Characterizing a Small Regulatory RNA in *Brucella abortus* Linked to Outer Membrane Stress Resistance

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ABSTRACT

Brucella abortus is a bacterial species that infects cattle, elk, and bison herds worldwide and is a causative agent of brucellosis. *B. abortus* is a common form of zoonosis, as incidental spillover into the human population results in millions of infections annually. Current treatment options are limited to culling infected animals and treating humans with a rigorous antibiotic regimen, which still results in up to a 30% relapse rate. Detection of the pathogen is difficult due to the replicative niche residing within the host's immune cells, specifically macrophages and dendritic cells. Numerous small regulatory RNAs (sRNAs) were found to be expressed by *B. abortus*, and it was hypothesized that they may be important for virulence. One sRNA, when deleted, was shown to be linked to outer membrane stress resistance and was named MssR (membrane sensitivity sRNA). When the Δ*mssR* strain was tested in both macrophage and mouse models of infection, there were no virulence defects. Additionally, proteomic and transcriptomic studies of the Δ*mssR* strain showed very few dysregulated targets. Expression of *mssR* was tested under numerous biologically relevant conditions, and it was shown to be expressed significantly more during exponential phase of growth, compared to stationary phase. Initial microscopical analysis of mutant cells after treatment with sodium dodecyl sulfate (SDS_ did not reveal any morphological differences. It is unknown what contributes to the observed phenotypes and additional experiments are required to determine what is causing the perturbations in the outer membrane of the Δ*mssR* strain.

GENERAL AUDIENCE ABSTRACT

Brucella abortus is a bacterial species that causes the disease brucellosis in cattle and humans worldwide. To understand how *B. abortus* establishes infection, we are studying how the bacteria control the expression of genes during the process of infection. One method of bacterial gene regulation is the use of small regulatory RNAs (sRNAs). These small transcripts are similar to mRNAs but are shorter in length and typically do not encode for a protein. One such sRNA in *B. abortus* was shown to be linked to sensitivity to outer membrane stress and was named Membrane Sensitivity sRNA (MssR). After engineering a strain of *B. abortus* that does not produce MssR, there were no differences in the ability of the bacteria to infect macrophages or mice. Additionally, there were no noticable differences in the structure of the bacterial cells. When sRNAs regulate gene expression, differences can be seen at the mRNA and protein levels when the sRNAs are deleted. Very few targets were found be dysregulated at the transcript and protein level within the Δ*mssR* mutant. It is unknown what is causing the mutant to be more sensitive to outer membrane perturbations and additional tests are necessary to determine how MssR is linked to this phenotype.

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Chapter 1
Introduction – *Brucella* and small Regulatory RNAs

Despite their small size, bacterial pathogens present a growing threat to global health, both for humans and the herds of domesticated livestock our economy depends on. This is further exacerbated by the steady climb in antibiotic resistance exhibited by these pathogens. This review focuses on the genetic regulation of a specific bacterial species: *Brucella abortus*.

The brucellae are closely-related to the soil-dwelling bacteria *Ochrobactrum* and *Sinorhizobium*, perhaps sharing a common ancestor, which eventually evolved to infect a variety of mammalian hosts [3, 50]. Many *Brucella* species exhibit host preference, with *B. abortus* (cattle), *B. melitensis* (goats), and *B. suis* (pigs) being commonly encountered due to their respective hosts comprising much of the world's domestic livestock [16, 41]. Additionally, the number of reported cases of *B. canis* (canines) has risen in recent years [38, 39]. The infectious dose can range from 10-100 bacteria, depending on the species, and an established infection can persist for the remainder of the animal's life [4]. Infection results in a variety of clinical manifestations within the animals, including arthritis, mastitis, and infertility. *Brucella* has a tropism for host macrophages and dendritic cells, a niche where the bacteria can readily survive and replicate. Detecting *Brucella* infections via conventional serological tests is inconsistent due to transient levels of antibody titers in infected animals [51]. Outside of the host, the brucellae can be found in dairy products from infected females, coating the corpse of the aborted fetuses, or in the seminal fluid of infected males in the case of *B. melitensis* [42]. All these sources are potential transmission routes to the rest of the herd. Due to the threat infected animals pose to the rest of the herd, the primary strategy for handling *Brucella* outbreaks is screening and culling infected animals, but this can be very costly to individual farmers if support from local governments is limited [43]. Additionally, reservoirs of *Brucella* are found in wild animal populations, which is a health concern for those wild herds and a potential for spillover into domestic herds. In the United States, *B. abortus* can be found in wild bison and elk, which presents numerous logistical challenges for diagnosis and treatment [44]. In some European countries, *B. melitensis* has established itself within herds of wild ibex, a species of mountain goats that are federally protected [5,6]. *B. abortus* is a worldwide problem that is only made worse by the lack of an efficient vaccine for our cattle. There are limited live-attenuated *B. abortus* vaccines approved for use in cattle, but the most predominant vaccines, S19 and RB51, have notable drawbacks and need further improvement. Specifically, S19 inoculated animals test positive via agglutination tests, and RB51 can be shed in the mother's milk [7]. Improving *B. abortus* cattle vaccines has been a worldwide effort, one that is aimed at improving the welfare of the animals, but also an effort that will have direct impacts on human health. This is because humans are an incidental, dead-end host for *B. abortus*.

