tumefaciens* revealed little nucleotide conservation, except in their -10 and -35 sites. These promoter regions, including *B. abortus abcR1*, share -10 and -35 motifs that resemble a sigma32 promoter sequence, which is designated RpoH, and interestingly, RpoH is known to be involved in the regulation of sRNAs in *S. meliloti* (Gruber and Gross, 2003; Barnett *et al.*, 2012). If the sRNAs are truly

dependent on this heat-shock sigma factor, this may add to the working model that the AbcRs are involved in aiding the bacterium during adaptive response to different environmental conditions. However, this observation is based solely on bioinformatics, and it will be interesting to experimentally define the transcriptional regulation of the *abcR* genes in each bacterium. Overall, the genetic organization and regulation of the AbcR sRNAs are well conserved, and major differences in the sRNAs underscore their diverse regulation in each bacterium.

The AbcR sRNAs have diverged immensely in their regulatory capacity

ABC transport systems are critical for organisms to import essential nutrients and export excess and/or toxic components (Davidson *et al.*, 2008; Wilkens, 2015), and the AbcR sRNAs are largely involved in the regulation of ABC transporters in *S. meliloti*, *A. tumefaciens* and *B. abortus* (Wilms *et al.*, 2011; Caswell *et al.*, 2012; Torres-Quesada *et al.*, 2013; Overlöper *et al.*, 2014; Torres-Quesada *et al.*, 2014). A handful of these AbcR target systems are conserved between two of the three bacteria. Only one of the AbcR targets, encoding an ABC transporter periplasmic binding protein (i.e., *S. meliloti* LivK, *A. tumefaciens* Atu2422 and *B. abortus* BAB1_1794) is conserved in all three bacteria. Moreover, the complementary AbcR M1 motif is also conserved. Another AbcR target, encoding a putative ABC transporter amino acid-binding protein, is conserved in *A. tumefaciens* (Atu1879) and *B. abortus* (BAB2_0612). However, AbcR regulation of this target has diverged. In *A. tumefaciens*, AbcR1 utilizes the M1 regulatory motif to bind Atu1879. In contrast, *B. abortus* AbcR1 and AbcR2 utilize the M2 regulatory motif to bind BAB2_0612. Although the AbcRs share similar targets among bacteria, they have diverged in how they mechanistically regulate these transcripts.

Aside from the different AbcR-regulated targets, there are also differences in their regulatory capacity. In *B. abortus* and *S. meliloti*, AbcR1 and AbcR2 are involved in the negative regulation of targets (Caswell *et al.*, 2012; Torres-Quesada *et al.*, 2013). In contrast, *A. tumefaciens* AbcR1 positively and/or negative regulates targets (i.e., FrcB and ChvE) (Overlöper *et al.*, 2014). In some cases, this regulation is dependent on the

phase of growth of *A. tumefaciens*. This growth-phase dependent regulation would prove to be beneficial, as AbcR1 can turn on and off targets at precise times, such as in soil versus during infection. In *Brucella*, it is hypothesized that some macrophage-derived signal drives the expression of the AbcR sRNAs, potentially directly through VtlR (Sheehan *et al.*, 2015). Going forward, it will be interesting to fully elucidate AbcR regulation in *S. meliloti*, as only three targets have been experimentally confirmed to date. Altogether, the AbcR sRNAs are involved in the regulation of numerous ABC transport systems that, in turn, allows the bacteria to precisely control the uptake of essential nutrients.

AbcR1 as the primary AbcR sRNA in the *Rhizobiaceae*

The most interesting molecular differences between the AbcRs are the two regulatory motifs, M1 and M2 (Fig. 1). These motifs were first identified in *A. tumefaciens*, where AbcR1 contains M1 (CUCCCA) in the first hairpin and M2 (GUUCCC) in the single-stranded region between the first and second hairpin (Wilms *et al.*, 2011; Overlöper *et al.*, 2014). Interestingly, *A. tumefaciens* AbcR2 is not involved in the regulation of transcripts, and while AbcR2 does contain an intact M2 motif, M1 is absent. This small difference may explain why AbcR2 is non-functional in *A. tumefaciens*. It will be interesting to learn if M1 could be incorporated into AbcR2 and restore its regulatory activity.

In *S. meliloti*, AbcR1 and AbcR2 both have regulatory capabilities, as both sRNAs contain M1 and M2 in their respective locations (Torres-Quesada *et al.*, 2013; Torres-Quesada *et al.*, 2014). However, M1 in AbcR2 has one slight difference: CUCCCA is changed to CUCCCC. Although small, this may explain why AbcR1 is the major regulatory AbcR in *S. meliloti*. Identification of additional AbcR-regulated targets, and subsequent examination of putative complementary motif sequences would reveal if this M1 mutation in AbcR2 is hindering the activity of the sRNA in *S. meliloti*.

Overall, the AbcR sRNAs are major players in the regulation of ABC-type transport systems and virulence factors (Wilms *et al.*, 2011; Caswell *et al.*, 2012; Torres Quesada *et al.*, 2013, 2014; Overlöper *et al.*, 2014). Although similar in nucleotide sequences and secondary structures, they have diverged among the rhizobiales to allow organisms to adapt to their specific environmental niches. The AbcR sRNAs are a perfect example of how seemingly conserved molecular systems have evolved greatly based on the lifestyle of an organism.

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Figures/Figure Legends

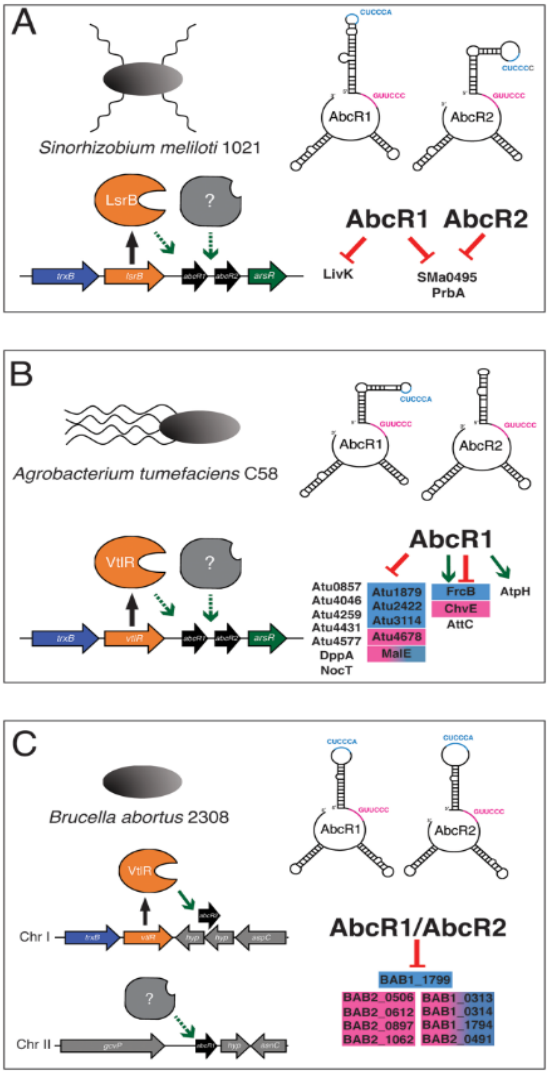

Figure 4.1. The AbcR sRNAs and their regulatory profiles in (A) *Sinorhizobium meliloti*, (B) *Agrobacterium tumefaciens* and (C) *Brucella abortus*

The AbcR sRNAs are encoded either in tandem (*S. meliloti* and *A. tumefaciens*) or on separate chromosomes (*B. abortus*). VtlR binds the promoter region of *abcR2*, and ongoing studies in our laboratory (represented by dashed arrows) support the hypothesis that Atu2186 (VtlR) and LsrB bind the promoter region of *abcR1*, but not *abcR2*. The second transcriptional regulator of the *abcRs* is currently unknown. The AbcR sRNAs all fold into similar three hairpin structures. M1, colored in blue, has the sequence CUUCCA. M2, colored in fuchsia, has a sequence of GUUCCC. Experimentally confirmed AbcR-targets in each organism are listed to the right. Targets in blue are regulated by M1. Targets in fuchsia are regulated M2. Targets in blue/fuchsia are regulated by M1 and M2. Targets in black have not been experimentally confirmed to be regulated by a specific AbcR-motif.

