Discovery and Characterization of a Novel Regulatory Small RNA, VcrS, Required for Virulence in *Brucella abortus.*

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Abstract

Brucella abortus is a facultative, intracellular, zoonotic pathogen that resides inside macrophages during infection. This is a specialized niche where *B. abortus* encounters various stresses, such as acidic conditions and reactive oxygen species, as it navigates through the macrophage. In order to survive this harsh environment, *B. abortus* utilizes post-transcriptional regulation through the use of small regulatory RNAs (sRNAs). sRNAs bind to messenger RNA (mRNA) targets via complementary base pairing. sRNAs are a class of regulatory molecules in bacteria that elicit rapid post-transcriptional regulation. sRNA-mRNA binding can positively or negatively influence gene expression. Positive regulation can occur through sRNA binding to protect the mRNA from RNases. sRNA binding can also alleviate the secondary structure and reveal the ribosomal binding site. Alternatively, sRNA-mRNA interactions can have negative consequences on gene expression through degradation via RNases or sRNA binding can occlude the ribosomal binding site. Although some sRNAs have been discovered in *B. abortus*, few have been characterized in regards to virulence.

In this study, *B. abortus* was stressed in conditions relevant to the macrophage, including, including low pH, oxidative stress, and nutrient limitation. Transcriptomic analysis revealed high levels of transcripts in intergenic regions, a hallmark of sRNAs, which led to the discovery of VcrS for virulence and cell wall regulating sRNA. A Δ*vcrS* was engineered and this mutant was used to infect both naïve murine macrophages, as well as BALB/c mice. Both virulence studies demonstrated significantly decreased

bacterial recovery of Δ*vcrS* compared to the wildtype strain. Quantitative proteomics revealed that one protein, BAB1_1454, is 30-fold over-produced in ΔvcrS compared to wildtype. This essential protein encodes MurF, which catalyzes the final cytoplasmic step of generating the mura-pentapeptide precursor for peptidoglycan synthesis. VcrS is hypothesized to interact with murF mRNA and interfere with translation initiation. Sequence data indicates a putative 6 nucleotide motif in VcrS that has complementarity to the ribosomal binding site of murF. Identification of the binding site and further characterization of VcrS will showcase the importance of sRNA regulation in the virulence of *B. abortus.*

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General Audience Abstract

Brucella abortus is a bacterial pathogen that primarily infects cattle but is also transmitted to humans. Human disease most commonly results from the consumption of unpasteurized milk and milk products. Human brucellosis has very limited treatment options, with a high incidence of disease relapse. *B. abortus* survives and replicates within immune cells, which create a harsh environment. However, the bacteria are able to sense and adapt to survive and replicate within these immune cells, maintaining a chronic infection. A better understanding of the adaptation process *B. abortus* utilizes to survive within the human host can lead to improvement of treatment options. The present work characterizes a novel regulatory small RNA- VcrS, which was found required for survival and replication inside immune cells

Dedication

I would like to dedicate this work to my parents Stephen King and Karen King, who have allowed me to find my passion and become a research scientist. I would also like to dedicate this work to my siblings Leslie and Blake King, and my handsome nephew Beckham King, thank you for always being supportive and letting me teach you about science. I would lastly like to dedicate this work to my family, especially my grandparents Thomas and Margret King and Don and Janice King. I can't say thank you enough for giving me the opportunity and pushing me to follow my dreams.

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As soon as I could, I adopted a cat and I can easily say that was the best decision of my life. Stevie has been the best distraction during this journey. She is very entertaining: talking to birds, playing fetch with hair ties, and don't get me started on the cuddles. She is the best emotional support cat and I am forever grateful I adopted her.

I wanted to give a huge thank you to my friends and family. You all have continuously supported me and gladly listened to every presentation I had. I am grateful

to be surrounded by such supportive people who push me to follow my dreams. Thank you to my parents for giving me the ability to go to college and follow my passion of science. I am so grateful you always said yes and allowed me to reach for the moon.

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Chapter 1: A comprehensive review of the regulatory small RNA molecules in *Brucella* spp.

Kellie A. King and Clayton C. Caswell

What are sRNAs?

Regulatory small RNAs (sRNAs) are single stranded RNA molecules ranging between 50-500 nucleotides long, and sRNAs have two distinct classes based on genetic location, *cis*-encoded and *trans*-encoded[1]. *Cis*-encoded sRNAs are encoded on the DNA strand opposite of their regulatory targets. These sRNA-mRNA interactions often involve longer, high complementarity base-paring regions. *Trans*-encoded sRNAs are encoded independently of their regulatory targets, often being located within intergenic regions. These interactions comprise of short, imperfect base-pairing segments, most often requiring the RNA chaperone Hfq[2].

Due to sRNAs being single stranded, they have the potential to base-pair within themselves and therefore create secondary structures. These structures reveal single stranded regions, known as seed regions, of the sRNA that have potential to interact with mRNA targets[3,4]. sRNA-mRNA interactions can influence gene expression in a positive manner by stabilizing the mRNA and providing protection against RNases, or sRNA binding can alleviate secondary structure to make the ribosomal binding site (RBS) more accessible[5]. Alternatively, sRNA binding can negatively impact gene expression, as binding can create a double stranded RNA molecule (dsRNA) and be targeted for degradation through RNases. Additionally, sRNA binding can influence secondary structure and either occlude the RBS or directly bind to and block the RBS, therefore inhibiting translation[1].

Hfq: The Riboregulator

Hfq is considered a major virulence factor in many bacteria, as an Δ*hfq* mutant has pleiotropic phenotypes including delayed growth rate, small colony phenotype, increased sensitivities to acidic conditions, and decreased virulence[6–9] . Hfq was first discovered as a host factor that was essential for Qβ phage replication in *Escherichia coli* [10]. This Sm-like family protein is found in all three domains of life, showcasing the evolutionary role this RNA chaperone encompasses[11]. Hfq forms a homohexamer structure with three binding faces: the distal face, the proximal face, and the outer ring or rim surface[12]. Each binding face has specific affinity for sRNAs or mRNAs. The distal face of Hfq preferentially binds single stranded ARN or ARNN motifs, (R signifies a purine and N represents any base), commonly found on mRNA sequences. The proximal face, has high affinity for RNA molecules enriched with uracil residues, which are commonly found in Rho-independent terminator sequences of sRNAs, followed by stem loop structures [13]. The rim or lateral side of Hfq is an important interaction face that binds UA-rich RNA molecules through positively charged residues[14]. Hfq facilitates RNA regulation by increasing local concentrations of sRNA and mRNA targets. This chaperone also causes structural changes and even restructuring of larger mRNA molecules to assist in sRNA binding[15]

sRNAs in *Brucella*

Brucella species (spp) are Gram-negative, facultatively intracellular bacteria[16]. *B. melitensis*, *B. suis*, *B. abortus* and *B. canis* are the most common causes of brucellosis, infecting goats, pigs, cattle, and canines, respectively[17]. Zoonotic brucellosis results in

spontaneous abortions, and sterility. The transmission to humans occurs via aerosols and ingestion or direct contact with contaminated animal products[18]. Human brucellosis often presents with undulant fever accompanied with "flu-like" symptoms and can lead to neurological defects, hepatomegaly, splenomegaly, and endocarditis if left untreated[19,20]. Currently, there are approved livestock vaccines for *B. abortus*, but no human vaccine exists.

Brucella is an intracellular pathogen that resides within macrophages and dendric cells. It's rapidly engulfed and contained within a *Brucella* containing vacuole (BCV)[21]. This vacuole is trafficked through the macrophage, and *Brucella* is sensing and adapting to the harsh conditions to maintain survival. Eventually, the BCV is trafficked to the endoplasmic reticulum, become *Brucella's* specialized niche where it is able to survive and replicate[22]. *Brucella* is a stealthy pathogen and lacks many classical virulence factors known to mediate immune evasion, instead, *Brucella* utilizes post-transcriptional regulation to adapt to the changing conditions of the macrophage. Hfq, best known as a riboregulator that facilitates RNA-RNA interactions, is required for proper adaptation to host conditions in *B. abortus*[9]. Hfq regulates major virulence factors, including the LuxR-type regulator BabR and the type IV secretion system proteins VirB[23]. This RNA chaperone works with sRNAs to adapt to the harsh conditions of the macrophage.

The first sRNAs discovered in *B. abortus* were AbcR1 and AbcR2. These redundant sRNAs are Hfq-dependent and are essential for virulence in both macrophages and mice[24]. A double deletion of *abcR1* and *abcrR2* resulted in 25 transcripts being overexpressed and 16 proteins being overproduced compared to the wild-type strain, showing the diverse regulon of the AbcR sRNAs. AbcR1 and AbcR2 promote the

degradation of target mRNAs predominantly encoding ABC-type transport systems. Sheehan *et al*. identified VtlR, the virulence associated transcriptional LysR-type regulator of *abcR2*[25]. This protein is crucial for virulence and controls the expression of *abcR2* in addition to three genes encoding small hypothetical proteins. This direct regulation was confirmed using an electrophoretic mobility assays. The VtlR binding site was identified using DNase footprinting. Another study by Sheehan *et al.* revealed two regulatory binding motifs found in both AbcR1 and AbcR2 and showcased the importance of the M2 regulatory motif for virulence[26].

Since the initial discovery of sRNAs in *Brucella*, numerous bioinformation prediction programs have been implemented to search for additional *Brucella* sRNAs. Various pipelines have since predicted numerous sRNAs in various *Brucella* species. Although many of these findings remain as predictions, some sRNA candidates have been validated, and very few have been functionally characterized.

Dong and colleagues[27] used the bioinformatic program SIPHT, which focuses on Rho-independent terminators within intergenic regions, paired with NAPP, detecting sRNA elements, to predict 129 sRNA candidates. These sRNA candidates developed a nomenclature of BASRC for *B. abortus* sRNA candidate followed by chromosome number and candidate number. Of these 129 sRNA candidates, only 7 out of 20 were verified using RT-PCR. The regulatory targets of those 7 sRNAs were predicted using another bioinformatic software, sTarPicker. These interactions were then tested using a two-plasmid reporter system in *E. coli*. However, further investigation on the regulatory mechanisms of these sRNAs is needed to gain insight into the regulatory network encompassing these sRNAs.