Human *B. abortus* infections progress and present clinical manifestations very similar to cattle infections, with a few exceptions. There are isolated reports of *B. abortus* transmission between humans, but no direct link has been made infected females and aborted pregnancies [15]. Those who are infected can suffer from an undulant fever and arthritis, which can persist for the rest of the person's life if left untreated [4]. With a dual antibiotic regimen of doxycycline and rifampicin, most people will clear the infection, however, there is still a relapse rate of up to 30% [40]. The people that are at the greatest risk of infection are those in direct proximity to the

infected materials, such as farmers, butchers, laboratory workers, and those who choose to consume unpasteurized dairy products [8, 45]. To further complicate the matter, there is no approved vaccine to prevent brucellosis in humans. To efficiently target this insidious pathogen, an understanding of how it survives within the host and evades the immune system is necessary.

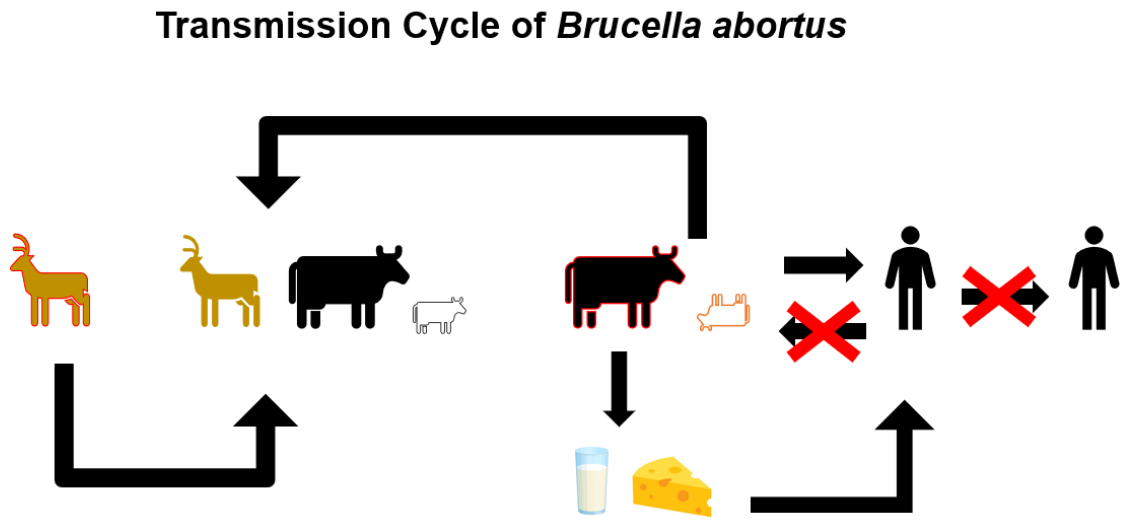

Figure 1. *Brucella abortus* exhibits a diverse host range with multiple routes of infection. Infected domestic cattle, highlighted in red, shed *B. abortus* in numerous ways. Infected females can transmit to their young (shown as smaller, white cows) via milk during nursing. Other members of the herd can be exposed if an infected pregnant female suffers an abortion, which results in a fetal corpse covered with the bacteria (shown in red) in proximity to cattle that are prone to sniffing or licking newborns. Humans who come into contact with these contaminated sources, as well as unpasteurized dairy products, are also at risk of infection. Humans represent a dead-end host, as there are isolated reports of human-to-human, or human-to-animal, transmission. Wild herds of elk and bison, shown in lighter colors, also represent a reservoir of *B. abortus* that contributes to its persistence.

Infiltrating a mammalian host and establishing residence within the very cells designed to kill foreign bodies involves numerous steps and careful regulation of gene expression. The process begins when *B. abortus* is phagocytized by cells such as macrophages or dendritic cells. Once inside the immune cells, the *Brucella*-containing vacuole (BCV) must prevent the complete fusion of the incoming lysosome, a vacuole containing chemicals intended to destroy any material that was phagocytized [4]. Transient fusion of the lysosome caused a rapid decrease in pH within the BCV, which then signals the expression of the type-IV secretion system within the brucellae. This secretion system has be shown to be essential for the virulence of *Brucella* species and has been studied extensively [9]. Many effectors secreted by the type-IV system have been identified, with roles ranging from host immune evasion to bacterial trafficking [4, 52, 53]. The other virulence factors expressed by the brucellae are similar to some of the more classical examples, such as lipopolysaccharides (LPS), with notable differences. For example, the LPS of most *Brucella* species are weakly antigenic due to variations in the Lipid A region of the core [66]. Additionally, expression of a polar flagellum has been shown to be essential for *Brucella* virulence, but the bacteria exhibit no motility [67]. After the phagocytized *Brucella* avoid fusion with the lysosome, the BCV is trafficked to the endoplasmic reticulum, where it incorporates elements of the host's ER on the exterior of the vacuole [10]. This event signifies the beginning of the replicative phase of the *Brucella* infection. The timing of these events is