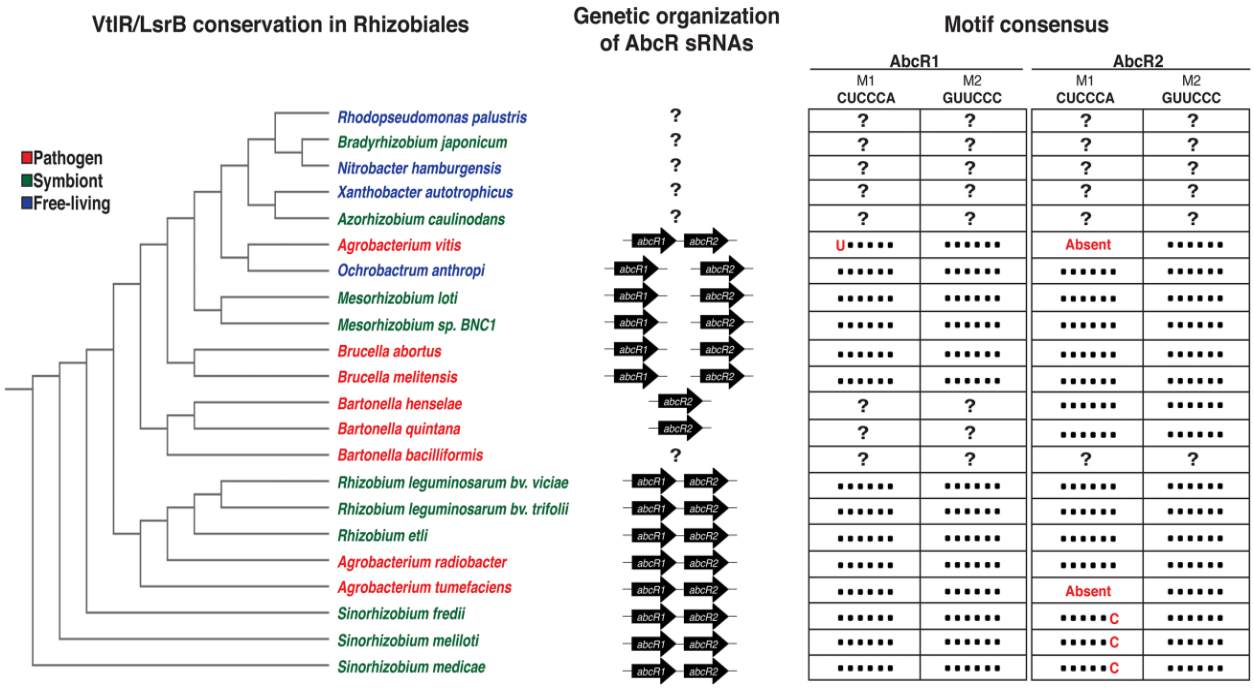

Figure 4.2. Conservation of the AbcR system in *Rhizobiales*
Left. Phylogenetic tree analyzing the conservation of a LysR-type transcription regulator commonly found upstream of one or both *abcR* sRNAs. This regulator, called VtlR for virulence-associated transcriptional LysR-family regulator, has been previously shown to positively regulate *abcR2* in *B. abortus* (Sheehan *et al*., 2015). Preliminary evidence has found *S. meliloti* LsrB (LysR-type symbiosis regulator) and *A. tumefaciens* VtlR to positively regulate *abcR1*.
Middle. The AbcR sRNAs can be found in tandem or separated in the *Rhizobiales*. For *R. palustris*, *B. japonicum*, *N. hamburensis*, *X. autotrophicus*, *A. caulinodans*, *B. henselae*, *B. quintana,* and *B. bacilliformis*, bioinformatics failed to identify the location(s) of one or both of the *abcRs*.
Right. The AbcR sRNAs contain two regulatory motifs called M1 (CUCCCA) and M2 (GUUCCC). Black circles represent consensus with these motifs. Differences are denoted by red text.

Chapter 5. Uncovering the regulatory mechanism of VtlR in the plant pathogen *Agrobacterium*

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* J.A.B and L.M.S contributed equally to this work, and first authorship was determined by rock, paper, scissor (best of three).

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 of *Alphaproteobacteria*, a conserved LTTR named VtlR is critical to the establishment of host-microbe interactions. In the pathogen *Brucella abortus*, VtlR is important for pathogenesis in a mouse model of infection and regulates the AbcR sRNA *abcR2* and three genes encoding small proteins. In the plant symbiont *Sinorhizobium meliloti*, the ortholog of VtlR, named LsrB, is involved in the symbiosis of the bacterium with alfalfa. A close relative of both, *B. abortus* and *S. meliloti*, is *Agrobacterium tumefaciens*, the causative agent of crown gall disease. Although VtlR is present in *A. tumefaciens*, it was unknown what role, if any, it has in gene regulation and/or pathogenesis. In the present study, we revealed how: 1) VtlR is essential for tumorigenesis; 2) VtlR regulates 250 genes in *A. tumefaciens*; 3) VtlR binds to and activates a novel small RNA named VrsA (VtlR-regulated sRNA). Altogether, although this LTTR is present and structurally conserved in *Alphaproteobacteria*, these data suggest that VtlR has diverged in its regulatory capacity to fit the needs of an organism's environmental niche.

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 and 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 N-terminal domain is most commonly found as a helix-turn-helix, and has a 'classic' LTTR-binding box sequence of TTA-$N_{7/8}$-TAA (Maddocks and Oyston, 2008). The C-terminal domain is the domain which separates LTTRs from one another. It is this domain that a specific substrate can bind to 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; Taylor *et al*., 2012; Liu *et al*., 2016; Privett *et al*., 2017), BenM and CatM from *Acinetobacter baylyi* (Ezezika *et al*., 2006; Craven *et al*., 2009; Ruangprasert *et al*., 2010), CbbR from *Rhodobacter sphaeroides* (Smith and Tabita, 2002; Tichi and Tabita, 2002; Dangel *et al*., 2005), and OccR from *Agrobacterium tumefaciens* (Wang *et al*., 1992; Akakura and Winans, 2002). Alternatively, LTTRs can also undergo conformational changes without binding of a substrate. This is the case with OxyR, a redox-sensing LTTR responsible for activating genes important for defending the bacterium against oxidative damage (Kullik *et al*., 1995; Zheng *et al*., 1998). Overall, LTTRs have been shown to play important regulatory roles in bacteria, allowing

organisms to sense environmental cues and, in turn, swiftly adjust gene expression through transcriptional activation and/or repression.

In the class of *Alphaproteobacteria*, one highly conserved LTTR has been shown to have a key part in an organism's lifestyle. 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*., 2015). In *B. abortus*, this LTTR, which is 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 mice and macrophages. Microarray analysis revealed *B. abortus* VtlR to activate the expression of three genes encoding for small proteins. In addition, VtlR was also found to positively regulate *abcR2*, a sibling small regulatory RNA (sRNA). The AbcR sRNAs have been well documented to be involved in nutrient acquisition in the *Rhizobiales* (Wilms *et al*., 2011; Caswell *et al*., 2012; Torres-Quesada *et al*., 2013; Becker *et al*., 2014; Overlöper *et al*., 2014; Torres-Quesada *et al*., 2014; Sheehan *et al*., 2015; Sheehan and Caswell, 2017).

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 (reviewed in Storz *et al*., 2011). Two sRNAs found throughout the order of *Rhizobiales* are the sibling AbcR sRNAs named AbcR1 and AbcR2 (reviewed in Sheehan and Caswell, 2018). The AbcR sRNAs regulate targets encoding ABC-type transport systems, many of which are responsible for obtaining nutrients in certain environmental conditions (Wilms *et al*., 2011; Caswell *et al*., 2012; Torres-Quesada *et al*., 2013; Becker *et al*., 2014; Overlöper *et al.*, 2014; Torres-Quesada *et al*., 2014; Sheehan and Caswell, 2017). Several of these mRNA targets encoding transport systems can be 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 extended back to the transcriptional regulation of the sRNAs (i.e., through VtlR/LsrB).

The present study aimed to characterize the LTTR VtlR in the plant pathogen *Agrobacterium tumefaciens*. Until now, it was unknown what virulence-associated role, if any, VtlR has in *A. tumefaciens*, and moreover, if VtlR is responsible for regulating one or both of the *abcR* sRNAs. Finally, although the regulatory capacity of the AbcR sRNAs has been studied in *A. tumefaciens*, this study asked the question of whether these sRNAs are necessary for host-microbial interactions. Altogether, the present study reveals how VtlR is necessary for the lifestyle of *A. tumefaciens* and is responsible for regulating over 250 genes in the pathogen. However, it is interesting to note that the regulatory role of VtlR differs dramatically from one bacterium to another, suggesting VtlR has evolved to meet the regulatory requirements of a particular microbe.

Results

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

The *vtlR* gene, designated as *atu2186,* is located on the circular chromosome of *A. tumefaciens* strain C58 (Fig. 1A). Directly upstream of *vtlR* is *trxB* (*atu2185*), a gene encoding a thioredoxin reductase. It is important to note that the genetic organization of *trxB* and *vtlR* is well conserved in the family of *Alphaproteobacteria*. Downstream of *vtlR* are the genes encoding the AbcR sRNAs, *abcR1* and *abcR2*.

In *B. abortus*, VtlR has been shown to positively regulate the tandemly encoded sRNA *abcR2* (Sheehan *et al*., 2015). In *A. tumefaciens*, both *abcR1* and *abcR2* are located directly downstream of *vtlR* (Fig. 1A). To test whether VtlR regulates *abcR1* and/or *abcR2*, northern blot analysis was performed to measure expression of AbcR1 and AbcR2 within wild-type *A. tumefaciens*, the isogenic deletion strains of *abcR1* and abcR2, a double deletion strain of *abcR1 and abcR2,* and an isogenic deletion strain of *vtlR* (Fig. 1B). These data revealed AbcR1 expression to be abolished in the *vtlR* mutant, while AbcR2 expression was unaffected, indicating that VtlR positively affects the expression of AbcR1.

In the plant symbiont *S. meliloti*, a deletion of *lsrB* results in a significant growth defect (Luo *et al*., 2005; Tang *et al*., 2014). To assess the affect a deletion of *vtlR* has on the growth of *A. tumefaciens*, the *vtlR* deletion strain, along with the *abcR* deletion strains, were grown in a nutrient-rich medium, and 12-hour time-points were taken to examine growth over time (Fig. 1C). Similar to *S. meliloti*, *A. tumefaciens vtlR* displays a growth defect and small colony phenotype when compared to the parental strain C58 (Fig. 1C

and 1D). Isogenic deletions of *abcR1* and *abcR2*, and a double deletion of *abcR1* and *abcR2,* resulted in no differences in *A. tumefaciens* growth or colony sizes compared to wild-type (Fig. 1C and 1D). The *A. tumefaciens vtlR* growth defect can be rescued by in-trans complementation with the plasmid pSRK-Km (Khan *et al*., 2008) harboring an IPTG-inducible wild-type *vtlR* gene (Fig. 1D). As a control, *A. tumefaciens vtlR* harboring an empty pSRK-Km plasmid showed no difference in growth compared to *A. tumefaciens vtlR* (Fig. 1D). Altogether, these data revealed how VtlR positively regulates the sRNA AbcR1, and that VtlR is critical to the growth of *A. tumefaciens*.

VtlR is critical to the virulence of *A. tumefaciens*

VtlR was previously shown to be necessary for the symbiosis and pathogenesis of *S. meliloti* (Luo *et al*., 2005) and *B. abortus* (Sheehan *et al.*, 2015). However, it was unknown what impact VtlR has on *Agrobacterium* tumorigenesis. To assess this, *A. tumefaciens vtlR* was tested for its ability to form tumors in experimentally-infected potatoes (Fig. 2). Compared to wild-type, the *vtlR* deletion strain caused significantly less tumors post-infection. In addition, no other strains tested (*abcR1* isogenic mutant, *abcR2* isogenic mutant, or *abcR1/2* double mutant) were attenuated compared to wild-type. These data, which highlight the importance of VtlR for microbe-host interactions, are in agreement with previous studies on VtlR in other *Rhizobiales* (Luo *et al.*, 2005; Sheehan *et al*., 2015).