This study led to the further characterization of one sRNA candidate, named BsrH[28]. This *cis*-encoded sRNA was of interest due to its location antisense of *hemH (bab2_0075 / bab_rs26720)*, which is involved in heme biosynthesis. This target interaction was tested using a two-plasmid reporter system in *E. coli*, which verified the negative regulation of BsrH on *hemH*. Overexpression of BsrH had no impact on virulence *in vitro* or *in vivo*. Moreover, BsrH is a *Brucella*-specific sRNA and predicted to have a role in acidic, oxidative, and iron limiting stress conditions, however, the mechanism to this involvement has yet to be characterized.

Wang *et al.*[29]also employed similar computation predictions limited to intergenic regions of the genome. Using RNA motif and sRNAPredict programs to predict possible promoters and terminators within intergenic regions, 21 candidate *Brucella* sRNAs named BSR for *Brucella* small-noncoding sRNA followed by the gene number of the downstream protein-encoding gene were predicted. Northern blot analysis was used to confirm 15 of the predicted sRNAs. One Hfq-dependent sRNA, BSR0602 showed the highest abundance during stationary phase of growth and is also predicted to play a role in acidic and oxidative stress conditions. This sRNA also appears to play a role in virulence, as a deletion mutant resulted in decreased bacterial counts following 24 hours post-infection in a mouse model of infection. TargetRNA was used to predict targets of BSR0602, leading to discovery of the target BMEI0106, a global transcriptional regulator GntR. qRT-PCR data revealed increased expression of BMEI0106 in a *bsr0602* deletion mutant, and expression was almost abolished in a *bsr0602* overexpression strain. A two-plasmid reporter system in *E. coli* confirmed the negative regulation by BSR0602, and subsequently the binding motif of this interaction was revealed. Additional investigation

is required to confirm this direct regulation and further define the regulatory network of BSR0602.

Saadeh et. al[30] took an alternative approach to identify sRNAs in *B. suis.* This group utilized the RNA chaperone Hfq as bait to enrich for sRNAs using coimmunoprecipitation coupled with strand-specific cDNA library generation and deep sequencing. Instead of using prediction programs, they visually mined transcriptomic data, concentrating on intergenic regions of the genome. This technique led to the discovery of 33 candidate Hfq-associated sRNAs, all of which were confirmed using RT-PCR or northern blot analysis. The study further showcases the importance Hfq plays in sRNA activity in *Brucella* species.

Another deep-sequencing approach taking into account gene model and location between genes was used to identify 1321 sRNA candidates in *B. melitensis*[31]. TargetRNA2 was employed predict the target genes of each sRNA. One candidate, BSR0441 was chosen for future characterization due to its predicted targets involvement in virulence. This sRNA was confirmed via RT-PCR and was shown to be growth-phase dependent and involved in heat and acidic stress responses. A *bsr0441* deletion mutant had decreased survival in murine macrophages but had altered levels throughout a mouse model of infection. The regulatory targets BMEII1007/BAB_RS30865, a GntR transcriptional regulator and BMEII1118/BAB_RS29970, the multidrug resistance protein A were confirmed using qRT-PCR. Further investigation is needed to define the regulatory mechanism of BSR0441.

In a study exploring RNaseIII, 126 sRNAs were predicted in *B. melitensis* strain 027 from deep sequencing[32]. A representative sRNA BM-sr00117 was confirmed via

northern blot analysis. However, no further information involving this sRNA was provided. To further understand how this sRNA contributes to *Brucella* it is crucial to identify regulatory targets and understand the role this sRNA plays in *Brucella* biology.

RNA sequencing of *B. melitensis* 16M under acidic conditions followed by analysis revealed 94 *trans*-encoded sRNAs and 948 *cis*-encoded sRNAs[33]. These sRNAs were named BsnR for *Brucella* small non-coding RNA. Regulatory targets of these sRNAs were predicted using TargetRNA2, which revealed many predicted sRNAs regulate various VirB transcripts and the VjbR transcript. sRNA-mRNA interactions were investigating using IntaRNA software. However, these sRNAs-mRNA interactions need to be validated before the regulatory networks can be mapped and understood.

Another study validated an additional 43 sRNAs via RT-PCR from Dong *et. al.,*[27]. Overexpression of one of these sRNAs, named BASI74 led to decreased survival in a macrophage model of infection[34]. The target prediction program, sTarPicker, was used to predict regulatory targets (BAB1_0097/N/A: hypothetical protein, BAB1_0343/BAB_RS17575: hypothetical protein, BAB1_0847/BAB_RS19970: hypothetical protein, BAB1_ 1154/BAB_RS214500: SAM-dependent methyltransferase, BAB1_1335/N/A: hypothetical protein, and BAB1_1361/N/A: hypothetical protein) of this sRNA, and these targets were evaluated via qRT-PCR. However, the mechanisms behind this regulation remain unknown.

Xu *et al.* predicted 14 sRNAs in *B. melitensis* M28, using two bioinformatic prediction programs Softberry and ARnold focusing on conserved promoter and terminator elements within intergenic regions[35]. Among these predicted sRNAs, seven were confirmed via RT-PCR and northern blot, further confirming the presence of four

new sRNAs, named *Brucella melitensis* small RNAs. One sRNA, Bmsr1, encoded downstream of a transcriptional repressor (*bab1_1111/bab_rs07835)*, appeared important for virulence, as a deletion led to attenuation in macrophage and mice models of infection. TargetRNA2 was used to predict the regulatory targets of Bmsr1, and quantitative proteomics using iTRAQ revealed 314 differentially produced proteins, with hits on VirB proteins, as well as VjbR. However, further investigation is required to confirm these regulatory targets and better define the regulatory mechanism of Bmsr1.

Recent work investigating the endoribonuclease RNaseE uncovered a new sRNA named Bsr4 for *Brucella* small RNA. This sRNA was found to be highly abundant in a *rnaseE-trunc* mutant strain, indicating RNaseE aids in the degradation of Bsr4. Sheehan *et al.* also confirmed that Bsr4 is dependent on the RNA chaperone Hfq for full stabilty[36]. Further work is required to elucidate the regulatory role of Bsr4 and how this sRNA contributes to the biology of *B. abortus.*

Contribution of regulatory sRNAs to *Brucella*

Throughout the hundreds of sRNAs that have been predicted in *Brucella* spp., the seven that have been further validated and investigated have showcased the importance of regulatory RNA molecules. Many of these sRNAs are required for virulence and regulate transcripts that are key virulence factors of *Brucella*. This suggests that sRNAs are virulence factors themselves, as they heavily contribute to the adaption process once inside the macrophage. However, many questions are still left unanswered regarding the predicted regulatory targets of these sRNAs. Therefore, experimental approaches are needed to validate these target mRNA predictions. Furthermore, transcriptional regulators controlling the expression of the sRNA molecules are unknown? Experimental

approaches utilizing streptavidin-biotin binding affinity can be employed to identify the transcriptional regulator bound to the sRNA promoter. This review has highlighted the *Brucella* sRNAs that have been identified and starts to shed light on the regulatory mechanisms these sRNAs employ. It is clear the RNA chaperone Hfq plays a major role in virulence and as the regulatory networks of sRNAs are better defined, we can begin to shed light on the major roles these relatively short RNA molecules play in gene regulation and the adaptation process. Therefore, undercovering how sRNAs contribute to the pathogenesis of *Brucella* spp.

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Tables

Table 1.1: Confirmed *Brucella* sRNAs summarized in this review

Name of sRNA	Organism	Orientation	Flanking genes	Predicted Size (nt)	Confirmed with?	Citation
AbcR1	*B. abortus*	→ ← →	*bab2_0515-bab2_0516*	116	Northern	Caswell, 2012
AbcR2	*B. abortus*	→ ← →	*bab1_1514-bab1_1516*	110	Northern	Caswell, 2012
BsrH	*B. abortus*	← → ←	*bab2_0075-bab2_0076*	150	RT-PCR	Peng, 2015
BSR0602	*B. melitensis*	← → ←	*bmeII0601-bmeII0602*	169	Northern	Wang, 2015
BSR0441	*B. melitensis*	← → ←	*bmeII0445-bmeII0446*	250	RT-PCR	Zhong, 2016
BM-sr00117	*B. melitensis* (027)	N/A	*N/A*	N/A	Northern	Wu, 2016
BAS I 74	*B. abortus*	N/A	*babrs_32375-babrs_19410*	86	RT-PCR	Dong, 2018
Bmsr1	*B. melitensis* (M28)	→ → ←	*rsS07835-rsS07840*	121	Northern	Xu, 2018
Bsr4	*B. abortus*	→ ← →	*bab1_0839-bab1_0842*	124	Northern	Sheehan, 2020

Chapter 2: A regulatory small RNA, VcrS, is required for virulence and controls production of an essential cell wall biosynthesis enzyme in *Brucella abortus*

King, K.A.[1], Benton, A.H.[1], Kang, L.[2], Michalak, P.[2], Dunman, P.M. [3], Jutras, B.L.[4] and Caswell, C.C[1,a].

Abstract

Brucella abortus is a facultative, intracellular, zoonotic pathogen that resides inside macrophages during infection. This is a specialized niche where *B. abortus* encounters various stresses, such as acidic conditions and reactive oxygen species as it navigates through the macrophage. In order to survive this harsh environment, *B. abortus* utilizes post-transcriptional regulation of gene expression through the use of small regulatory RNAs (sRNAs). In this study, we identified 9 novel sRNAs expressed by *B. abortus*, and one of these *Brucella* sRNAs, called VcrS is required for virulence in both a macrophage and a chronic mouse model of infection. VcrS (for virulence and cell wall regulating sRNA) requires the RNA chaperone Hfq for stability, and while transcriptomics determined that no mRNAs exhibited differential levels in the deletion strain, quantitative proteomics revealed that only one protein, BAB1_1454, is >30-fold over-produced in Δ*vcrS* compared to wildtype. BAB1_1454 is the essential protein encodes MurF, which catalyzes the final cytoplasmic step of generating the UDP-MurNac-pentapeptide precursor for peptidoglycan synthesis. Dysregulation of MurF production leads to increased resistance to H_2O_2 stress and decreased cell envelope length. The present study underscores the importance of sRNA regulation in the physiology and virulence of *B. abortus*.