well-studied, but little is understood about what triggers the phagocytosis of the bacteria [4,34]. It is also unknown what signals the egress of the bacteria from the host cell, but the act of exiting the cell seems to be a non-lethal event, as there are minimal pro-inflammatory or cell death markers seen during *in vitro* macrophage infections [11]. There are many unknowns about all of the specific genes involved in the infection process, but it is believed that rapid and precise gene regulation is critical for the establishment of *B. abortus* within the host's immune cells. To this end, it is hypothesized that small regulatory RNAs play a role in the virulence of *B. abortus*.

Small RNAs are short (30-300 nt) transcripts that often do not contain open reading frames (ORFs), meaning no protein is translated from the message [19]. Because of this, the term "non-coding RNA" has been used synonymously with "small RNA", which may be too broad of a generalization. sRNAs can in fact contain ORFs, leading to the translation of small peptides and these peptides may have regulatory functions entirely separate from the sRNA that encode them [46]. While these small peptides seem to be an uncommon occurrence, the paradigm for sRNA regulation involves direct binding of the sRNA to mRNA targets. This binding facilitates post-transcriptional regulation in both positive and negative manners [17]. For example, negative regulation occurs if the sRNA binds to a region of the mRNA that inhibits translation, such as the ribosomal binding site. Conversely, if the sRNA binds to the mRNA and relieves inhibitory secondary structure, the binding will promote the translation of the targeted message. These sRNA:mRNA interactions often involve imperfect base pairing and require the mediation of a protein chaperone, such as Hfq.

Hfq is a protein that forms a homohexamer and facilitates the binding of sRNAs to their mRNA targets, but there are many cases where the chaperone is dispensable [13]. The ring-like structure this protein forms is characterized by multiple faces; a distal face which has preferential binding to poly-A regions, and the proximal face, which has preferential binding to poly-U regions [1]. There are two other regions within Hfq, the rim and the C-terminus tail, which also serve as binding pockets for specific RNA motifs. Many sRNAs rely on Hfq to maintain stability, since their short lengths often do not allow for the formation of stabilizing secondary structures. Another major function of the RNA chaperone is the ability to facilitate the binding of sRNAs to mRNAs with limited base pairing complementarity. Since sRNAs can be encoded *in trans* of their target, promiscuous base pairing allows for multiple mRNA targets to be regulated by a single sRNA [17]. An example of this in *Brucella* are the AbcR sRNAs.

AbcR1 and AbcR2 (ATP-binding cassette regulator) were the first regulatory sRNAs recorded in *B. abortus* by Caswell *et al.* [14]. These sRNAs negatively regulate multiple outer membrane proteins and are highly redundant in their regulatory functions. The AbcRs share many similarities on the sequence and structure levels as well, including two conserved binding motifs and similar stem-loop secondary structures [47]. Since their discovery, it was hypothesized that additional sRNAs exist within the intergenic regions of *B. abortus* and that they may have important regulatory roles. RNA sequencing experiments have uncovered the transcriptome of *B. abortus* in conditions ranging from a nutrient rich brucella broth to conditions that mimic the harsh interior of a macrophage. This collection of data was screened for transcript reads that did not align to any annotated genes, indicative of a sRNA being encoded in that region. Confirmation of these transcripts is then carried out via northern blot analysis. Using this approach, numerous novel sRNAs were found to be encoded throughout the *B. abortus* genome.

To efficiently characterize these regulators, experiments need to be performed to uncover the regulon of each sRNA, determine when they are expressed, and study their impact on virulence. To that end, one of the novel sRNAs found by our lab was studied using a variety of *in vitro* and *in vivo* assays. This sRNA, initially annotated as Bsr7 (*Brucella* sRNA 7), was shown to be linked to resistance of the outer membrane stress. This link was established after experimentation showed that the Δ*bsr7* mutant was significantly more sensitive to agents such as the detergent sodium dodecyl sulfate (SDS) and the antibiotic polymyxin B, which both disrupt the outer membrane. Due to this, Bsr7 was renamed to MssR (Membrane sensitivity sRNA) and studied further.