AbcR1 primarily regulates ABC-type transport systems

To better understand the role of VtlR in *A. tumefaciens* pathogenesis, we sought next to define the regulons of VtlR, AbcR1, and AbcR2. RNA-sequencing (RNA-seq) was carried

out to compare RNA expression from *vtlR*, *abcR1*, and *abcR2* to the parental strain C58. Although AbcR1 is despensible for *A. tumefaciens* pathogenesis, the regulon of AbcR1 is robust with almost 100 genes differentially expressed ≥3 fold when compared to wild-type (Table 1). In comparison, the AbcR2 regulon was much smaller, with only 8 genes differentially expressed in the deletion strain, half of which encode for hypothetical proteins (Table 2). The importance of AbcR1 over AbcR2 regulation is in line with previous studies showing AbcR1 as the primary regulatory sRNA in *Agrobacterium* (Wilms *et al*., 2011; Overlöper *et al.*, 2014). Overall, a majority of genes differentially expressed in *abcR1* were components of ABC-type transporters and membrane proteins (56%), followed by hypothetical proteins (22%), and genes involved in enzymatic processes (18%) (Fig. 3A). Since VtlR is the activator of *abcR1* in *A. tumefaciens*, the AbcR1 regulon was then compared to the VtlR regulon to gain insight into the phenotypic differences between the strains.

The *A. tumefaciens* VtlR 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 genes dysregulated in the *A. tumefaciens vtlR* deletion strain. Genes differentially expressed ≥3 fold in *vtlR* can be found in Table 3, and an outline of functional distribution of differentially expressed genes is depicted in Fig. 3B. Of these dysregulated genes, one third represented membrane proteins and transport systems, one third represented hypothetical proteins, and roughly a fifth represented genes involved in enzymatic processes (Fig. 3B).

A comparison of the VtlR and AbcR1 regulons shed light on the dependent and independent regulatory functions each possessed (Fig. 3C). Importantly, the VtlR regulon was found to be significantly larger than the AbcR1 regulon, with 254 dysregulated genes in *vtlR* compared to 95 dysregulated genes in *abcR1*. Approximately half of the genes differentially expressed ≥3-fold in *abcR1* were also differentially expressed in *vtlR*. However, lowering this threshold to ≥2-fold results in the vast majority of dysregulated targets from the AbcR1 regulon to be also found in the VtlR regulon. Altogether, these data reveal how >150 genes could potentially be regulated by VtlR in an AbcR1-independent manner.

A. tumefaciens VtlR binds to and activates the sRNA *abcR1* and a hypothetical protein *atu1667*

To further characterize the regulatory mechanism of *A. tumefaciens* VtlR, electrophoretic mobility shift assays (EMSAs) were employed with putative targets. As expected, VtlR bound to the promoter region on *abcR1* in a concentration-dependent manner (Fig. 4A), which demonstrated VtlR to be the bonafide activator of the sRNA *abcR1* in *A. tumefaciens*.

In *A. tumefaciens*, the gene *atu1667* encodes for a small hypothetical protein, which is orthologous to *bab1_0914* and *bab2_0512* in *B. abortus*. Since both *bab1_0914* and *bab2_0512* are transcriptionally activated by VtlR in *B. abortus*, it was hypothesized that *A. tumefaciens* VtlR also activates the expression of *atu1667* (Sheehan *et al.*, 2015). To test this, northern blot analysis analysed the expression of Atu1667 in *A. tumefaciens vtlR* deletion strain (Fig. 4B). It was found that Atu1667 expression was significantly

decreased in *A. tumefaciens vtlR* compared to the parental strain, suggesting that VtlR activates expression of this gene. To test the specificity of the interaction between VtlR and *atu1667*, EMSAs were performed with the promoter region of *atu1667* and recombinantly purified *A. tumefaciens* VtlR (rVtlR) (Fig. 4C). EMSAs revealed *A. tumefaciens* rVtlR to bind directly to the promoter region, demonstrating VtlR as the activator of *atu1667*.

To identify other genes directly regulated by VtlR, EMSAs were performed on promoter regions of genes most differentially expressed in *vtlR*. EMSAs were performed on the promoter regions of *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*. As a positive control, the promoter of *abcR1* was utilized. However, *A. tumefaciens* rVtlR did not directly bind to any of these promoter regions aside from *abcR1* (data not shown).

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

Due to the failure of identifying direct regulation by VtlR, a bioinformatics approach was employed to determine putative binding regions using a putative VtlR-binding consensus sequence identified upstream of *abcR1* and *atu1667* (Fig. 4D). This hypothetical VtlR consensus sequence was utilized to identify other potential VtlR binding sites in the *A. tumefaciens* genome. Putative VtlR sites were identified through the use of the online service Virtual Footprint (Münch *et al*., 2005). Surprisingly, an intergenic region flanked by *atu4669* and *atu4670* displayed a match to the *abcR1/atu1667* VtlR-binding consensus

sequence (referred to as *IG69/70*; Fig. 5A and 5B). Subsequently, an EMSA was performed with rVtlR and intergenic DNA, which encompassed the *atu4669* and *atu4670* intergenic region (Fig. 5C). rVtlR was shown to bind to this intergenic region (*IG69/70*).

Importantly, the promoter regions of *atu4669* and *atu4670* are not in this intergenic region; in contrast, this intergenic region is located between the 3' ends of both *atu4669* and *atu4670* (Fig. 5B) 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 a gene was transcribed in this region, northern blot analysis was carried out with RNA isolated from C58 and *vtlR.* Northern blot analysis revealed the presence of a ~300 nucleotide transcript antisense to *atu4670* (Fig. 5D). Furthermore, the expression of this transcript was abolished in *vtlR*. The evidence of direct binding of rVtlR to *IG69/70* and decreased expression of the newly identified transcript in the *vtlR* mutant suggested *IG69/70* to be under the direct regulation of VtlR in A. *tumefaciens*. Thus, we have named this new small RNA VrsA, for VtlR regulated small RNA.

Heterologous complementation of *A. tumefaciens* Δ*vtlR* with *S. meliloti lsrB* or *B. abortus vtlR* restores small colony phenotype, but only *lsrB* fully restores Δ*vtlR* growth defect

Although VtlR/LsrB are conserved in the *Rhizobiales*, it was unknown if this LTTR was functionally orthologous in all organisms. Upon comparison of the protein sequences of *A. tumefaciens* VtlR and *S. meliloti* LsrB, the proteins share over 88% identity in amino acid sequence, whereas *A. tumefaciens* VtlR and *B. abortus* VtlR only share 68% amino

acid sequence identity. Furthermore, there have been differences noted in the growth of an organism when *vtlR*/*lsrB* is absent. In *S. meliloti*, a deletion of *lsrB* resulted in a severe growth defect in rich medium (Luo *et al*., 2005). In contrast, there is no difference in the growth kinetics of *B. abortus* when *vtlR* is deleted (Sheehan *et al*., 2015). Because of these differences, it was hypothesized that heterologous complementation of *A. tumefaciens vtlR* with *lsrB* from *S. meliloti*, but not *vtlR* from *B. abortus,* would result in restoration of wild-type growth kinetics.

vtlR from *A. tumefaciens*, *vtlR* from *B. abortus,* and *lsrB* from *S. meliloti* were individually cloned into the expression vector pSRK-Km, and the *A. tumefaciens vtlR* deletion strain was transformed with the newly constructed plasmids. When plated on AT agar, wild-type colony size was restored with complementation of all three *vtlR* genes (Fig. 6A). This revealed the ability of *vtlR* from *A. tumefaciens*, *vtlR* from *B. abortus,* and *lsrB* from *S. meliloti* to successfully complement the small colony phenotype of *A. tumefaciens vtlR*.

To assess the ability of VtlR orthologs to complement the A. *tumefaciens vtlR* growth defect, growth curves were conducted in rich medium with *A. tumefaciens* heterologously complemented strains (Fig. 6B). As expected, the growth defect displayed by *A. tumefaciens vtlR* was not complemented by the presence of an empty pSRK-Km plasmid or the pSRK-Km plasmid harboring *vtlR* from *B. abortus*. However, *A. tumefaciens vtlR* harboing the pSRK-Km plasmid with *lsrB* from *S. meliloti* restored the strain to parental growth (Fig. 6B). Current experiments are examining the ability of these heterologous complements to restore the ability of *A. tumefaciens* Δ*vtlR* to cause tumorigenesis in experimentally-infected potatoes.

Discussion

In the present study, we have characterized a conserved LTTR named VtlR in the plant pathogen *A. tumefaciens* by showing its necessity for virulence, determining its regulon, and defining its mechanism of regulation (Fig. 1-5; Table 3). Furthermore, this study revealed similarity and differences amongst three VtlR/LsrB orthologs from *A. tumefaciens*, *S. meliloti*, and *B. abortus* (Fig. 6). This study also aimed to further characterize the regulatory capabilities of the AbcR sRNAs and to determine what role, if any, they have in *Agrobacterium* pathogenesis (Fig. 2-3; Tables 1-2).

The requirement of *vtlR* in *A. tumefaciens* pathogenesis resembles what was previously reported in two other rhizobiales, *B. abortus* and *S. meliloti* (Fig. 2; Luo *et al*., 2005; Sheehan *et al*., 2015). However, the dispensability of *A. tumefaciens abcR1* and *abcR2* during host-microbe interactions and during growth differs from what has been observed in *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). In *A. tumefaciens*, AbcR1 and AbcR2 are not functionally redundant, and moreover, only AbcR1 is responsible for regulating transcripts. In *A. tumefaciens* AbcR1, two RNA-binding motifs, named M1 (for motif 1) and M2 (for motif 2), are utilized by the sRNA for interactions with target mRNAs (Overlöper *et al*., 2014). The reason AbcR2

lacks regulatory functionality may have to do with the absence of M1 (Sheehan and Caswell, 2018). Altogether, data from this study supports previous work, where RNA-seq analysis found a deletion of *abcR1*, not *abcR2*, to lead to massive gene dysregulation (Overlöper *et al*., 2014; Fig. 3; Tables 1-2).