Introduction

Brucella is a leading zoonosis worldwide. These Gram-negative bacteria infect cattle, goats, and swine causing brucellosis, a disease resulting in spontaneous abortions and infertility. Brucellosis in humans results from the ingestion or direct contact of contaminated animal products, such as unpasteurized milk, or through the inhalation of aerosols[1]. *Brucella* is a facultative intracellular pathogen, infecting and replicating within macrophages and dendritic cells[2]. Macrophages phagocytize the brucellae, enclosing the bacteria within a *Brucella* containing vacuole (BCV), and traffic the BCV towards the phagolysosome [3]. Along this route, the macrophage subjects the BCV to a variety of stressful conditions aimed at destroying the bacterium, including low pH, reactive oxygen species, and nutrient deprivation[4]. However, *Brucella* can sense and quickly adapt to these various stress conditions, allowing it to avoid fusion with the lysosome. Once fusion with the lysosome is avoided, the BCV is trafficked to the endoplasmic reticulum, and within this specialized niche, *Brucella* readily survives and replicates [5].

Rapid regulation of gene expression is critical for surviving the aforementioned stressful conditions. Post-transcriptional regulation allows for gene expression to be altered very quickly, and one way to achieve rapid gene regulation is through the use of regulatory small RNAs (sRNAs). sRNAs are single-stranded RNA molecules that bind via complementary base pairing to other messenger RNAs (mRNAs), which can influence mRNA stability and translation[6]. There are two main classes of sRNA molecules, *cis*-encoded and *trans*-encoded. *Cis*-encoded sRNAs are located on the DNA strand opposite of its target gene, and therefore these interactions involve longer regions of complementarity base-pairing. Alternatively, *trans*-encoded sRNAs share limited

regions of base-pairing with target mRNAs and often are encoded at distal regions on the chromosome from its regulatory targets. These interactions usually require the RNA chaperone protein Hfq to mediate sRNA-mRNA binding[7] .With this simple mechanism of complementary base pairing, regulatory sRNAs can rapidly influence gene expression, allowing the bacteria to adapt to the stressful conditions within the macrophage and lead to a chronic infection.

The first sRNAs discovered in *B. abortus* were AbcR1 and AbcR2. These sRNAs negatively regulate ABC transport systems, including the transport of amino acids[8] and the transport of a GABA under nutrient limiting conditions[9]. Following this discovery, sRNA prediction programs were implemented to search for additional sRNAs in *Brucella* spp. Hundreds of sRNAs have been predicted [10–14] and new sRNAs have been discovered, such as BsrH which is predicted to regulate HemH[15], and BSR0602 which is highly abundant during stationary phase[11]. Bsr4 was discovered and found to depend on Hfq for its stability[16]. However, further characterization is necessary to understand the collective roles these sRNAs play in *B. abortus*. Better understanding of the sRNAs *B. abortus* utilizes will further elucidate the regulatory mechanisms this bacterium uses to survive the harsh environment of the macrophage.

In the present study, seven new sRNAs in *B. abortus* have been identified and authenticated through northern blot analyses. One of the newly-discovered sRNAs, VcrS, for virulence and cell wall regulating sRNA, is crucial for survival and replication within the macrophage and is required for chronic infection in mice. VcrS negatively regulates the transcript encoding MurF, a protein directly involved with peptidoglycan synthesis.

The work outlined in this study characterizes the role of VcrS in the genetic regulation, biology, and virulence of *B. abortus.*

Results

Discovery of new regulatory small RNAs (sRNAs) in *Brucella abortus*.

To screen for novel *B. abortus* sRNAs, *B. abortus* 2308 was grown to late exponential phase in brucella broth. The bacteria were washed with PBS and inoculated into brucella broth, GMM (nutrient deprivation), brucella broth supplemented with 5 mM H_2O_2, and acidic brucella broth at pH 4.5. Following 20 minutes of incubation, the cells were killed and stabilized, RNA was extracted, and RNA sequencing was performed. We visually mined the RNA-sequencing data searching for high levels of transcript located within intergenic regions, a typical location for *trans*-encoded sRNAs (Fig. 1A). Northern blot analyses were performed to authenticate new sRNAs in *B. abortus* (Fig. 1B). Each sRNA was depicted with its chromosomal organization and relevant information regarding size and nucleotide position (Table 1). These sRNAs are found in "classical" species of *Brucella* (*B. melitensis, B. suis, B. abortus, B. canis, B. ovis, and B. neotomae*) and have been added to the Rosetta Stone for *Brucella* sRNAs that our lab has created to maintain a database for newly discovered sRNAs in *Brucella spp.* (caswelllab.com).

A novel sRNA, VcrS is crucial for *Brucella* virulence

To further investigate *Brucella* sRNAs, the focus shifted to the first sRNA discovered named VcrS for virulence and cell wall regulating sRNA. An unmarked deletion was constructed and designated Δ*vcrS*, along with a reconstruction of *vcrS* back on the chromosome, designated Δ*vcrS*-RC. Strains were confirmed via northern blot analysis to ensure *vcrS* was deleted and reconstructed (Fig. 2A). *B. abortus* strains 2308, Δ*vcrS,* and Δ*vcrS*-RC were used to infect bone-marrow derived macrophages (Fig. 2B).

The Δ*vcrS* strain exhibited a significant reduction in survival and replication at 24- and 48-hours post-infection. Reconstruction of the *vcrS* locus restored the levels of bacteria recovered from the macrophages to levels similar to the parental strain 2308. These data showcase the defect in the ability of the Δ*vcrS* strain to survive and replicate within macrophages. These three strains were also used in a chronic mouse infection model where both female and male BALB/c mice were infected intraperitonially (Fig. 2C) Four- and eight-weeks post-infection, the Δ*vcrS* strain was significantly attenuated compared to the wild-type strain 2308. Again, reconstruction restored spleen colonization to wild-type levels. Together, these data demonstrate that VcrS is required for wild-type virulence of *Brucella* both in a macrophage and chronic mouse model of infection.

VcrS is conserved in *Brucella* species and requires Hfq for stability

VcrS is located on chromosome II of *B. abortus* in the intergenic region between an endonuclease V (*bab2_0621/babRS_29285)*, and a pseudogene predicted to contain a GGDEFF domain (*bab_RS29290*) (Figure 3A). Rapid amplification of cDNA ends (RACE) was employed to determine the transcriptional start site of the sRNA, and mFold was used to predict the secondary structure of VcrS (Supp. Fig 2). Genome sequence analysis revealed VcrS is conserved in "classical" species of *Brucella* (*B. melitensis, B. suis, B. abortus, B. canis, B. ovis, and B. neotomae*). Northern blot analysis further confirmed that VcrS is present in *B. abortus* 2308*, B. melitensis* 16M*,* and *B. suis* 1330 (Fig. 3B). Given the importance of Hfq in sRNA-mediated regulatory pathways, northern blot analysis for VcrS was performed using RNA isolated from *B. abortus* 2308 and *B. abortus* Δ*hfq* strains. VcrS expression was abolished in the *hfq* deletion strain, and

expression was restored in the *hfq*-complemented strain (Fig. 3C). These results confirm that VcrS is a conserved *Brucella* sRNA and requires Hfq for stability.

Identifying regulatory targets of VcrS

The significant virulence attenuation in the absence of *vcrS* raised questions about the identity of VcrS regulatory targets, and in order to determine the targets of VcrS, microarray analysis and quantitative proteomics using iTRAQ labeling was employed to compare the transcriptomes and proteomes, respectively, of *B. abortus* strains 2308 and Δ*vcrS.* Quantitative proteomics identified one protein, annotated as MurF (BAB1_1454/BAB_RS22860), was approximately 30-fold over-produced in Δ*vcrS* compared to the parental strain 2308 (Fig. 4A). Interestingly, microarray analysis revealed that no transcripts, including the MurF transcript, were significantly altered between the two strains. As confirmation, MurF mRNA levels were analyzed directly by qRT-PCR, and we determined that indeed MurF mRNA is not significantly altered in the Δ*vcrS* strain (Supp. Fig. 3). In order to verify the proteomic findings, a 3xFLAG tag was fused to the carboxy-terminal end of MurF on the chromosome in *B. abortus 2308,* Δ*vcrS,* Δ*vcrS*-RC, and Δ*hfq*, and western blot analysis was performed to determine MurF levels, as well as SodC protein levels as a control (Fig. 4B). Overall, these data demonstrate that MurF is a regulatory target of VcrS, and VcrS regulates MurF protein production but not MurF mRNA levels.

Deletion of *vcrS* results in increased resistance to oxidative stress

MurF is essential in *B. abortus*[17] and involved in peptidoglycan biosynthesis. MurF is an ATP-dependent amino acid ligase, catalyzing the addition of the final two D-

alanine residues onto the growing muropeptide chain in the cytoplasm[18]. Dysregulation of MurF could alter cell wall properties, which in turn can alter sensitivities to cell wall targeting stressors. Therefore, we utilized a disk diffusion assay to test the effects of cell wall and cell membrane targeted stressors on the growth of *B. abortus* strains 2308 Δ*vcrS,* and Δ*vcrS*-RC*,* and we demonstrated a significant decrease in the zone of inhibition of Δ*vcrS* when stressed with H_2O_2 (Fig. 5). Importantly, reconstruction of the *vcrS* locus restored the zone of inhibition to wild-type levels. These results led us to hypothesize that deletion of *vcrS* leads to elevated MurF levels, which in turn, alters the cell wall making the bacteria more resistant to H_2O_2. This suggests the overproduction of MurF is responsible for the increased resistance to H_2O_2.