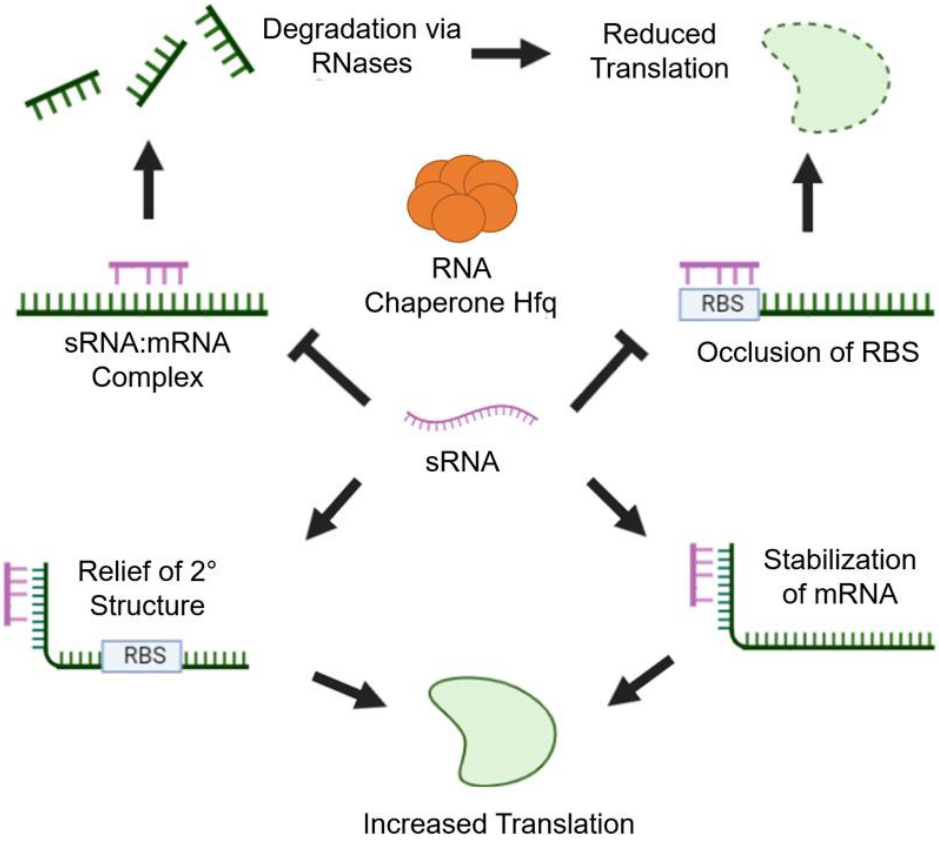

Figure 2. Small Regulatory RNAs have both positive and negative regulatory capabilities. sRNAs can bind to mRNA targets via imperfect base pairing, often facilitated by the RNA chaperone Hfq, and regulate the expression of the mRNA in a number of ways. The resulting sRNA:mRNA complex may be targeted for degradation by RNases or the sRNA could occlude the ribosomal binding site, both of which results in decreased protein product. The sRNA binding may also relieve the secondary structure of the mRNA, potentially exposing the RBS and promoting translation.

Chapter 2
Characterization of MssR, a small RNA linked to outer membrane resistance in *Brucella abortus*

Abstract
Small regulatory RNAs can have profound impacts on the fitness and virulence of bacterial pathogens. In *Brucella abortus*, numerous uncharacterized sRNAs have been identified, with the deletion of the *mssR* (membrane sensitivity sRNA) gene leading to the increased sensitivity to outer membrane perturbations, specifically exposure to the detergent sodium dodecyl sulfate (SDS). It was also observed that MssR is present during exponential phase of growth and not during stationary phase. The mutant showed no growth defect in nutrient-replete or -deplete media, and there were no virulence defects when tested in macrophage and mouse models of infection. Both the transcriptome and the proteome of the Δ*mssR* strain were analyzed, but the data from these experiments did little to elucidate the regulon of MssR or the links to the observed sensitivity to SDS. Further experiments are needed to determine the biological relevance of MssR and how it is linked to the perturbation of the outer membrane.

Introduction
Brucella are Gram-negative, intracellular bacteria capable of infecting a wide range of mammalian hosts, including incidental spillover into the human population [4]. This zoonosis primarily occurs in regions where unpasteurized dairy products are common, but this is not to say that *Brucella* infections are limited to areas of limited resources, as consumption of unpasteurized dairy in the United States still occurs [57, 58]. *B. abortus* was officially eliminated from domestic cattle herds in the U.S. through rigorous culling programs and extensive monitoring thereafter. However, these programs are expensive and simply not feasible for many countries dealing with this pathogen. To make matters worse, there is also no approved human vaccine to prevent disease [16]. Human brucellosis results in fatigue, joint pain, arthritis, and a recurring, undulant fever that can be debilitating to the host [3]. Treatment of human brucellosis consists of a harsh antibiotic regiment of doxycycline and rifampicin, and even then, the relapse rate can be as high as 30% [32,33]. A key reason that *Brucella* is so hard to combat within the host is where it resides and persists: the macrophage [34].

Despite being a cell designed to phagocytize and clear infections, the macrophage is the main replicative niche for *Brucella* species [4]. To survive within this harsh environment, *B. abortus* utilizes numerous secreted effectors, transported by a type-IV secretion system, to prevent the vacuole containing the bacterium from completely fusing with a lysosome [14]. Expression of this secretion system is rapid and can be induced by external signals, such as a decrease in pH during the initial fusion of the lysosome [21]. One method to rapidly alter gene expression in response to these stimuli is through small regulatory RNAs (sRNAs) [26]. This method of regulating gene expression is quick and energetically efficient for the bacterial cells [18,20]. sRNAs may also require a protein chaperone, such as Hfq, to help facilitate the often-imperfect base pairing of the sRNA with the mRNA target [19,27]. These sRNA:mRNA interactions can result in both positive and negative regulation, but this can be difficult to predict through bioinformatic means alone [20].