Although deletion of *abcR1* did not result in any phenotypic differences with regards to growth, virulence, biofilm formation, or motility, we sought to further analyze the AbcR1 regulon (Fig. 3; Table 1). RNA-seq uncovered 95 differentially expressed genes (≥3-fold) in the *abcR1* deletion strain compared to C58. Previously, Overlöper and colleagues utilized proteomic and bioinformatics analyses to characterize 16 targets of AbcR1, the majority of which were components of ABC-type transport systems (e.g., *chvE*, *malE*, *atu2422*, *atu4678*, and *atu1879*) (Overlöper *et al*., 2014). Our transcriptomic data largely resembles previous proteomic data (Table 1). However, aside from the ABC-type transport systems, RNA-seq revealed additional AbcR1 targets such as genes encoding transcriptional regulators, chemoreceptors, and enzymes. Remarkably, several genes previously shown to be necessary for *A. tumefaciens* virulence, including *chvE* and *attC*, are dysregulated in the *abcR1* strain (Kemner *et al.*, 1997; Matthysse *et al*., 2008) (Table 1). Yet, a deletion of *abcR1* does not affect the ability of *A. tumefaciens* to form tumors in experimentally-infected potatoes (Fig. 2). A possible explanation for this may reside in the expression of these virulence-associated targets in the *abcR1* deletion strain. Expression of these targets in *abcR1* may be enough to sustain infection, thus resulting in a lack of attenuation. Further investigation of the direct regulation and sRNA-mRNA interactions between AbcR1 and the newly identified targets in *Agrobacterium* is necessary to fully answer this question.

In *Agrobacterium*, VtlR is the transcriptional activator of *abcR1*, and binds to the promoter region to exert its regulatory activity (Fig 1, 4). Following identification of the gene dysregulation in both *A. tumefaciens abcR1* and *vtlR*, we sought next to compare the transcriptome profiles of the two (Fig. 3). Importantly, several genetic systems differentially expressed in *vtlR* showed no difference in *abcR1*, suggesting these systems to be key for *Agrobacterium* pathogenesis. Of note, a type IV secretion system (*avhB*), a conjugation system (*tra*), genes necessary for polysaccharide biosynthesis (*exo*), as well as 10 transcriptional regulators are all differentially expressed in *vtlR*. However, to date, none of these genes have been shown to contribute to or are associated with the virulence of *A. tumefaciens* (Chen *et al*., 2002).

The difference in size of the VtlR regulons in *Brucella* and *Agrobacterium* is striking, where the *B. abortus* VtlR regulon is comprised of 10 genes, and the *A. tumefaciens* VtlR regulon is comprised of >100 genes (Sheehan *et al*., 2015) (Table 3). The complete regulon of LsrB in *S. meliloti* is currently unknown, although several genes involved in LPS synthesis, glutathione synthesis, and oxidative stress have been shown to be 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 (Tang *et al*., 2014), it was hypothesized that the homologous *lrp3-lpsCDE* system in *A. tumefaciens* may be similarly regulated. However, EMSAs performed in our lab showed no binding of *A. tumefaciens* rVtlR to the promoter region of *lrp3-lpsCDE* (Fig. S1). Furthermore, the VtlR/LsrB systems have diverged in their regulation of the *abcR* sRNAs between organisms, where *S. meliloti* LsrB does not seem to regulate the abcR sRNAs under the conditions tested in our laboratory (Fig. S2). Regarding glutathione production, Tang and

colleagues found the genes *gshA* and *gshB* to be regulated by OxyR (Tang *et al*., 2014). OxyR, a LTTR responsible for combating 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 (Nakjarung *et al.*, 2003; Eiamphungporn *et al.*, 2003). In contrast to *S. meliloti*, *oxyR* does not seem 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). Overall, these findings suggest 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 vtlR* with *lsrB* from *S. meliloti* or *vtlR* from *B. abortus* was carried out (Fig. 6). Interestingly, *lsrB* from *S. meliloti*, but not *vtlR* from *B. abortus,* could complement the growth defect of *A. tumefaciens vtlR*. Without knowing the complete regulon of *S. meliloti* LsrB, it is difficult to determine why *S. meliloti* LsrB and *A. tumefaciens* VtlR may be more functionally similar than *B. abortus* VtlR. Since *Sinorhizobium* and *Agrobacterium* are both soil-dwelling bacteria, these two rhizobiales share more similarities in genomic content and regulatory characteristics as compared to the facultative intracellular pathogen *B. abortus*, which may partially explain successful heterologous complementation by *lsrB* from *S. meliloti*. However, current experiments in our laboratory are examining the ability of these orthologous genes to complement *A. tumfaciens vtlR in vivo*. It will be interesting to determine if one or both orthologous genes are capable of restoring the ability of *A. tumefaciens vtlR* to cause tumorigenesis.

Of particular interest, a bioinformatics approach led to the identification of a novel sRNA regulated by VtlR in *A. tumefaciens* (Münch *et al.,* 2005). A genome-wide search of the predicted VtlR-binding box in *A. tumefaciens* identified a new sRNA, named VrsA (VtlR-regulated sRNA), that is directly activated by VtlR (Fig. 4-5). Currently, aside from its transcriptional regulation, nothing is known about VrsA. Since VtlR has not been shown to directly regulate any other genes in the RNA-seq dataset aside from *abcR1* and *atu1667*, it is plausible that VrsA may have some regulatory role with the dysregulated genes identified in the *vtlR* RNA-seq dataset. Additionally, *atu1667* encodes a putative hypothetical protein, which has not yet been characterized in *Agrobacterium*. It is also possible for Atu1667 to have some putative role in regulation. Current efforts in our laboratory are aimed at further characterizing VrsA and Atu1667 in *A. tumefaciens*.

Recently, a study describing the necessity of VtlR in *Agrobacterium* pathogenesis was published (Tang *et al*., 2018). There are several similarities observed between the present study and the study published by Tang and colleagues, but also many differences with regards to experimental approach and results observed. One major difference between the previously published study and the present study is the *Agrobacterium* strain utilized. Our study used *A. tumefaciens* str. C58, which is a naturally occurring strain of *A. tumefaciens* found, whereas Tang and colleagues utilized *A. tumefaciens* strain C58C1 (Tang *et al*., 2018), which 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, while the *A. rhizogenes* virulence plasmid is lacking the *virB* operon (Berger and 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 *vtlR* dataset, there are also 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 present study, RNA was isolated from *A. tumefaciens* grown in LB broth, whereas Tang and colleagues isolated RNA from bacteria grown in TY broth (Table 3; 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 the *abcR1* and *vtlR* deletion strains *(*Table 1 & 3; Overlöper *et al*., 2014). None of these genes appear to be differentially regulated in the VtlR regulon previously described (Tang *et al.*, 2018). Tang and colleagues do not address dysregulation of AbcR1 expression in their *A. tumefaciens vtlR* mutant, thus it is currently unknown why there are so many discrepancies between the two studies.

In conclusion, the present work has demonstrated how VtlR is necessary for the growth and tumorigenesis of the plant pathogen *Agrobacterium*. *A. tumefaciens* VtlR has been shown to directly bind to a conserved binding box in three promoter regions and is involved in regulation of over 250 genes (Fig. 7). Moreover, the VtlR regulon not only encompasses the AbcR1 regulon, but also over 100 genes independent of AbcR1 regulation. Importantly, this work uncovered a novel sRNA, VrsA, that is directly activated by VtlR. Finally, this study demonstrated that while VtlR orthologs are present in

Sinorhizobium and *Brucella*, only *lsrB* from *S. meliloti* is able to complement the phenotypes of *A. tumefaciens vtlR*, revealing functional differences of an orthologous protein between genetically related bacteria.

Materials and Methods

Construction and complementation of *A. tumefaciens vtlR* deletion strain

(i) Mutagenesis of *A. tumefaciens vtlR*

The *vtlR* gene (*atu2186*) was mutated utilizing an unmarked gene excision strategy previously described (Sheehan *et al*., 2015; Sheehan and Caswell, 2017). 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. 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 (Spratt *et al.*, 1986) and T4 DNA ligase (Monserate Biotechnology Group). The resulting plasmid was introduced into *A. tumefaciens* C58 by electroporation.

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

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. 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}).

(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. 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.

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. 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.

Potato disk tumor assay

Tumorgenesis of *A. tumefaciens* strain C58 and *vtlR* was evaluated by assays with potato slices (Kim *et al*., 2013). Potato cores were washed with distilled water, incubated with bleach, and UV-irradiated prior to infection. Subsequently, cores were placed on 1.5% agar plates and overnight *A. tumefaciens* cultures were diluted to OD_{600} 0.6 and serially diluted. 50 μl of each dilution were inoculated on potato surfaces, and tumors were counted on day 28. Each strain was tested in three independent experiments.

Northern blot analysis

A. tumefaciens RNA was isolated from cultures using the methodology previously described (Sheehan *et al*., 2015; Sheehan and Caswell, 2017). 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 an 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. 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). 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. Following 3 hours of constant shaking at 37°C, cultures 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.

Electrophoretic mobility shift assays (EMSAs)

EMSAs with rVtlR were carried out as previously described (Sheehan *et al.*, 2015). 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). Fragments were then run 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 run 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 *et al*., 2015; Sheehan and Caswell, 2017). *Agrobacterium* strains were grown up to an OD_{600} nm of 0.15 with constant shaking at 27°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 cleaned up by phenol-chloroform extractions and subsequent ethanol precipitation. RNA samples were resuspended in nuclease-free H_2O and 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 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 to get a minimum of 25 million reads.

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

(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 1). The remaining reads were mapped to the gene reference using BWA (Li and Durbin, 2009) with default parameters. The 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.