Impact of MurF overproduction on *B. abortus* cell wall organization

Transmission electron microscopy (TEM) was performed to visually investigate how the deletion of *vcrS* that leads to an overproduction of MurF alters cell wall organization. Bacterial cells from *B. abortus* 2308 and Δ*vcrS* were grown to stationary phase in brucella broth, and cells were prepared for transmission electron microscopy. Images revealed a visible difference in cellular envelope organization. In the absence of *vcrS*, there is a decrease in the cell envelope length compared to wild-type cells (Fig. 6A). In order to determine the cell wall architecture and composition, bacterial cells from *B. abortus* 2308 and Δ*vcrS* were grown to stationary phase and peptidoglycan was isolated and analyzed liquid chromatography and mass spectrometry. Surprisingly, this analysis revealed no altered chemical composition or abundance in specific muropeptides between these two strains (Fig. 6B). This suggests the alteration in cell envelope size is

independent of peptidoglycan organization. Collectively, the deletion of *vcrS* results in altered cellular envelope length, while interestingly maintaining similar cell wall architecture composition to wild-type.

Discussion

The importance of sRNAs in regulating bacterial gene expression in response to changing environmental conditions has been evident[19,20]. Within *Brucella*, 77 sRNAs have been confirmed, with several of these sRNAs regulating importance virulence mechanisms including amino acid transport[8], heme transport[15], and predicted control of *vjbR* and *virB* gene expression[21]. This study has identified seven novel sRNAs in *B. abortus*, all of which are conserved between *Brucella* spp. (Fig. 1). Moreover, we have characterized one of these sRNAs that is essential for the full virulence of *B. abortus*, and we have named this sRNA VcrS. (Fig.2)

VcrS is a *trans*-encoded, Hfq-dependent sRNA located on chromosome II of *B. abortus* (Fig. 3) Deletion of *vcrS* resulted in an approximate 30-fold overproduction of a single protein, MurF (BAB1_1454/BAB_RS22860). MurF is involved in peptidoglycan synthesis, where it joins the final two D-alanine residues on the growing muropeptide chain. Since *trans*-encoded sRNAs often negatively regulate their targets, either by promoting degradation of target mRNAs or interfering with translation[6], we examined MurF mRNA levels and found no significant difference in transcript levels in the Δ*vcrS* mutant (Supp. Fig. 2) Translation interference through blockade of initial ribosomal binding is the canonical regulatory mechanism of *trans*-encoded sRNAs [22]. Structural analysis of VcrS using mFold reveals four single-stranded seed regions, one of which contains sequence complementary to the ribosomal binding site of MurF. We propose, therefore, that VcrS is directly interfering with MurF translation, rather than repressing MurF mRNA transcription or stability. Our lab is currently investigating this binding motif to confirm direct interaction between VcrS and MurF ribosomal binding site.

Since VcrS seemingly regulates only MurF, we attribute the attenuation of the Δ*vcrS* mutant in both macrophage and mouse infection assays to overproduction of MurF (Fig. 2). Transmission electron microscopy shows that the Δ*vcrS* mutant has a shorter cell envelope, however, this difference in length is not due to the chemical composition of peptidoglycan or the architecture of cross-linking (Fig. 6). The mechanism by which increased MurF results in cell envelope shortening is not clear at this time. Furthermore, the Δ*vcrS* mutant exhibits increased resistance to hydrogen peroxide (Fig, 5A), indicating the reduction in the size of the periplasmic space may have physiological consequences for the bacteria.

While it is clear that Δ*vcrS* mutants are attenuated, the underlying physiologic mechanisms resulting in loss of fitness during infection remain obscure. It is possible that the shortening of cell envelope displaces outer membrane proteins resulting in greater immune recognition and host clearance. It is also possible that the decreased periplasmic space results in alterations in protein folding and trafficking. Our lab is currently exploring the consequences of the deletion of *vcrS* and how the overproduction of MurF impact the biology of *Brucella*.

It is interesting, yet not surprising, that VcrS has a single regulatory target. The *E. coli* sRNA, MicF, has been extensively studied and shown to regulate the translation of a single transcript, OmpF [23]. Since VcrS is only regulating MurF production, it is imperative that VcrS RNA levels are maintained to avoid dysregulation of MurF. The question arises, however, what transcriptional regulator is interacting with the promoter to control *vcrS* expression. This regulation is likely crucial to not only maintain proper

RNA levels of VcrS, but also proper protein levels of MurF. The regulation of VcrS expression if currently being explored in our laboratory.

Overall, the work above has identified new *Brucella* sRNAs and has further characterized the regulatory role of VcrS. This sRNA is required for virulence, and regulates the production of an essential protein, MurF. It is hypothesized that VcrS is interfering with proper ribosomal binding needed for translation initiation. These findings expand the understanding of how *Brucella* adapts to the harsh environment of the macrophage by utilizing regulatory sRNAs to quickly alter gene expression.

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) or in brucella broth (BD). For cloning, *Escherichia coli* strains (DH5α) were grown on tryptic soy agar (BD) or in Luria-Bertani (LB) broth. When appropriate, medium was supplemented with kanamycin (45 μg/ml) or carbenicillin (100 μg/ml). All experiments utilizing *Brucella* strains were performed in a biosafety level 3 (BSL-3) facility.

Construction of *B. abortus* mutants, genetic complementation, 3xFLAG tag, and overexpression strains

The strain containing a nonpolar unmarked deletion of *vcrS* was constructed using the strategy previously stated[8,16,24,25]. Briefly, a 1-kb fragment immediately upstream of *vcrS* was amplified using *vcrS*-Up-For and *vcrS*-Up-Rev primers (Table 2.1), and a 1-kb fragment downstream of *vcrS* was amplified using *vcrS*-Dn-For and *vcrS*-Dn-Rev primers and genomic *B. abortus* 2308 DNA and *Phusion* polymerase (Monserate Biotechnology Group). The upstream fragment was digested with BamHI, and the downstream fragment was digested with PstI. Both fragments were phosphorylated using polynucleotide kinase in the presence of ATP. The upstream and downstream fragments were then ligated to each other and then into a BamHI/PstI digested pNPTS138 vector (M. R. K. Alley, unpublished data) using T4 DNA ligase (Monserate Biotechnology Group, San Diego, CA). The deletion construct (pKK001) (Table 2.2) was introduced into *B. abortus* 2308, and merodiploid transformants were selected on SBA containing kanamycin.

Merodiploid transformants were then incubated at 37°C for 7 h and then plated onto SBA plus 10% sucrose. Colonies that were sucrose resistant and kanamycin sensitive were screened via PCR for a loss of the genes of interest. The unmarked *vcrS* mutant derived from *B. abortus* 2308 was named KK001.

Reconstruction of the *vcrS* gene was achieved using the *sacB* counter-selection strategy described above, but with modification. The *vcrS* locus was amplified using *vcrS*-Up-For and *vcrS*-Dn-Rev primers and genomic *B. abortus* 2308 DNA. The amplified product was digested with BamHI and PstI, phosphorylated and ligated together and ligated into the pNPTS138 plasmid. This plasmid (pKK002) (Table 2.2) was then introduced into *B. abortus* KK001 and selection and screening were carried out as described above. The reconstructed strain was named KK014.

The addition of the C-terminal 3xFLAG tag (5' GACTACAAAGACCATGACGGTGATTATAAAGATCATGACATCGACTACAAGG ATGACGATGACAAG 3') to *murF* (*bab1_1454/bab_rs22860*) DNA sequence was incorporated as previously described[25] primers Up-Rev and Down-For were designed to include half of the 3xFLAG tag. The upstream region was amplified using *bab1_1454*-Up-For and *bab1_1454*-Up-Rev-3xFLAG, and the downstream region was amplified using *bab1_1454*-Down-For-3xFLAG and *bab1_1454*-Down-Rev. These fragments were digested using BamH1 for the upstream fragment and Pst1 for the downstream fragment, phosphorylated and ligated together into the pNTPS138 plasmid. This plasmid (pKK003)

was introduced into *B. abortus* strains 2308, KK001, KK014, and B8 (*hfq* deletion) and resulting strains were named KK024, KK025, KK026, KK027, respectively.

For the construction of overexpression of *murF* (*bab1_1454/bab_rs22860*) was amplified using *bab1_1454*-Up-For-OE and *bab1_1454*-Down-Rev-OE from genomic DNA isolated from KK024, which has a C terminal 3xFLAG tag on *murF*. This fragment was digested using Nde1 and Nhe1 and subsequently ligated with digested pSRKK [26]. This plasmid (pKK004) was then introduced into *B. abortus* 2308 and following PCR screening, the resulting strain was named KK028.

RNA-sequencing analysis

All RNA extractions were carried out as previously described[8,24,25,27] *Brucella* strains were grown to an OD_{600} nm of 0.15 with constant shaking at 37°C. Cells were then washed with PBS, and cells were stressed with either 5mM H_2O_2, pH 4, GMM, or grown in brucella broth, at a concentration of 10^9 CFU/ml. Cultures were incubated for 20 min at 37°C with shaking. Following incubation, each culture received equal amounts of 1:1 ethanol-acetone and cultures were stored at -80°C. The cultures were then centrifuged at 10,000 × *g* for 10 minutes. RNA was isolated from cells with TRIzol reagents (Invitrogen) followed by ethanol precipitation. Genomic DNA was removed with DNase I and samples were further purified by phenol-chloroform extractions and precipitated with ethanol. RNA samples were then resuspended in nuclease-free H_2O, and the purity of the samples 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.

Stranded RNA library construction for prokaryotic RNA-seq.

One microgram of total RNA with an RNA integrity number (RIN) of ≥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 Illumina's TruSeq stranded mRNA high-throughput (HT) sample prep kit (catalog no. RS-122-2103; Illumina). This is followed by second-strand synthesis using polymerase I and RNase H, and dinucleotide triphosphates (dNTPs) that contain dUTP instead of dTTP. The cDNA fragments then go through end repair, the 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 a stranded template. The template molecules with the adapters are enriched by 10 cycles of PCR to create the final cDNA library. The library generated was validated using Agilent 2100 Bioanalyzer and quantitated using the Quant-iT double-stranded DNA (dsDNA) HS kit (Invitrogen) and quantitative PCR (qPCR). Sixteen individually indexed cDNA libraries were pooled and sequenced on an Illumina NextSeq platform.

Illumina NextSeq sequencing.