Through RNA sequencing, numerous sRNAs have been found within the intergenic regions of *B. abortus*, but only a subset has been characterized so far [36]. One sRNA, initially designated

Bsr7 (*Brucella* sRNA 7), was confirmed to be encoded at the locus indicated by RNA sequencing and appeared to be highly conserved across multiple *Brucella* species, as well as *Ochrobacterum* and *Mesorhizobium* species. Bsr7 is one of numerous confirmed sRNAs in *B. abortus*, with the others being cataloged in a public database maintained by our lab (https://caswelllab.com/#resources). Due to individual researchers having their own naming conventions for sRNAs, this database was also constructed as an effort to consolidate all of the published sRNAs in *Brucella* and to best ensure the novelty of the sRNAs we study. Bsr7 was also detected in an Hfq ChIP-seq experiment in *Brucella suis*, indicating there is a direct interaction between the sRNA and the RNA chaperone in *B. suis* [21]. To date, this is the only other study that has detected Bsr7, but no further characterization has been done. Sequence homology did little to indicate the regulatory function of the sRNA, so deletion of the *bsr7* locus was carried out, and a variety of phenotypic analyses were performed. The first phenotype that was observed after deleting *mssR* was an increased sensitivity to the detergent SDS and the antibiotic polymyxin B. Because of this, the sRNA will now be referred to as MssR, or Membrane sensitivity sRNA.

Materials and Methods

Strains, media, and culturing conditions

The *Brucella abortus* strains were grown on either Shaedler agar (BD, Franklin Lakes, NJ) supplemented with 5% defibrinated bovine blood (Quad Five) or in brucella broth (BD) and were then incubated at 37°C with 5% CO_2. When appropriate, media were also supplemented with kanamycin (45 µg/mL) or 10% (w/v) sucrose. For cloning, *Escherichia coli* strain DH5α was grown on tryptic soy agar (BD) or in Luria broth.

Generation of mutant and complementary strains

Chromosomal mutations were generated by allelic exchange via homologous recombination as described previously [36]. Briefly, two 1-kb regions flanking the locus of interest were amplified by PCR with Taq polymerase (Monserate Bio.). The upstream fragment was digested with the restriction enzyme BamHI, and the downstream fragment was digested with PstI. Both fragments were treated with polynucleotide kinase in the presence of ATP and then ligated with T4 ligase. The resulting 2-kb fragment was then amplified via PCR with Phusion polymerase (ThermoFisher) and electrophoretically separated on an agarose gel to confirm the correct size. The 2-kb band was purified from the gel, digested with both BamHI and PstI, and ligated into the pNPTS138 vector (M.R.K Alley, unpublished). *E. coli* DH5α was transformed with this new construct for propagation. Successful construction was confirmed via PCR and Sanger sequencing before electroporation into *B. abortus* 2308 and the resolution of mutant strains. All primers used to generate this construct are listed in Table 1.

Isolation of RNA and northern blot analysis

RNA isolation. Cultures of *B. abortus* were routinely grown in brucella broth and total RNA was isolated as previously described [25]. When necessary, SDS (0.01% m/v), H_2O_2 (1 M), and deoxycholate (0.1% w/v) were supplemented to the medium or the pH was adjusted to 4.0 using HCl. Additionally, Gerhardt's Minimal Medium was used when limited nutrients were necessary [54].

Northern blot analysis. Northern blot analyses were performed as previously described [47]. Signal was registered on both autoradiography film and phosphor imaging screens (Cytiva, Amersham Typhoon). Signal intensity was quantified by densitometry in FIJI (www.fiji.sc).

Outer membrane stress assays

Disk diffusion assay

To test sensitivity to various diffusible agents, a 0.6% concentration of agar was mixed with the nutrient-rich brucella broth and autoclaved. The molten agar was allowed to cool to 60°C before $1x10^7$ CFU/mL of each strain was added. Four milliliters of this suspension was evenly added to tryptic soy agar (TSA) plates and allowed to solidify. A small circular disk of Wattman paper was added to the center of each plate, and 7 µL of each diffusible agent was pipetted onto the disk. These agents included 10% SDS, carbenicillin (100 mg/mL), polymyxin B (100 mg/mL), 10% Triton X_{100}, and 10% Zwittergent. Three days later, the diameter of each zone of inhibited growth was measured.