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Figures/Figure Legends

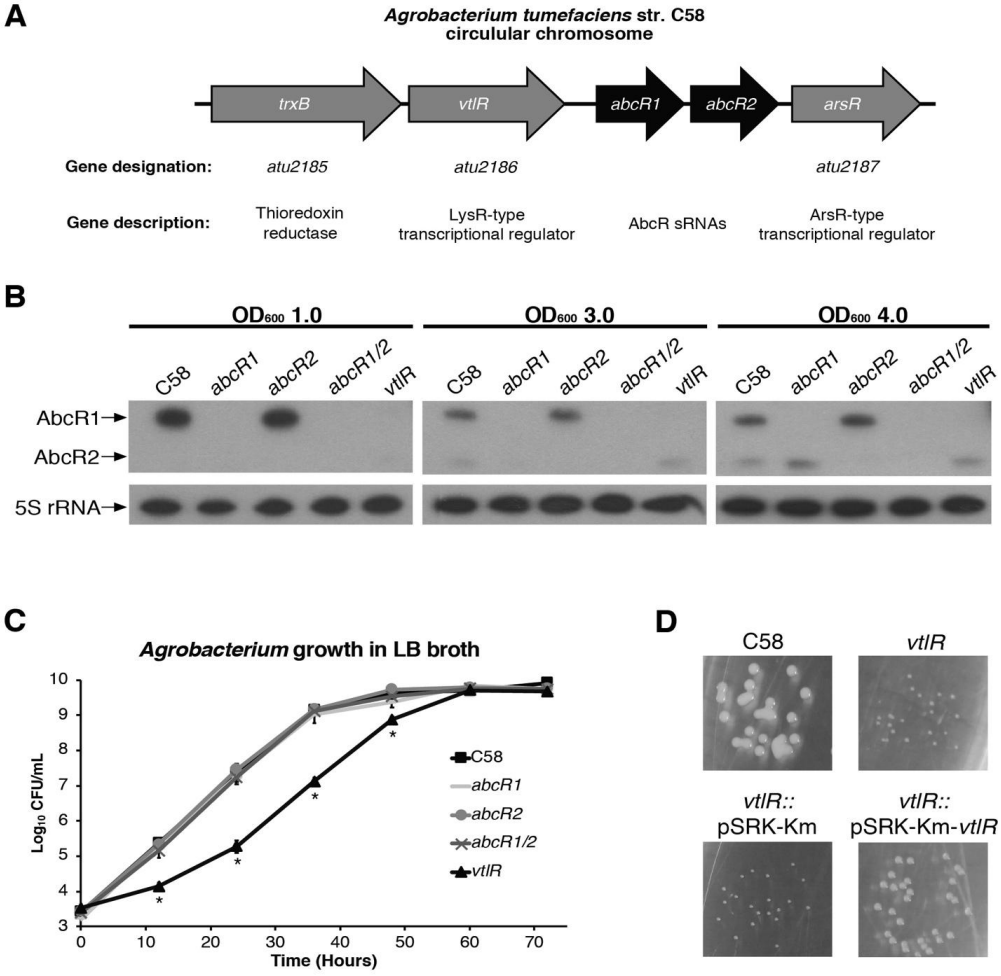

Figure 5.1. VtlR in *Agrobacterium tumefaciens* str. C58

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 analysis was employed to determine if VtlR activates the *abcR* sRNAs. RNA from *A. tumefaciens* C58, *vtlR*, *abcR1*, *abcR2*, and *abcR1/2* were isolated from cultures grown to optical densities (OD_{600}) of 1, 3, or 4, separated on a denaturing polyacrylamide gel and transferred to a nitrocellulose membrane. 5S ribosomal RNA was blotted for as a loading control.

C. The *A. tumefaciens* C58, *abcR1*, *abcR2*, *abcR1/2*, and *vtlR* strains were grown in broth, and at specified timepoints, samples from each culture were taken, serial diluted and plated out 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 agar after 72 hours of growth.

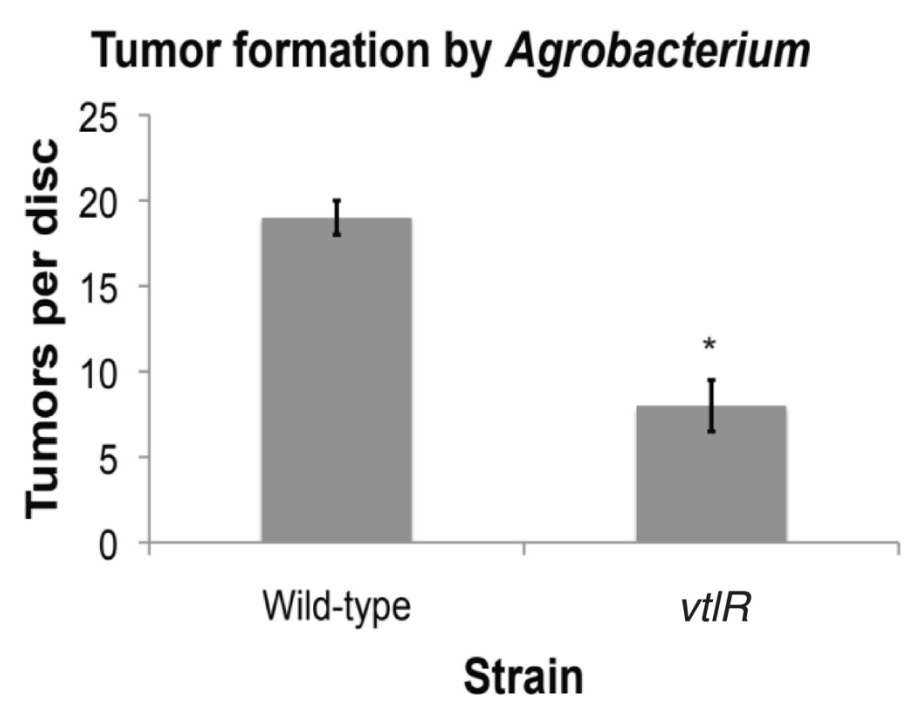

Figure 5.2. Contribution of *vtlR*, *abcR1*, and *abcR2* on *A. tumefaciens* virulence
Tumor formation of *A. tumefaciens* str. C58 and *vtlR* in experimentally-infected potatoes. Sterile potato discs were inoculated with C58 or *vtlR*, and the number of tumors were counted 28-days post infection. Statistical significance is denoted by an asterisk (*) (*t*-test; $P < 0.05$). Data not shown for *abcR1*, *abcR2*, or *abcR1*/*2* mutants. All experiments are in the process of being repeated with complements.

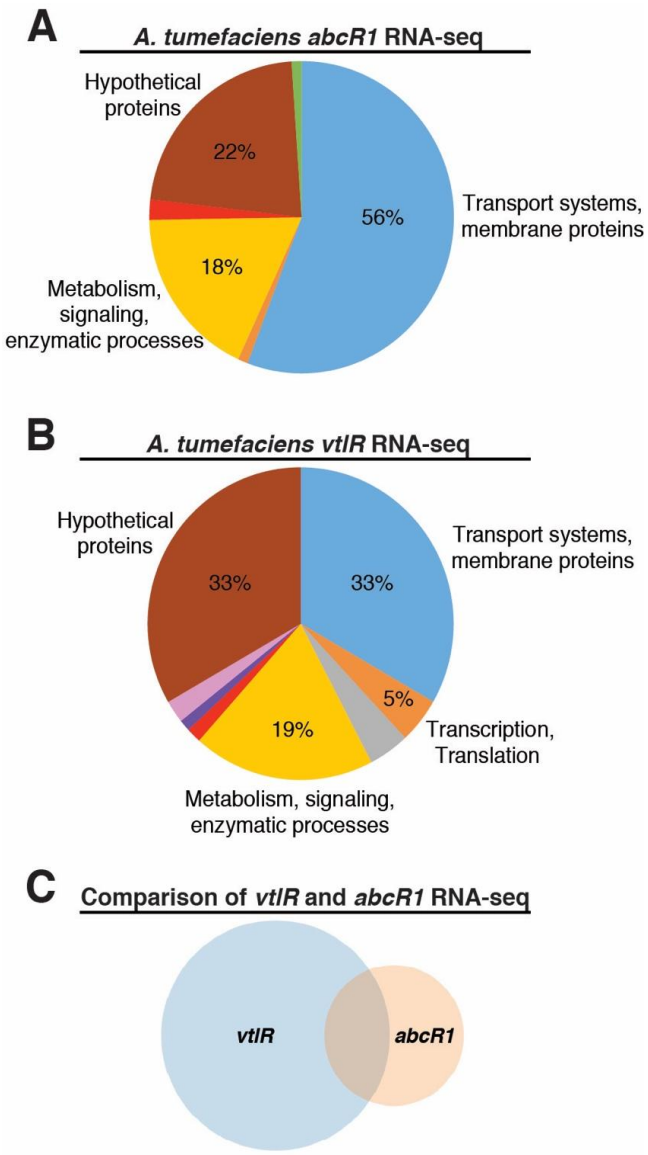

Figure 5.3. Overview of RNA-seq analysis of *A. tumefaciens abcR1* and *vtlR*
A. Pie chart summarizing genes differentially expressed (≥3-fold) in *abcR1* mutant. Blue: Transport systems and membrane proteins (56%); Brown: Hypothetical proteins (22%); Yellow: Metabolism, signaling, enzymatic processes (18%); Red: Phage-related (2%); Orange: Transcription, translation (1%); Green: Chemotaxis, motility (1%).
B. Pie chart summarizing genes differentially expressed (≥3-fold) in *vtlR* mutant. 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 *vtlR* and *abcR1* mutants.