The libraries were clustered and sequenced using the NextSeq 500/550 high-output kit V2 (150 cycles) (P/N FC-404-2002) to 2 × 75 cycles to generate paired-end reads. The

Illumina NextSeq control software version 2.1.0.32 with Real Time Analysis (RTA) version 2.4.11.0 was used to provide the management and execution of the NextSeq 500 kit and to generate BCL files. The BCL files were converted to FASTQ files and demultiplexed using bcl2fastq conversion software version 2.20.

RNA-Seq data processing and analysis.

The *Brucella abortus* strain 2308 gene and genome sequences, as well as corresponding annotations from NCBI (https://www.ncbi.nlm.nih.gov/), were used as the references. Raw reads were quality controlled and filtered with FastqMcf [28], resulting in an average of 1,971 Mbp (1,730 to 2,305 Mbp) of nucleotides per read The remaining reads were mapped to the gene reference using BWA[29] with default parameters. Differential expression of genes was calculated using the edgeR package [30] in the R software (http://www.r-project.org/), with Benjamini-Hochberg-adjusted *P* values of 0.05 considered to be significant.

Northern blot analysis

RNA was isolated from *Brucella* cultures as described previously [8]. Ten micrograms of RNA were 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-32P]-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™-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 h at ~45°C in a rotating hybridization oven. The oligonucleotide probes [VcrS-Northern, Bsr19-Northern, Bsr20-Northern, Bsr21-Northern, Bsr22-Northern, Bsr23-Northern, Bsr24-Northern, and 5S-Northern] (Table S1) were end-labeled with [γ-32P]-ATP and polynucleotide kinase. The radiolabeled probes were incubated with the prehybridized membranes at ~45°C in a rotating hybridization oven overnight (~12 h). The next day, membranes were washed four times for 30 min 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.

Rapid Amplification of cDNA Ends (RACE)

RNA was isolated from *Brucella abortus* 2308 as described previously, and contaminating genomic DNA was removed by treatment with RNase-free DNase I (Ambion). 5'-RACE was carried out using the 5' RACE system kit (Invitrogen) according to a previously described method [31]. Primers utilized for this procedure VcrS-5'RACE- GSP1 and VcrS-5'RACE- GSP2 are found in in Table S1. The 5'- RACE products were gel purified and cloned into pGEM-T Easy (Promega). Plasmid DNA was purified from potential clones, and DNA sequencing was performed to identify the transcriptional start site of VcrS.

Microarray analysis

RNA was isolated from *Brucella* cultures grown to late exponential phase in brucella broth [8], 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 vcrS* mutant, were reverse transcribed, fragmented and 3′ biotinylated as previously described [32]. 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. abortu*s 2308 and the *vcrS* 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.

Isobaric tag for relative and absolute quantitation (iTRAQ) proteomic analysis

iTRAQ was performed as described previously [33]. Briefly, *B. abortus* 2308 and *B. abortus* KK001 (Δ*vcrS*) were grown to early stationary phase in brucella broth, and the cells were collected by centrifugation (15,000 X *g* for 10 min at 4°C) and suspended in ~700 µl of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH7.5). The cells were then boiled

for 1 hour, followed by 20 cycles (setting 4; 20 seconds per cycle) of disintegration in Lysing Matrix B (MP Biomedicals, Solon, OH) using a BIO101 FastPrep FP120 cell disintegrator (Thermo Savant [Thermo Fisher Scientific Inc.], Waltham, MA). Approximately 100 µg of protein from each sample was digested with sequencing grade modified porcine trypsin (Promega), and the tryptic peptides were labeled with isobaric tags according to the guidelines in the iTRAQ® Reagents Multiplex Kit (AB SCIEX, Foster City, CA, USA). The labeled peptides were then purified using strong cation exchange (SCX) high performance liquid chromatography (HPLC) before being analyzed via liquid chromatography and tandem mass spectrometry (LC-MS/MS). ProteinPilot V2.0.1 software (AB SCIEX, Foster City, CA, USA) was used to search the annotated *Brucella melitensis* biovar *abortus* 2308 genome sequence, and differences in the amounts of identical peptides between the parental strain 2308 and the *vcrS* mutant were determined using the software.

Reverse transcriptase quantitative PCR (qRT-PCR).

Total RNA was isolated from *B. abortus* strains and treated with DNase I as described above. cDNA was generated from the final RNA preparation using a SuperScript III cDNA synthesis system (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol, and this cDNA was used for real-time PCR employing a SYBR green PCR supermix (Roche, Mannheim, Germany). For these experiments, primers for 16S rRNA were used as a control, and gene-specific primers were used for evaluating relative levels of MurF mRNA. The steps for PCR consisted of a single denaturing step for 5 min at 95°C, followed by 40 cycles (denature for 15 s at 95°C, anneal for 15 s at 51°C, and

extend for 15 s at 72°C) of amplification. Fluorescence from SYBR green incorporation into double-stranded DNA was measured with an iCycler machine (Bio-Rad), and the relative abundance of mRNA was determined using the Pfaffl equation[34].

Virulence of *B. abortus* strains in murine macrophages and experimentally-infected mice

To test the virulence of *Brucella* mutant strains, experiments utilizing bone-marrow derived murine macrophages were carried out as previously described [8,16,25,27]. Briefly, macrophages were harvested from mice and seeded into 96-well plates in Dulbecco's modified Eagle's medium with 5% fetal bovine serum. The next day, macrophages were infected with opsonized *brucellae* at a multiplicity of infection (MOI) of 100:1. Following incubation for 2 hours, infected macrophages were treated with gentamicin (50 μg/ml) for 1 hour. Macrophages were the lysed with 0.1% deoxycholate in PBS, and serial dilutions were plated on SBA. For other timepoints, macrophages were washed with PBS following gentamicin treatment, and fresh cell culture medium containing gentamicin (20 μg/ml) was added to the monolayer. At indicated timepoints, macrophages were lysed, serial diluted and plated on SBA in triplicate.

The infection and colonization of mice by *Brucella* mutant strains was performed as previously described [8,16,25,27]. 6-week-old BALB/c mice (six mice: three male and three female per *Brucella* strain) were infected intraperitoneally with 5×10^4 CFU of each *Brucella* strain in PBS. Mice were then sacrificed at 4- and 8-weeks post-infection,

spleens were homogenized, and serial dilutions were plated on SBA. All data was analyzed by the statistical software JMP 11.0.0 (SAS Institute, Cary, NC).

Electron microscopy

Brucella strains were grown to the appropriate phase of growth in brucella broth with constant shaking (200 rpm) at 37°C. When cells reached the stationary phase, cultures were killed and preserved by treatment with 1:1 ethanol:acetone (mixed 1:1 with the culture), and the cultures were centrifuged at 16,000 × *g* for 10 min. Supernatants were discarded, and pellets were washed once with cold dH_2O followed by vigorous vortex mixing. Cells were then centrifuged at 16,000 × *g* for 10 min, and the supernatants were discarded. The pellets were then fixed in 0.1 M sodium cacodylate. Fixed samples of *brucellae* were then submitted to the Electron Microscopy Services at the Virginia-Maryland College of Veterinary Medicine (VMCVM) for transmission electron microscopy. Samples were fixed in0.1% OsO_4 in 0.1 M sodium cacodylate buffer, and dehydrated with 15%, 30%, 50%, 70%, 95%, and 100% ethanol. Dehydration was continued by submersion in propylene oxide and infiltrated with a 50:50 solution of propylene oxide:Poly/Bed812 followed with complete infiltration with 100% mixture of Poly/Bed812. Samples were embedded onto molds which cured at 60°C for at least 48 hours. Cells were then viewed using a Carl Zeiss 10/CA microscope.

Peptidoglycan purification and analysis

B. abortus peptidoglycan was purified as described previously [35]. Briefly, cells were harvested at 3,500 x g, washed three times with PBS, and resuspended in PBS. Cell

suspensions were added dropwise to 10% of boiling SDS. The final concentration of SDS 5%. After boiling for 1-hour, insoluble material was collected by ultra-centrifugation at 275,000 x g for 1 hour and washed five times with 20mL of 35°C water. PG was resuspended in PBS and treated with 1000 U Benzonase Nuclease (Sigma-Aldrich) for 4 hours at 37°C, followed by overnight digestion with 300 µg/mL chymotrypsin (Sigma-Aldrich) at the same temperature. SDS (1%, final concentration) was added to each and boiled briefly. Insoluble material was once again harvested as described above and washed 3 times. After removing undigested material by centrifugation, supernatants containing muropeptides were passed through a YM-10 filter and flow through dried. The concentration of the purified *B. abortus* peptidoglycan was determined using dry weight. *B. abortus* peptidoglycan was re-suspended in phosphate buffered saline (Thermo-Fisher) prior to use.

Acknowledgements

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Tables

Table 2.1: Confirmed Brucella small RNAs

Name of sRNA	Organism	Orientation	Flanking genes	Predicted Size (nt)	Confirmed with?
VcrS	Abortus	→ ← →	*bab2_0621-(bab2_0622)*	160	Northern
Bsr19	Abortus	→ ← →	*bab1_0323-(bab1_0324)*	150	Northern
Bsr20	Abortus	← → →	*bab1_1550-bab1_1551*	300	Northern
Bsr21	Abortus	← → →	*bab1_0653-bab1_0655*	150	Northern
Bsr22	Abortus	→ ← →	*bab_RS32205-bab1_0449*	300	Northern
Bsr23	Abortus	← ← ←	*bab1_1119-bab1_1121*	150	Northern
Bsr24	Abortus	← ← →	*bab2_1026-bab_RS33505*	300	Northern