Liquid culture stress assays

To determine the concentrations of each inhibitory agent to use, a 96-well plate format was employed. In the first row of wells, 200 µL of each stress condition was mixed with $5x10^7$ CFU ($1x10^7$ CFU/mL) and statically incubated at 37°C for 24 hours. Cultures are then serially diluted using the remaining wells and plated on TSA to enumerate colonies. The ideal concentration of each agent resulted in a notable decrease in viable wild-type cells without completely eliminating the CFUs. Once the appropriate concentration of each agent was determined, the experiment was repeated in 5 mL liquid cultures containing the agent and $1x10^7$ CFU/mL of each strain. Cultures were grown in 15 mL pop-cap tubes and were incubated shaking at 37°C for 24 hours. After incubation, aliquots of each culture were serially diluted and plated on TSA to enumerate CFUs.

Isolation of total protein, mass spectrometry and iTRAQ analysis

Duplicate cultures of *B. abortus* 2308 or Δ*mssR* were grown to an OD_{600} of 1.0 before being pelleted via centrifugation and then resuspended in PBS. The resuspended cultures were then added to tubes containing glass beads (BeadBug, Benchmark Sci.) and boiled for an hour. Every 10 minutes the samples were removed, vortexed for 30 seconds, and placed back into the water bath. Total lysates were then shipped to the University of Victoria's mass spectrometry core. Data was analyzed via iTRAQ and protein abundancies were comparison between the two strains.

RNA sequencing and analysis

Total RNA was isolated as described previously in this study, with the additional step of depleting ribosomal RNA (Illumina Ribo-Zero Plus). The integrity of the RNA samples was visually verified via TapeStation. Verified samples were then sent to the Genomics Sequencing Core at Virginia Tech, where they were processed and run on the Illumina NovaSeq platform for 100 cycles. Raw data was demultiplexed and aligned in-lab and comparisons were made using Microsoft Excel. Benjamini-Hochberg correction was employed to reduce the number of false positives reported.

Cryo-electron microscopy
Duplicate cultures of *B. abortus* 2308 or Δ*mssR* were grown to an OD_{600} of 0.5 or 3.0 (exponential and stationary phase) in nutrient-rich broth. Two additional cultures were grown to an OD_{600} of 1.0 and then stressed with 0.01% SDS overnight. At each desired density and time point, the cells were pelleted via centrifugation and then resuspended in PBS with 2% glutaraldehyde as a fixative. The fixed cells were then prepared and imaged at the University of Virginia's cryo electron microscopy core.

Infection studies
Two mouse models of infection were used to determine if the Δ*mssR* mutant has any fitness defects *in vivo*. Strains were first grown on Shaedler blood agar (SBA) before being scraped and added to PBS for OD adjustment to $5x10^5$ CFU/mL. Two hundred microliters of this culture ($1x10^5$ CFU) were administered to the mice via intraperitoneal injections. A group of mice also received an injection of the PBS vehicle as a negative control. At four- and eight-weeks post-infection, the animals were euthanized, spleens were collected and completely homogenized, and the homogenate was serially diluted in PBS and plated on agar to enumerate CFU's. Six-week-old mice from the C57BL/6 and BALB/C mouse lines were used in two separate experiments. Each cohort of mice were also an even mix of male and female mice, to account for any sex differences.

Results

The mssR *locus encodes a novel, Hfq-dependent sRNA*
mssR (formerly *bsr7*) was first seen to be located in the intergenic region between a gene encoding a DUF-family protein (BAB_RS24130) and a gene encoding a conserved general secretion pathway protein (BAB_RS24135) by RNA sequencing (Fig. 1), and production of the MssR transcript was subsequently confirmed via northern blot analysis (Fig. 2). Sequence analysis using SeqBuilder (DNASTAR, https://www.dnastar.com/) does not show an open reading frame within the transcript, limiting the potential for the translation of a small peptide. The predicted *mssR* genetic sequence is conserved across many *Brucella* species, as well as *Ochrobacterum* and *Mesorhizobium* species, with identical gene syntony across all three genera (Fig. 3). The only published account of MssR is in *Brucella suis*, where it was shown to be bound to Hfq through ChIP-Seq experiments. In *B. suis*, the sRNA was given the annotation of Bsnc135 and not studied further. These findings prompted the question of whether *B. abortus* MssR was associated with Hfq as well. MssR abundance was quantified in *B. abortus* strains 2308 and the Δ*hfq* strain. Northern blot analysis confirmed that MssR is much less abundant in the Δ*hfq* mutant, indicating the chaperone likely has a role in preserving the stability of MssR (Fig. 4). Expression of MssR was measured after exposing the bacteria to various stress conditions and at specific growth phases. MssR was significantly more expressed during exponential phase of growth compared to stationary phase, but no other significant expression differences were observed (Fig. 4).

Deletion of mssR results in increased sensitivity to outer membrane stresses
To study any effects MssR has on the cell, the *mssR* was deleted using an unmarked gene deletion strategy. The resulting mutant showed no growth defect when grown in nutrient-rich broth and exhibited the same survival kinetics as the wildtype 2308 strain when grown in a defined, nutrient limited medium (Fig. 5). However, when stressed with the detergent SDS, the

Δ*mssR* mutant was substantially more sensitive, showing a significant increase in the size of the zone of inhibition via disk diffusion assay (Fig.6). Chromosomal reconstruction of the *mssR* locus rescues the observed sensitivity to SDS. Repeating these experiments with polymyxin B, an antibiotic that targets the outer membrane, revealed that this sensitivity was not specific to SDS but was rather a more general sensitivity to outer membrane perturbations.