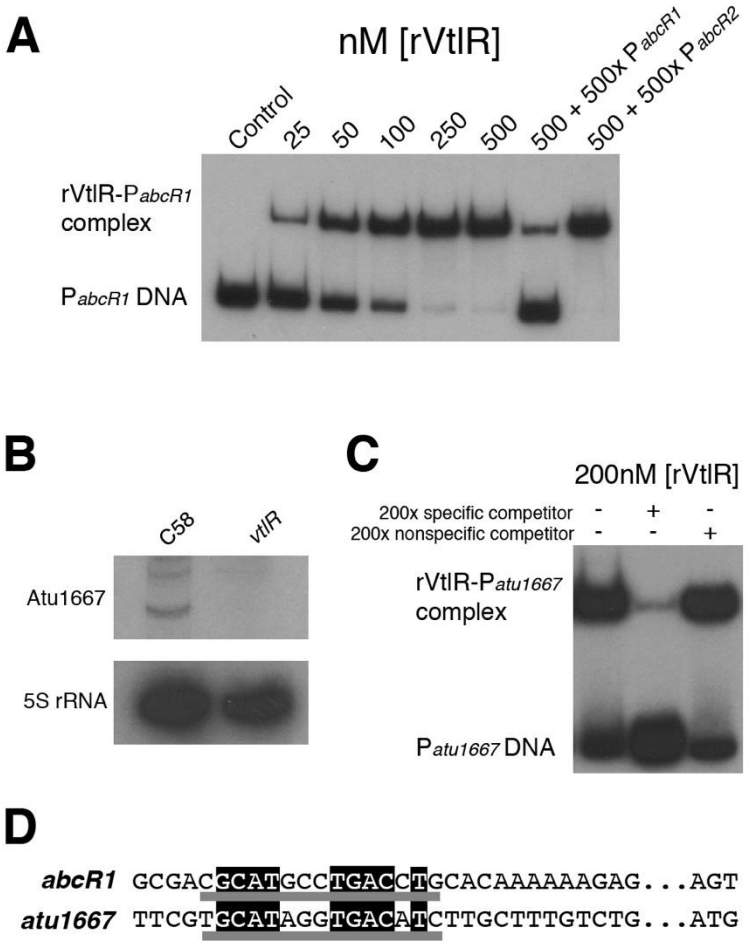

Figure 5.4. *A. tumefaciens* VtlR directly regulates the small RNA *abcR1* and the small hypothetical protein *atu1667*

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 set out 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 was employed to determine if VtlR activates *atu1667*. RNA from *A. tumefaciens* C58 and *vtlR* was isolated from cultures grown in LB broth to OD_{600} 0.6, separated on a denaturing polyacrylamide gel and transferred to a nitrocellulose membrane. 5S ribosomal RNA was used as a loading control.

C. EMSAs were carried out 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.

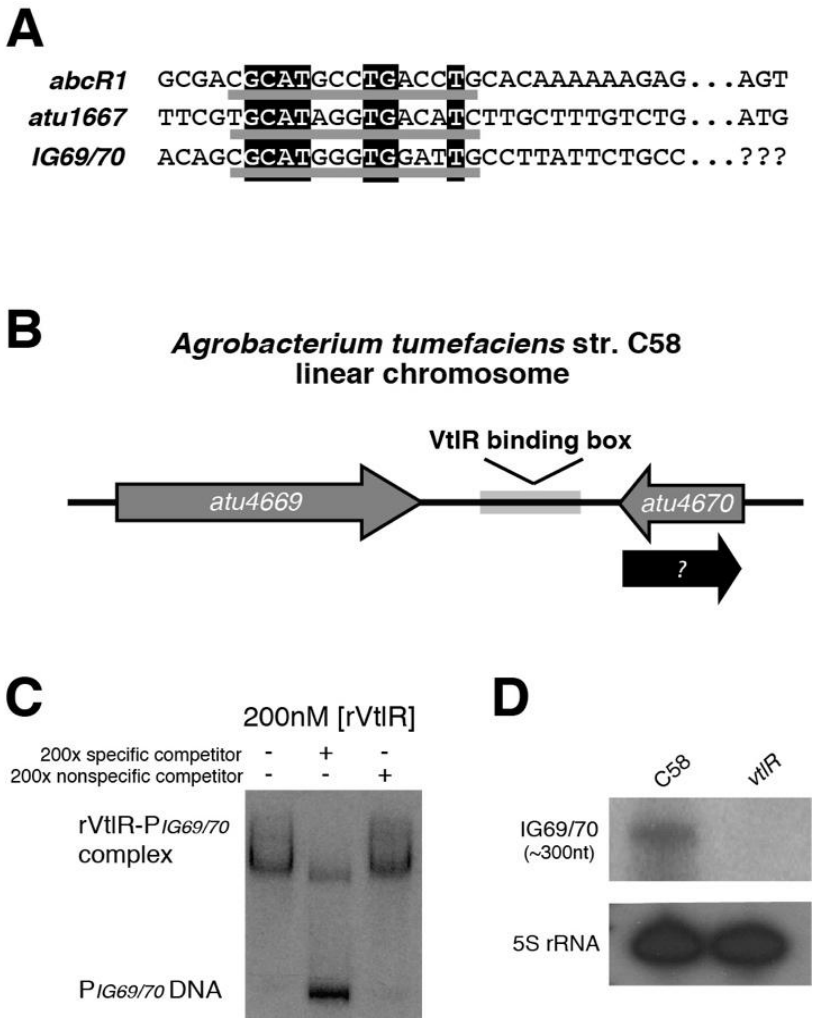

Figure 5.5. VtlR activates a novel transcript in *A. tumefaciens*

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 *IG69/70*) Nucleotide alignment of the promoter regions of *abcR1*, *atu1667*, and *IG69/70*. 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 *IG69/70* promoter region. To determine specificity of binding, specific (unlabeled P*IG69/70*) 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 *vtlR* was isolated from cultures grown in LB broth to OD_{600} 0.6, separated on a denaturing polyacrylamide gel and transferred to a nitrocellulose membrane. 5S ribosomal RNA was used as a loading control.

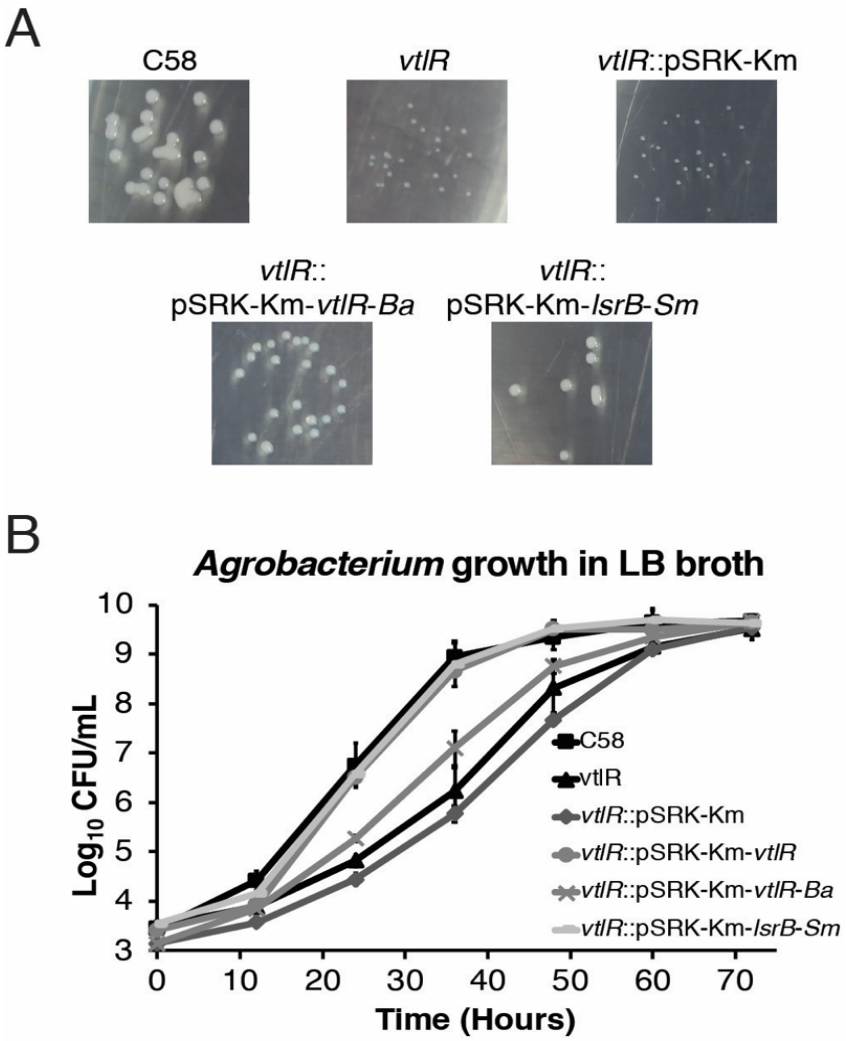

Figure 5.6. Heterologous complementation of *A. tumefaciens* Δ*vtlR* with *S. meliloti lsrB* and *B. abortus vtlR*