Table 2.2 Oligonucleotide primers used in this study

Primer name	**Sequence 5'→ 3'**
vcrS-Up-For	TAGGATCCTCGAATATCTGCCCTGTCTTAC
vcrS -Up-Rev	CTGCTCTGCGAGTGGCTGACG
vcrS -Down-For	TAGAGCGTTGCTCCACGACACG
vcrS -Down-Rev	TACTGCAGCGAGTTCCGAGAAAGTCCAGGG
bab1_1454-Up-For	GGATCCGCCCTGCGCCATGTTC
bab1_1454-Up-Rev-3xFLAG	**ATCTTTATAATCACCGTCATGGTCTTTGTAGTC**TTCCGCCGCTTCTACGGG
bab1_1454-Down-For-3xFLAG	**CATGACATCGACTACAAGGATGACGATGACAAG**TAGGCGATTATTGAGGGA
bab1_1454-Down-Rev	CTGCAGTGGATGGGGGCCATGAGA
bab1_1454-Up-For-OE	CATATGAGCAATCGGGAATTGAGCGATTGGCTCTGGA
bab1_1454-Down-Rev-OE	GCTAGCCTATTCCGCCGCTTCTACGGGCGGAAACT
VcrS-Northern	GGAGGGTGCTTCCACGGAGAAGATTGTCAAAA
Bsr19-Northern	GTTATTGCAGCATCAACCCGACGCTTAGAAA
Bsr20-Northern	CCGGTGCGGGTTTTGTTTATCTAAGTGTGAAT
Bsr21-Northern	GCCCACATGTCGCACGAAAGAATTTAACAG
Bsr22-Northern	ACTGTTCCGTTTGCCATATTGAATTCCT
Bsr23-Northern	CGCGAACGCATGTTGCGCAGCGTATCACGA
Bsr24-Northern	GTCGCCCGTGCCGCCTGCAACATCAAGCGA
5S-Northern	AGTTCGGAATGGGATCGGGTGCAGCC
VcrS-5'RACE-GSP1	AAATTCACATGTTAAAAAAT
VcrS-5'RACE-GSP2	CACGAGTTAAAAATGGATGGGCAAAAAGTACTAGTCGTAT
bab1_1454-qRT-For	GGATCCGCCCTGCGCCATGTTC
bab1_1454-qRT-Rev	GCGGCACGCCCCAATGATTGTTGAACGAGG

- underlined sequences represent restriction enzymes recognition site

-bolded sequences depict the 3X-FLAG nucleotide sequence

Table 2.3 Plasmids used in this study

Plasmid name	Description	Reference or source
pNPTS138	Cloning vector; contains *sacB* gene; Kan^{ra}	M. R. K. Alley, unpublished data
pSRKKm	pBBR1MCS-2 derived broad-host-range expression vector containg *lac* promoter and Kan^{R}	[25]
pKK001	Deletion of *vcrS* plus 1kb of each flanking region in pNPTS138	This study
pKK002	Wild-type *vcrS* plus 1kb of each flanking region in pNPTS138	This study
pK003	Intact *bab1_1454* gene plus coding sequence for 3xFLAG tag at C terminus and 1kb of each flanking region in pNPTS138	This study
pKK004	Intact *bab1_1454* gene plus coding sequence for 3xFLAG tag at C terminus in pSRKKm	This study

Figures and Figure Legends

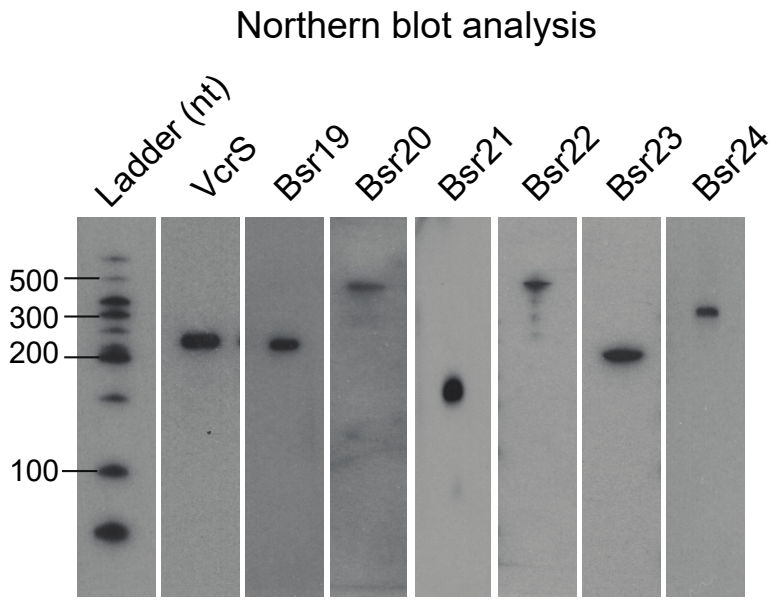
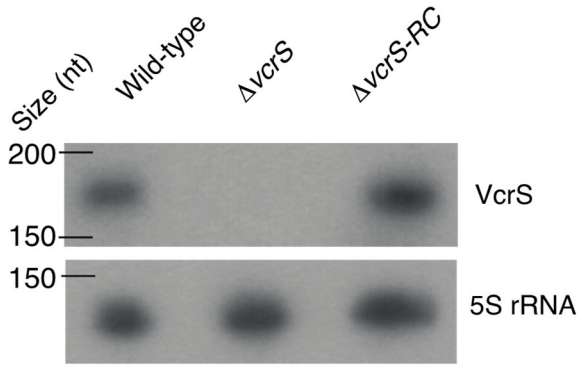


Figure 2.1: Discovery of sRNAs in *Brucella abortus* 2308

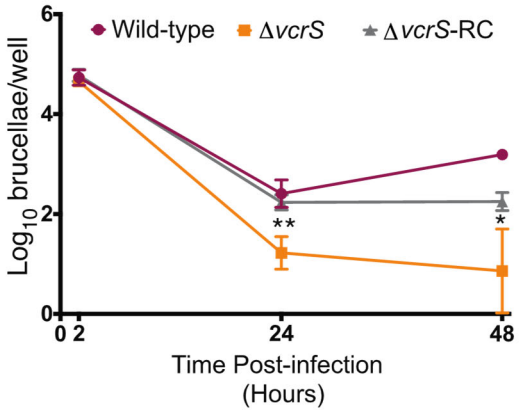
Total RNA from *B. abortus* strains 2308 was separated on a denaturing polyacrylamide gel and transferred to a nitrocellulose membrane. Probes were designed to detect predicted sRNAs (Table S1). The size (in nucleotides) for confirmed sRNAs are shown with a ladder on the left side of the panel

A



Northern blot analysis

B



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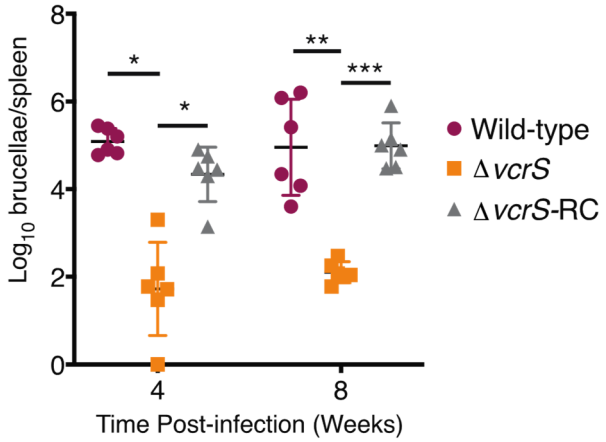
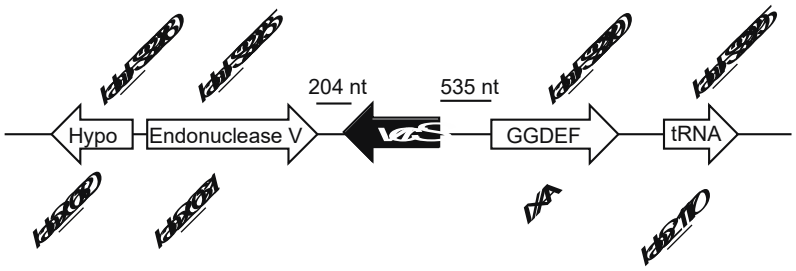


Figure 2.2: VcrS is essential for wild-type virulence of *Brucella abortus* 2308

A. Northern blot detection of VcrS and absence in mutant strain

Total RNA from *B. abortus* strains 2308, Δ*vcrS*, and Δ*vcrS*-RC was separated on a denaturing polyacrylamide gel and transferred to a nitrocellulose membrane. A probe was designed to detect VcrS. 5S rRNA was detected as a loading control. B. Survival and replication of the *B. abortus* Δ*vcrS* mutant in bone-marrow derived macrophages. Macrophages were collected from the bone marrow of male C57BL/6 mice and infected with the parental *B. abortu*s strain 2308, the Δ*vcrS* mutant strain, or the Δ*vcrS* reconstructed strain. 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; $P < 0.05$) are indicated by an asterisk (*). C. Spleen colonization of BALB/c mice infected with the *B. abortus* Δ*vcrS* mutant strain. Six-week-old male and female BALB/c mice were infected intraperitoneally with *B. abortus* 2308, Δ*vcrS*, or Δ*vcrS*-RC. Six mice (three male and three female) were used for each *Brucella* strain at each time point. At 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 determine the number of *brucellae* per spleen. Data are presented as average *brucellae* per spleen +/− the standard deviation from the six mice infected with each *Brucella* strain. Statistically significant differences (*t*-test; $P < 0.05$) are indicated by an asterisk (*).

A



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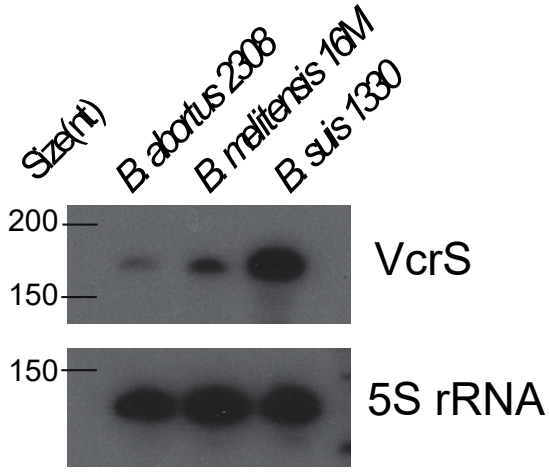
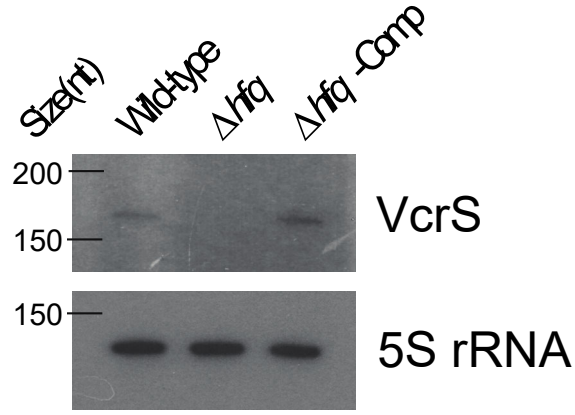


Northern blot analysis

C



Northern blot analysis

Figure 2.3: Characterization of VcrS in *Brucella abortus* 2308

A. Schematic of the loci encoding VcrS. VcrS is encoded on chromosome II in the intergenic region between endonuclease V (*bab2_0621/bab_RS29285)* and a pseudogene containing a GGDEF domain (*bab_RS29290).* B. VcrS is found in *Brucella spp.* Northern blot analysis confirmed the presence of VcrS in *B. abortus, B. melitensis,* and *B. suis*. C. Northern blot analysis was performed to determine the abundance of VcrS in *B. abortus* strains 2308, Δ*hfq*, and Δ*hfq*-Comp. 5S rRNA was used as a loading control.