Transcriptomic and proteomic studies uncover the MssR regulon

Small RNAs regulate at the post-transcriptional level, which can result in altered translation of the mRNA targets or a decrease in the target mRNA levels due to double-stranded RNA complexes being targeted for degradation by bacterial RNases [17]. Because of this, investigation of the transcriptomic and proteomic landscapes within the Δ*mssR* strain can elucidate what the sRNA may regulate. iTRAQ (isobaric tags for relative and absolute quantification) [59] and mass spectrometry analyses were able to identify 783 proteins within the samples and of those, only two appeared to be more than 2-fold dysregulated. The first was the protein TolB, which is found as a complex with Omp16 and bridges the outer membrane to the peptidoglycan layer. TolB is an integral part of the Tol-Pal system, which is responsible for maintaining the periplasm and repairing the peptidoglycan breaks during bacterial division [60]. TolB was shown to be approximately 10-fold less prevalent in the Δ*mssR* strain compared to the wildtype. The second dysregulated protein was ArcB, an ornithine carbyltransferase whose role within *Brucella* appears to be metabolic in function [61]. In the Δ*mssR* strain, ArcB appeared to be approximately 2-fold down-regulated. This experimental approach may not have captured all of the proteins that are normally present, but these data indicate very few targets dysregulated at the protein level in the Δ*mssR* strain.

Small RNA interactions with their mRNA targets can also influence the stability of the message, both in positive and negative manners [24]. Due to this, analysis of the transcriptome via RNA sequencing is a viable approach for screening putative regulatory targets. Total transcript levels in the Δ*mssR* mutant were compared to the wildtype 2308 strain, and numerous targets were shown to be significantly dysregulated, with the threshold for dysregulation being set at >2-fold differences and P values <0.01. To reduce the false-positive rate, Benjamini-Hochberg correction was also used. With these parameters set, six genes were shown to be upregulated and one was downregulated (Table 4). The upregulated genes ranged from 2.12- to 2.93-fold greater expression in the Δ*mssR* strain and encoded mainly hypothetical proteins or pseudogenes. The single downregulated target was a transcriptional regulator belonging to the GntR family (GntR21) and was expressed 3.27-fold less in the mutant.

Cryo-electron microscopy shows no major morphological differences in the ΔmssR strain

With Δ*mssR* being more sensitive to outer membrane stresses, it was hypothesized that the outer membrane or periplasm of the mutant may display architectural differences when compared to the wild-type strain. Cryo-electron microscopy was employed to visualize the cellular envelope of wild-type and Δ*mssR* cells collected at exponential and stationary phases of growth, as well as after being stressed overnight with 0.01% SDS. At exponential and stationary phases, no gross morphological differences were observed. However, when stressed with SDS, the mutant strain appeared to exhibit increased rates of bacterial chaining when compared to the wild-type strain. This phenotype was first observed in representative cryo-electron micrographs, but DIC microscopy was unable to confirm this phenotype (Fig. 7).

Δmssr does not have a virulence defect in mice or macrophage infection models
Two infection models were used to determine if MssR is linked to the virulence of *B. abortus*. The first model, which utilized bone marrow-derived macrophages from BALB/c mice, showed no virulence defect when comparing the wild-type and Δ*mssR* strains. It was confirmed that both strains were efficiently phagocytized by the macrophages and were cleared at similar rates (Fig. 8A). The second model utilized both male and female BALB/c mice. After intraperitoneal injections of *B. abortus*, the Δ*mssR* strain showed no virulence defects at 8-weeks post-infection (Fig. 8B). This is a common time point to model chronic *B. abortus* infections, as the bacteria have established residence in various organs, including the spleen. This splenic colonization and persistence are how we assess *B. abortus* virulence. The mouse infection study was also carried out using C57BL/6 mice and no virulence defect was seen at 4-weeks post-infection (data not shown).

Discussion

Over time, bacterial species have adapted a wide variety of surface-level defenses to cope with their environment or evade host immune responses [30]. These adaptations include outer membrane proteins or secreted effectors that alter the environments in which the bacteria reside. However, adaptations on the transcriptional level can be largely responsible for the successful infection of professional pathogens such as *B. abortus* [29]. Small regulatory RNAs are a powerful genetic tool employed by the *Brucella* to overcome stresses, ideally those associated with the host immune response designed to clear out the invading bacteria. It is believed that sRNAs are conditionally expressed in response to certain stimuli, but many sRNAs in *B. abortus* seem to be constitutively expressed in the conditions tested. MssR is expressed in significantly higher levels during exponential phase of growth, compared to stationary phase, but does not appear to have altered expression in *in vitro* conditions that mimic aspects of the macrophage environment. Perhaps MssR regulates a metabolic function or an aspect of bacterial division, as other sRNAs in other systems have been shown to be important for the transition into stationary phase and for regulating the stringent response [62, 63]. The increased sensitivity to agents that primarily affect the outer membrane led to the hypothesis that MssR regulates some aspects of the cellular envelope, specifically something that may contribute resistance to SDS and polymyxin B. The first step to testing this was to characterize the regulon of MssR by proteomic and transcriptomic studies. If specific genes are dysregulated in the absence of MssR, the functions of those genes may be responsible for the observed sensitivity in the mutant.