A. The *A. tumefaciens* strains C58, *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*

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

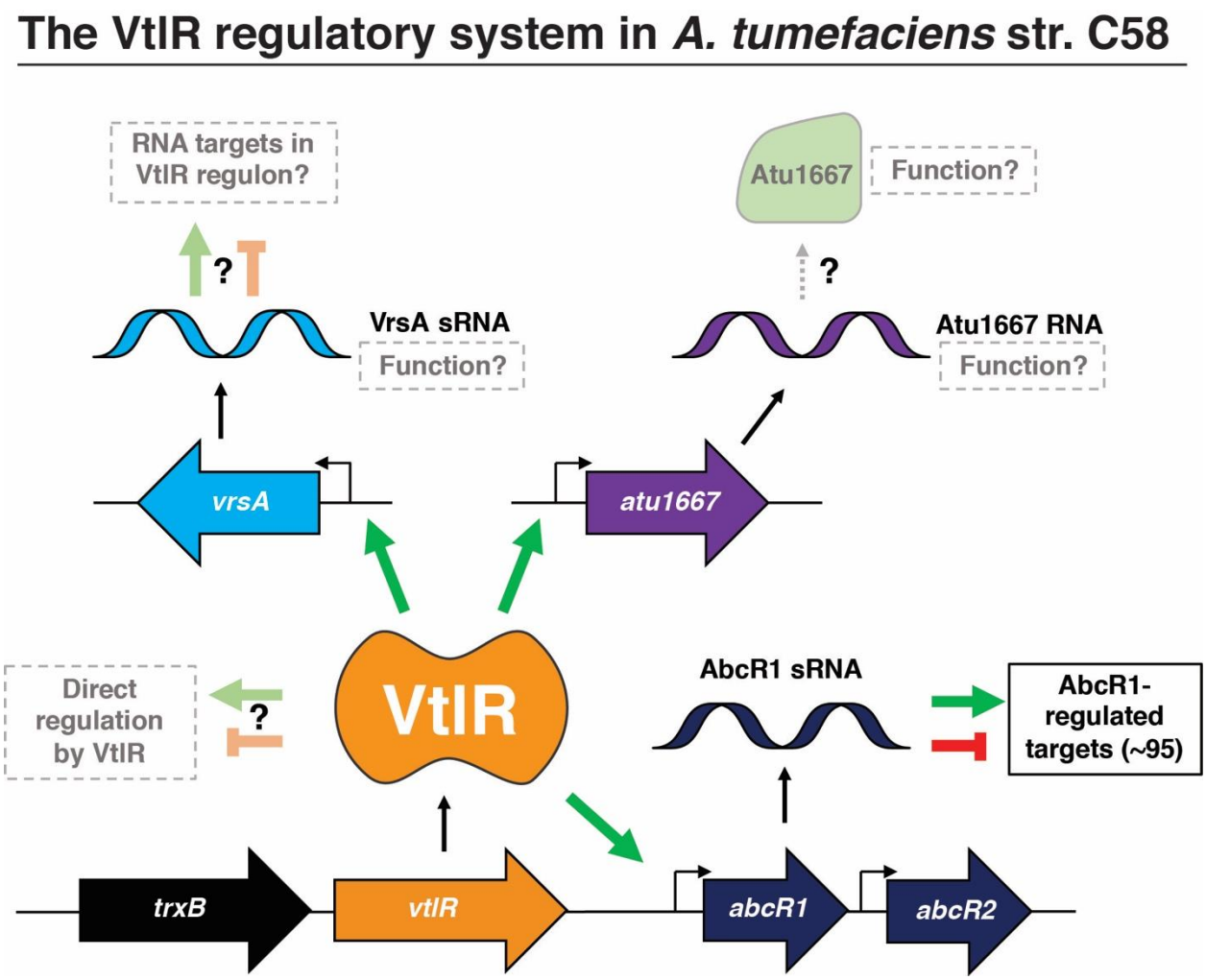

Figure 5.7. Working model of VtlR regulation in *A. tumefaciens*
vtlR (orange) is located downstream of the thioredoxin reductase, *trxB* (black) and upstream of the tandemly encoded sRNAs, *abcR1* and *abcR2*. VtlR directly activates *abcR1*, *vrsA* (blue) and *atu1667* (purple). AbcR1 regulated ~95 target independent of VtlR. The regulatory capacity of VrsA and Atu1667 are currently unknown.

Tables

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 5.1. 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 (*vtlR* RNA-seq dataset). Dataset sorted based on gene designation.

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 5.2. 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.

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 component	1.7
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 (iron)	-1.6
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 protein	-2.0
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 5.3. Summarized *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 1 (*abcR1* RNA-seq dataset). Dataset sorted based on gene designation.

Chapter 6. General Discussion

Discussion

The overall goal of this work was to identify and characterize genetic systems within *Brucella* that are important for the intracellular lifestyle of this pathogen. Initially, this work focused on further characterization of the AbcR sRNA system in *Brucella*, which was previously shown to be vital for pathogenesis and for regulating ABC-type transport systems (Caswell *et al*., 2012b). In the first study, a *B. abortus* LysR-type transcriptional regulator, named VtlR, was identified as the transcriptional activator *abcR2* in addition to three other genes (*bab1_0914*, *bab2_0512*, and *bab2_0574*) (Chapter 2: Table 1; Figures 1, 3-5). VtlR was also found to be essential for virulence, where a deletion of *vtlR* resulted in attenuation of *Brucella* in both a murine and macrophage model of infection (Chapter 2: Figure 2). It was hypothesized that it was the dysregulation of genes (i.e., *abcR2*, *bab1_0914*, *bab2_0512*, and *bab2_0574*) in the *vtlR* mutant that was responsible for the reduced virulence. However, a *Brucella* quadruple mutant of the four VtlR-regulated genes resulted in a wild-type infection (Chapter 2: Figure 6). A possible explanation for this is the presence of an alternate VtlR regulon. The microarray analysis done with *B. abortus vtlR* was performed in a nutrient-rich medium, which does not simulate the environment *Brucella* encounters during infection.

Since VtlR is critical for intracellular survival, it is plausible that VtlR may regulate a divergent set of genes in response to a certain intracellular stress (e.g., acidic pH, ROS, RNS, diminished oxygen, and nutrient limitation). Furthermore, LysR-family regulators commonly bind a specific co-inducer and can subsequently undergo a conformational change, allowing the protein to bind to and regulate genes in response to this stimulus

(Maddocks *et al*., 2008). Currently, two major questions still remain regarding VtlR in *Brucella*. First, why is a *B. abortus vtlR* mutant attenuated? The answer to this question likely lies in the unknown, putatively stress-induced alternate regulon of VtlR. Secondly, what is the co-inducer/signal VtlR is sensing and responding to? Preliminary work in *Brucella* and *Sinorhizobium* hints at the possibility of VtlR sensing oxidative stress (Fig. 1A; Tang *et al*., 2017). Bioinformatics analysis examining the predicted structure of *B. abortus* VtlR identified two cysteines in close proximity to one another, possibly capable of forming a disulfide bond. Site-directed mutagenesis of these cysteines in *B. abortus vtlR* resulted in attenuation of the bacterium in murine macrophages (Fig. 1B). We hypothesize that, similar to the LysR-regulator OxyR, VtlR undergoes a conformational change through disulfide bond formation in response to oxidative stress and regulates genes encoding products necessary for combating this stress (Zheng *et al*., 1998).

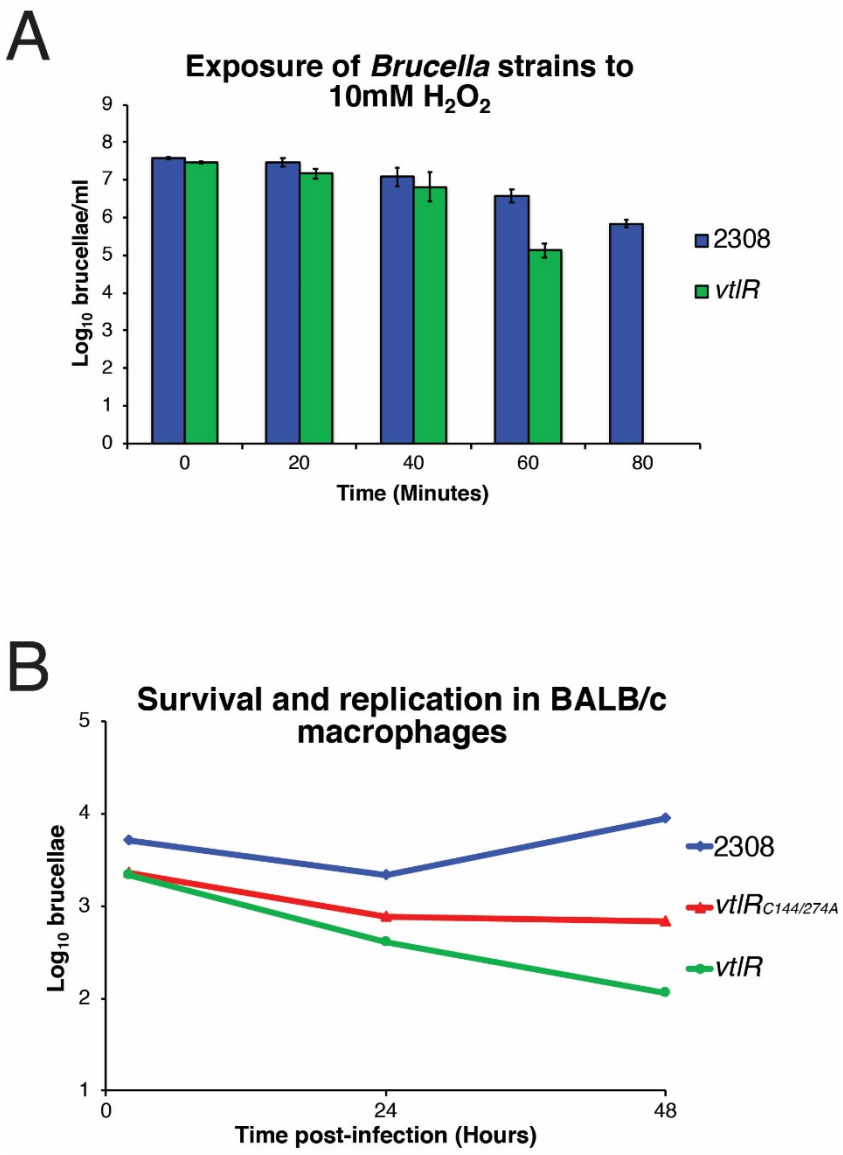

Figure 6.1. The putative role of *B. abortus* VtlR in sensing oxidative stress
A. *B. abortus* strains were grown to late exponential/early stationary phase in brucella broth, and stressed with a final concentration of 10mM H_2O_2. Following addition of H_2O_2, cultures continued to shake, and samples were taken every 20 minutes to monitor the ability of *Brucella* to survive over time. Samples were serial diluted, plated out on SBA plates, and colony forming units (CFUs) were counted. Bars represent mean CFU of triplicate samples.
B. Survival and replication of the *B. abortus* strains in primary murine macrophages. Primary macrophages were collected from the peritoneal cavity of female BALB/c mice and infected with the parental *B. abortus* strain 2308 (blue), Δ*vtlR* (green), or *vtlR* double cysteine mutant (red). At 2, 24 and 48 hours post-infection, macrophages were lysed, and serial dilutions of the lysates were plated on blood agar to determine the number of intracellular brucellae.