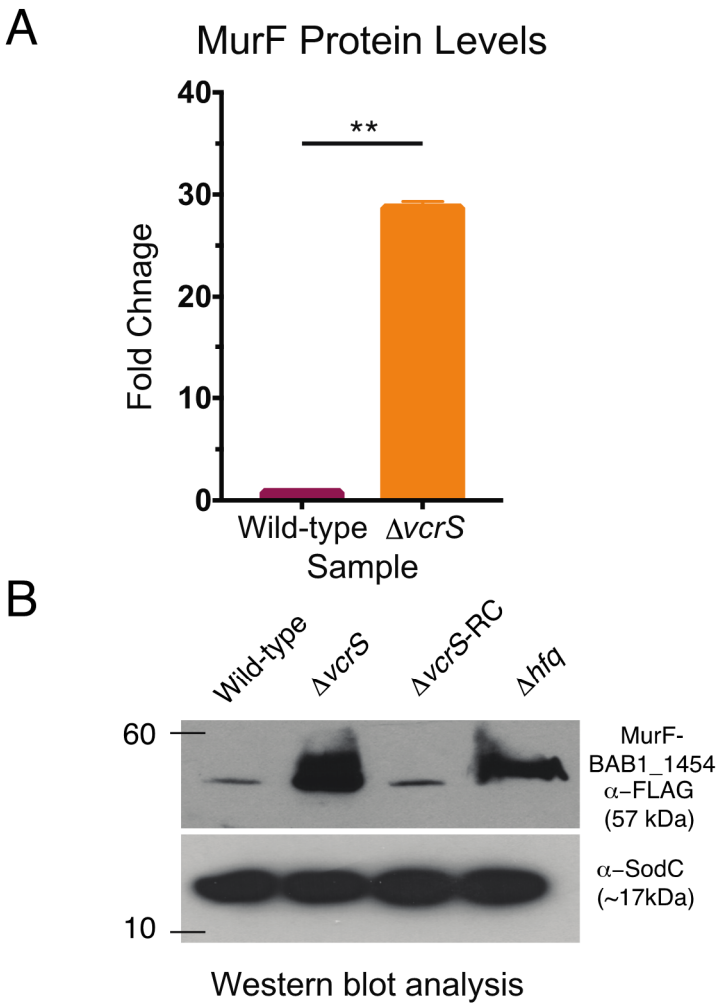


Figure 2.4: Identification of the targets regulated by VcrS

A. Quantitative proteomics analysis using iTRAQ labeling was performed on whole protein lysates from *Brucella* strains grown in brucella broth to early stationary phase. One protein, BAB1_1454 annotated as MurF was found to be 30-fold overproduced in the *vcrS* deletion strain compared to wild-type. B. Western blot analysis of total proteins lysates from *Brucella* strains with *bab1*_1454-3X-FLAG grown to exponential phase. Antibodies utilized include anti-FLAG and anti-SodC.

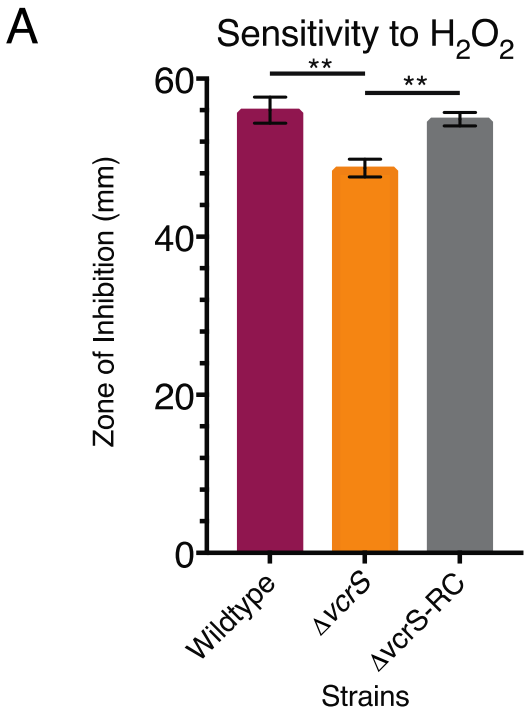


Figure 2.5: Deletion of *vcrS* increases resistance to oxidative stress

A. *Brucella* strains were grown on Schaedler blood agar at 37°C under 5% CO_2 for 48 to 72 h, and the bacterial cells were harvested into phosphate-buffered saline (PBS) and suspended at a concentration of ~3.33×10^7 CFU/ml in brucella broth containing 0.6% agar (maintained at 55°C). Four milliliters of this suspension were overlaid onto agar plates, and after solidification of the overlay, a sterile 7-mm Whatman disk was placed in the center of each plate. Seven microliters of a 9.97 M solution of H_2O_2 was applied to each filter disk, and the plates were incubated at 37°C with 5% CO_2 for 72 h. Zones of inhibition around each disk were then measured in millimeters.

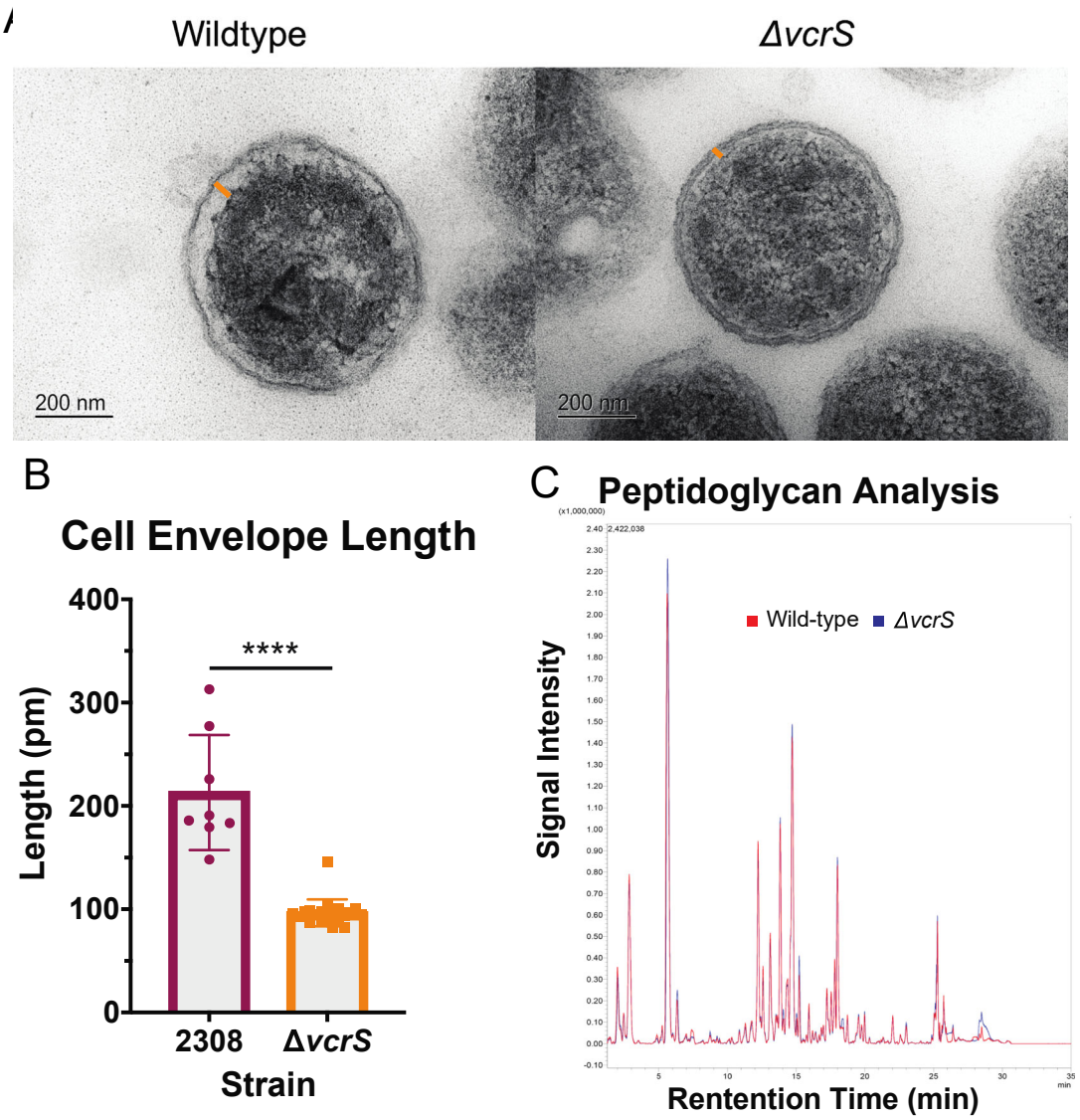


Figure 2.6: Impact of MurF overexpression on cell wall synthesis

A. Electron microscopy of *Brucella abortus* cells. Exponential- and stationary-phase cells of *Brucella* strains were fixed and viewed using transmission electron microscopy, Red bar depicts cell envelope measurement (magnification, ×20,000). Bars = 200 nm. B. C. Liquid chromatography spectra attained from muropeptides, isolated from strain wild-type (red) and Δ*vcrS* (blue). *B. abortus* 2308 and Δ*vcrS* strains were grown to early stationary phase and peptidoglycan was purified and subject to mutanolysin digestion. Muropeptide abundance was plotted as a function of retention time.

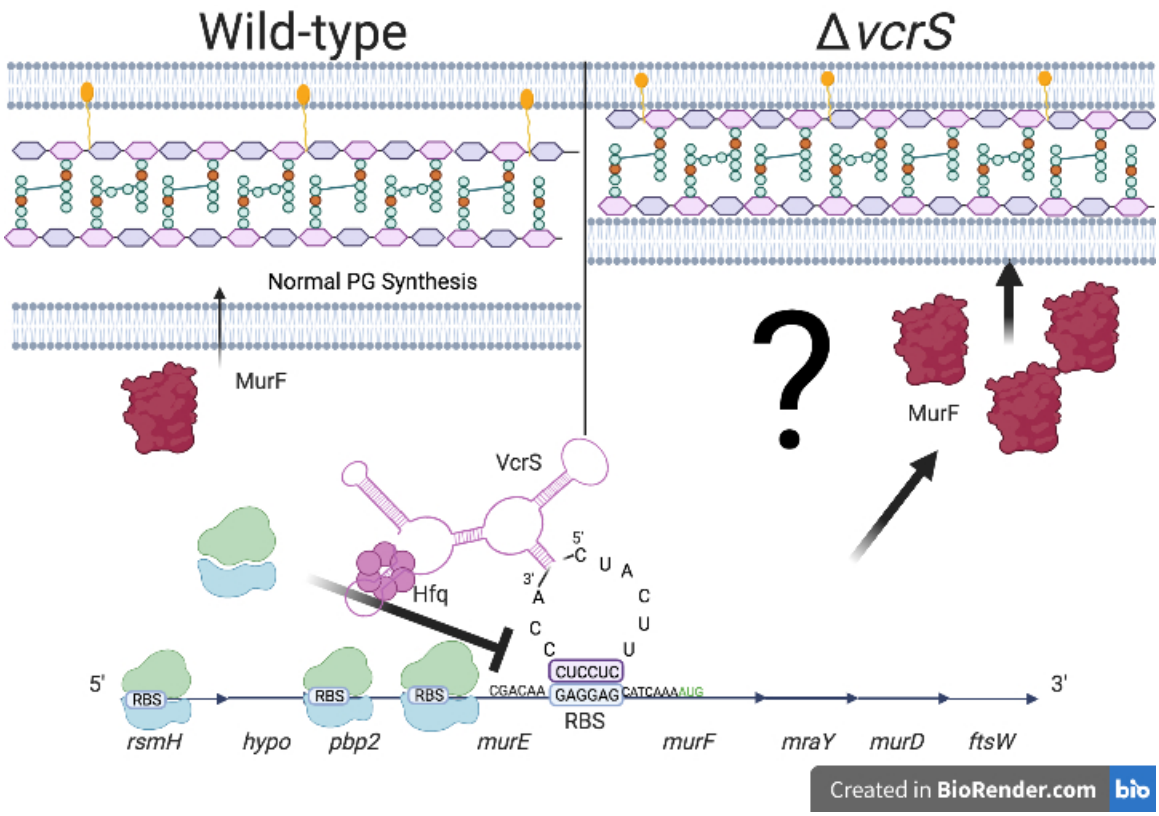


Figure 2.7: Working model of VcrS regulation of MurF

Chapter 4: Conclusions and Further Discussion

Conclusions and Further Discussion

The work presented in this thesis confirmed seven novel regulatory sRNA molecules conserved among "classical" species of *Brucella* (*B. melitensis, B. suis, B. abortus, B. canis, B. ovis, and B. neotomae*). This work supports the initial hypothesis that *Brucella* encodes and uses sRNA regulation as a way to cope with the harsh conditions within the macrophage. One sRNA, VcrS was required for virulence and was further functionally characterized. Throughout the chapters presented above, the regulatory network of VcrS is investigated through identifying the regulatory target and putative binding motifs utilized for regulation.

Brucella utilizes sRNAs to adapt to changing environmental conditions

sRNAs are key mediators between the outside environment and inside the bacterial cell[1]. Hfq, the RNA chaperone is important for the adaptation of bacteria to host conditions in α-proteobacteria, such as *Sinorhizobium*[2] and *Agrobacterium*[3]. In *B. abortus*, an *hfq* deletion mutant displayed increased sensitivity to oxidative stress, acidic stress, and nutrient deprivation and also resulted in decreased virulence[4]. The *hfq* deletion mutant also led to the discovery of the first sRNAs in *B. abortus*, AbcR1 and AbcR2 which are required for virulence[5]. *Brucellae* create a specialized niche within the endoplasmic reticulum of the macrophage, where the bacteria can sense and quickly adapt to the harsh conditions. It is not surprising that sRNAs are key players in this adaptation process. Since the initial discovery, hundreds of sRNAs have been predicted using bioinformatic prediction programs; however, less than 10 sRNAs have been functionally characterized in *Brucella*. In order to better understand the contribution

sRNAs play in the adaptation process, defining the regulatory networks of these sRNAs is fundamental.

In chapter 2, seven new sRNAs were confirmed in *Brucella* spp. Further characterization of one sRNA, VcrS for virulence and cell wall regulating sRNA revealed this sRNA is required for virulence. One striking result of the study was deletion of *vcrS* resulted in >30-fold overproduction of MurF (BAB1_1454) protein compared to wild type *B. abortus* 2308; surprisingly, MurF transcript levels remained consistent between strains. These findings indicate that VcrS is interfering with translation to affect MurF protein levels. The questions that remain are: what consequence arise due to the overproduction of MurF and how those consequences impact the bacterial cell? The results depict an increased resistance to hydrogen peroxide when *vcrS* is deleted. The initial hypothesis arose that if *vcrS* is absent, then MurF is overproduced, which causes altered peptidoglycan architecture or composition. Interestingly, following peptidoglycan analysis from *B. abortus* 2308 and *vcrS* deletion strain, no changes were detected in the architecture and chemical composition of the cell walls between strains. This was unexpected, as transmission electron microscopy depicts altered cellular envelope organization, with a decrease in cellular envelope length when *vcrS* is deleted compared to wild-type. With these results in mind, a new hypothesis arose that if overexpressed MurF alters the cellular envelope length, then this distortion in envelope length can interfere with placement of outer membrane proteins or could possibly alter protein folding and trafficking. These disturbances to the outer surface could explain the virulence defects we observed in a *vcrS* deletion mutant when MurF is overproduced.

In order to further investigate the effects of MurF overproduction on the cellular envelope, higher resolution microscopy would be useful to gain insight into the altered properties of the bacterial surface, such as atomic force microscopy. This would further define the biophysical properties, such as elasticity and rigidness and better visualize the peptidoglycan layer. Another interesting experiment would be to determine the lipid composition and properties of *B. abortus* 2308 and the *vcrS* deletion mutant. A detailed definition of cellular envelope properties will shed light on the effects of the overproduction of MurF.

Why did VcrS evolve and what is its purpose?

Sequence analyses reveal that VcrS is found in "classical" *Brucella* spp. Yet, this sRNA has not been identified in other α-proteobacteria. The current hypothesis is VcrS interacts with the ribosomal binding site of *murF* mRNA and therefore inhibits translation. This is a very specific regulatory event, since *murF* is encoded in the middle of an operon, and VcrS is solely regulating the translation of one transcript within this polycistronic mRNA molecule. This specificity indicates that regulation of MurF production is important for the intracellular lifecycle of *Brucella.*

VcrS forms a secondary structure containing four stem loops, which contain seed regions that may interact with mRNA targets. Sequence analysis of VcrS revealed a 6-nucleotide motif complementary to the ribosomal binding site on the MurF mRNA. In addition, VcrS also contains three other 6+ nucleotide motifs complementary to regions on MurF mRNA. This strongly indicates that VcrS direct binds to MurF mRNA and physically blocks the ribosome from binding and initiating translation. The number of putative binding motifs that VcrS shares with MurF mRNA supports the specificity of

this regulation. We hypothesize cu that VcrS binds to one or more of the motifs tin MurF mRNA to inhibit translation. To test this hypothesis, site-directed mutagenesis will be used to disrupt the putative binding motifs on VcrS, and measure MurF production via western blot analysis. This experiment will showcase the importance of each motif in regards to regulating the translation of MurF. Secondly, an RNA-RNA electrophoretic mobility shift assay will test the direct interaction of VcrS and MurF mRNA. One possible limitation with this experiment is the length of the MurF mRNA. To overcome this, only short regions of MurF mRNA encompassing the binding motif will be used.

MurF is an essential enzyme found in almost all bacterial species. This enzyme catalyzes the addition of the final D-alanine-D-alanine residues on the growing peptide chain within the cytoplasm [40]. Recently, this enzyme has been proposed as an attractive antibacterial target, due to its conservation throughout bacterial species and the lack of similar proteins in eukaryotes[7,8]. Instead of creating chemical MurF inhibitors[43,44], what if we could utilize sRNA regulation to target MurF production? The idea has been proposed to utilize or target *trans*-encoded sRNAs to employ regulation, which would ultimately lead to bacterial death[11]. It is a fascinating route to combat antibiotic resistance by utilizing sRNAs and their specific regulation to target key mRNA molecules, which are essential for bacterial growth.

Overall, this work has identified seven new sRNAs in *B. abortus* and that one of those, VcrS, is required for virulence. However, questions still remain regarding the regulatory network of VcrS. The first question is about the identity of the transcriptional regulator that drives the expression of *vcrS*? Secondly, is there an environmental signal that amplifies *vcrS* expression? As VcrS is further characterized, the role sRNAs play in

the adaptation process within the macrophage to alter trafficking and promote survival and replication becomes well-defined. sRNAs have the ability to relay environmental stressors and conditions and quickly alter regulation to adapt and survive within the harsh environment of the host. As the sRNAs in *Brucella* are identified and characterized, the crucial regulatory events will be revealed which are required for survival in macrophages, and the adaptation process to cope with environmental stressors will be better understood.

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