Although we have identified the transcriptional regulator of *abcR2* in *Brucella*, it remains unknown what regulates the sibling sRNA *abcR1*. Analysis of the -10 and -35 promoter region of *abcR1* revealed a putative RpoH binding box. However, northern blot analysis of a *B. abortus rpoH* mutant had no difference in the expression of either AbcR sRNAs. Preliminary work analyzing the expression patterns of the AbcRs found AbcR1 to be constantly expressed, whereas AbcR2 expression is significantly reduced in nutrient-limiting conditions and deoxycholate stress (Budnick, unpublished). Although these data suggests AbcR1 is constitutively expressed in *Brucella*, the AbcR sRNAs are likely are down-regulated at specific periods during intracellular trafficking to allow for expression of the ABC-type transport system, which are hypothesized to be important for nutrient acquisition. To this point, a mutant of the gene *bab2_0612*, an AbcR-regulated target encoding for a putative glutamate-binding protein, resulted in attenuation of *Brucella* in a mouse model of infection (Chapter 3: Figure 7). Overall, these data suggest that expression of the AbcR sRNAs is transient and is probably influenced by the environment of the bacterium. Ongoing studies are directed at identifying the transcriptional regulator of *abcR1* in *B. abortus*.

The RNA-mediated regulation by the AbcR sRNAs was previously defined, but it was unknown if this regulation was redundant, and moreover, what mechanism(s) the sRNAs utilized to bind target RNAs (Caswell *et al*., 2012b). To define regulatory redundancy, qRT-PCR analyzing expression of AbcR-specific targets was employed with isogenic *abcR* mutants and with a double *abcR1/2* mutant (Chapter 3: Figure 1). This

experiment confirmed that regulation by the AbcR sRNAs is indeed redundant, where only a double *abcR1/2* mutant showed overexpression of AbcR-targets. Redundancy of sRNAs is not unique to *Brucella*, and multiple copies of sRNAs are present in numerous organisms (Waters and Storz, 2009). In *Brucella*, this redundancy may act as a fail-safe mechanism, where if one AbcR sRNA is rendered non-functional, the other AbcR sRNA is present to take over regulatory duties. Moreover, only a double *abcR1/2* deletion resulted in attenuation of the bacterium, further suggesting regulatory and functional redundancy of the AbcR sRNAs in *Brucella* (Caswell *et al.*, 2012b).

Following bioinformatics analysis of the predicted secondary structures of the *Brucella* AbcR sRNAs, two 6-nucleotide motifs were identified in both sRNAs (named M1 and M2) (Chapter 3: Figure 3). Site-directed mutagenesis of these motifs revealed the AbcRs to utilize either one or both sequences to bind and regulate RNA targets (Chapter 3: Table 1; Figure 4-5). Since these motifs were hypothesized to be responsible for regulating the majority, if not all, of the AbcR-regulated targets, we sought next to determine if mutations of these motifs resulted in reduced virulence in *Brucella*. Interestingly, only changes to M2 negatively affected *Brucella* pathogenesis (Chapter 3: Figure 6). Since the AbcR sRNAs are involved in negatively regulating ABC-transport systems, it seems likely that the systems regulated by M2 are those most vital to the virulence of *Brucella*. As mentioned above, one of the AbcR-targets regulated solely by M2 is BAB2_0612, which has been implicated in pathogenesis (Chapter 3: Figure 7). Absence of M2 in the AbcR sRNAs lead to dysregulation of ABC-type transport systems. This dysregulation, specifically overexpression, of ABC-transport systems at incorrect times during infection may act as antigenic components and, subsequently, alert the

immune system of the presence of the pathogen. Current work is aimed at defining which M2-regulated systems are essential for pathogenesis, which could then lead to the development of therapeutics specifically targeting these M2-regulated systems. Overall, the virulence-associated M2-regulated targets can potentially be exploited in the future for combating brucellosis.

The AbcR sRNAs can be found throughout the *Rhizobiales* and are relatively conserved in their genomic organization, nucleotide sequences, and secondary structures (reviewed in Chapter 4). However, some interesting differences have arisen between organisms in this order (Chapter 4: Figure 1-2). In several organisms, the *abcR* sRNAs are located in tandem, directly downstream of the LysR-type regulator *vtlR* (e.g., *S. meliloti*, *A. tumefaciens*, *R. etli*). However, the *abcR* sRNAs can also be found on separate chromosomes in other bacteria, where only one sRNA is downstream of *vtlR* (e.g., *B. abortus*, *O. anthropi*, *M. loti*). Furthermore, a few *Rhizobiales* seem to only have one *abcR* sRNA located downstream of *vtlR* (*Bartonella* spp.). Although the exact reason for these differences in the *Rhizobiales* is unknown, it seems likely that the AbcR sRNAs originated from a gene duplication event early in evolution.

In *Agrobacterium*, although both sRNAs are present and located in tandem, only AbcR1 is functional (Wilms *et al*., 2011; Overlöper *et al*., 2014). Upon examination of the sequence and secondary structure of the AbcRs, only AbcR1 contains M1 and M2 (Chapter 4: Figure 1-2). M2 in AbcR2 is absent, which may explain why the sRNA is no longer functional in *Agrobacterium*. If gene duplication is the reason for the presence of the AbcRs, both sRNAs likely possessed regulatory roles early on in evolution. As time passed, AbcR2 may have developed mutations in its M2 region and, eventually, became

nonfunctional. Similar to *Agrobacterium*, the AbcR sRNAs in *Sinorhizobium* are located in tandem (Chapter 4: Figure 1-2). In contrast, both sRNAs have been reported to have some regulatory function in *S. meliloti*. However, AbcR1 seems to have the predominant role, where it regulates its own targets as well as AbcR2 targets (Torres-Quesada *et al*., 2013). AbcR2 has only been reported to regulate two transcripts, and this regulation is redundant to AbcR1. It seems likely that although both sRNAs are functional in *Sinorhizobium*, AbcR1 is the major regulator. Moreover, the M1 sequence in AbcR2 is mutated, which may be the driving force behind the lack of AbcR2 regulatory capacity (Chapter 4: Figure 1-2).

In contrast to both, *Agrobacterium* and *Sinorhizobium*, the *Brucella* AbcR sRNAs are located on opposite chromosomes and are functionally redundant (Caswell *et al*., 2012b; discussed in Chapter 3). The divergence in location of the *abcR* sRNAs is interesting and brings up the question of if the sRNAs were initially in tandem or divergently located. The *Rhizobiales* likely originated from a soil-dwelling bacterium, so it seems plausible that the AbcR sRNAs originated from a gene duplication event, which orientated them tandemly, and separation events of the sRNAs occurred later in evolution.

Another commonality in the AbcR system is the presence of the transcriptional regulator VtlR. *vtlR* is always located directly upstream of one of both of the *abcR* (Chapter 4: Figure 2). In *Agrobacterium* and *Brucella*, VtlR activates the expression of *abcR1* and *abcR2*, respectively (discussed in Chapters 2 and 5). In contrast, the ortholog of VtlR in *Sinorhizobium*, named LsrB, does not seem to regulate either one of the *abcR* sRNAs, which is perplexing given that electrophoretic mobility shift assays (EMSAs) have

shown LsrB binding to the promoter region of *abcR1*, and, moreover, a VtlR-binding site is present directly upstream of the -35 in the promoter region of *abcR1*. What transcription factor is involved in regulating the *abcRs* in *S. meliloti* remains to be determined.

From studies in various *Rhizobiales*, it can be concluded that VtlR/LsrB is a major factor in adaptation of the bacterium to a particular environmental niche (Luo *et al.*, 2005; Tang *et al.*, 2018; Chapters 2 and 5). Although this LysR regulator is conserved in *Rhizobiales* and is similar in amino acid sequence and predicted protein structure, major differences in the regulatory roles of VtlR have been described (Lu *et al*., 2013; Tang *et al*., 2014; Tang *et al*., 2017; Tang *et al*., 2018; Chapter2 2 and 5). Though one of the *abcR* sRNAs is regulated by VtlR in *Agrobacterium* and *Brucella*, the complete regulatory profile of VtlR differs between these organisms. In contrast to the four-gene regulon of *Brucella* VtlR, RNA-sequencing analysis of an *Agrobacterium vtlR* mutant found >100 genes to be dysregulated (Chapters 2 and 5). Although a large portion of the genes identified in the analysis can be attributed to the lack of AbcR1, numerous targets are independent of AbcR1 regulation, suggesting VtlR having an AbcR-independent regulatory role (Chapter 5). However, it is still unknown which targets are directly and indirectly regulated by VtlR in *Agrobacterium*. In *Sinorhizobium*, the LsrB regulon completely differs from *Agrobacterium* and *Sinorhizobium*, where LsrB regulates genes encoding for LPS and glutathione biosynthesis (Lu *et al*., 2013; Tang *et al*., 2014). Although this LysR-regulator was once thought to be relatively conserved in its function and regulatory capacity, we now know that VtlR is much more diverse than originally thought.

VtlR arose from a common ancestor of the *Rhizobiales*, but has diverged greatly in its regulatory capacity. This divergence in regulation is presumably key to allowing an

organism to reside and survive in certain environments (e.g., in the soil, within a plant, or intracellularly in a host). The putative signal resulting in a conformational change in VtlR still remains to be determined, but preliminary data suggest it is linked to coping with oxidative stress (Fig. 1; Tang *et al*., 2017). The previously mentioned *B. abortus* VtlR cysteines residues can also be found in *Agrobacterium* and *Sinorhizobium*. Moreover, protein modeling of VtlR from the human pathogen *Bartonella* revealed conservation of these cysteines, further suggesting that disulfide bond formation could trigger a conformation change and, subsequently, allow VtlR to regulate an alternate regulon.

In conclusion, the AbcR/VtlR system is essential for bacterial survival and allows for successful adaption to various environmental conditions. The AbcR sRNAs are vital components in the RNA-mediated post-transcriptional regulation of transcripts, which are involved in nutrient acquisition. VtlR is crucial to transcriptional regulation of numerous targets, in addition to playing a putative role in an organism's adaptation to stress signals. Although this work has made significant advances in understanding *Rhizobiales* genetics, specifically in transcriptional and post-transcriptional mechanisms in several organisms, further characterization is necessary to fully understand the importance of this conserved AbcR/VtlR regulatory system. Moreover, defining the co-inducer/signal of VtlR, identifying the stress-induced VtlR regulon, and determining which AbcR M2-regulated targets are important for virulence can be potential targets for future therapeutic development for use against animal and human pathogens.

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