The proteomic study showed only two proteins that are in lower abundance in the absence of MssR. Both proteins, TolB and ArcB, can be directly linked to the outer membrane and peptidoglycan layers, which may explain the sensitivity to the detergent. However, TolB is an essential protein in *B. abortus*, so it is surprising that it can be 10-fold under-expressed in the *mssR* mutant without resulting in more serious fitness defects. The mass-spectrometry data regarding the TolB reduction were limited, and the readings were very close to the lower threshold of detection, so it is possible our assumptions are incorrect. Validation of the mass-spectrometry data would then be required, ideally in the form of tagging the TolB protein in both the wild-type and the Δ*mssR* strains and performing western blot analyses. Analysis of the transcriptomic data also revealed few targets, with no obvious link to the increased sensitivity in the mutant strain. For both the proteomic and transcriptomic studies, there is the possibility that

some proteins and transcripts simply were not observed in their respective quantification approaches. Whether this is the case or not, the available data do little to determine what is causing the observed phenotypes.

There is no apparent link between MssR and the virulence of *B. abortus*. No fitness defect was observed for the Δ*mssR* strain in experimentally infected mice or macrophages. This, in combination with the lack of any major morphological defects, suggests that the outer membrane irregularities are subtle. There are documented cases of pronounced morphological defects in certain *B. abortus* strains, such as incomplete budding of daughter cells or the loss of the LPS O-chain, and in these cases the *Brucella* exhibit noticeable growth and fitness defects [64, 65]. It is possible that the permeability of the outer membrane is being affected just enough to allow the increased incorporation of SDS and polymyxin B, which would explain the increased sensitivity to those agents. This speculated change in permeability, not architecture, of the cellular envelope may also explain why no differences were seen via cryo-electron microscopy.

Numerous questions remain in linking MssR to the SDS and polymyxin B sensitivity. However, the limited number of targets identified by the transcriptomic and proteomic analyses still provide candidates for targeted genetic deletions. Additionally, assessment of the transcriptome in the wild-type and Δ*mssR* strains during stress with SDS would be beneficial. There could be an entire subset of genes that have altered expression when stressed with the detergent that were not identified in the initial experiments. While this is purely speculative in nature, additional experiments are necessary to understand the regulatory functions of MssR.

Chapter 2 Tables

Table 1. Primers used for the deletion of *mssR* and northern blot analyses

Name	Sequence
MssR_up_for	ATTAGGATCCTGCAGCGAATTGCCAAGCGATTC
MssR_up_rev	GCCTGTTTCTCTGGGAACCTGCG
MssR_down_for	GCGAAGGAAAAGCCGACCGA
MssR_down_rev	ATTACTGCAGTTGTCGCTGAAGATGTGGCG
MssR_con_for	GGCATCTGACCCTGTTCGCT
MssR_con_rev	GGCAAGAAGGGCGCGGTTCA
MssR_north	GTCAAACAGAGTGGCTAAACCACTCGGT
5S_north	AGTTCGGAATGGGATCGGGTGCAGCC

Table 2. Plasmids used in this study

Name	Description	Source
pNPTS138	non-replicating *B. abortus* vector, Kan_R, LacZ, SacB	M.R.K Alley (unpublished)
pBBR-1MCS4	Replicating *B. abortus* vector, $Carb_R$, LacZ	Roop et al [55]
pTS9	pBBR-1MCS4:*mssR*	This Study

Table 3. *B. abortus* strains used in this study

Name	Description	Source
2308	Wildtype *B. abortus* Strain	Provided by M. Roop II [56]
LS051	Δ*mssR*	This Study
LS053	Δ*mssR* Chromosonal Recontruction	This Study
TS007	2308:pBBR-1MCS4	This Study
TS009	LS051:MCS4-*mssR*	This Study
TS010	LS051:pBBR-1MCS4	This Study

Gene	Fold Change	Gene Product
BAB_RS26415	2.93	Hypothetical Protein
BAB_RS32150	2.84	Pseudogene
BAB_RS23460	2.80	Hypothetical Protein
BAB_RS27755	2.40	GntR15
BAB_RS24960	2.30	Ribosomal Protein L11 Methyltransferase
BAB_RS26450	2.12	Eukaryotic Molybdopterin Oxidoreductase
BAB_RS29795	-3.27	GntR21

Table 4. Numerous genes are dysregulated within the Δ*mssR* mutant. Transcriptomic analysis via RNA sequencing showed multiple genes either up- or down-regulated in the Δ*mssR* mutant. RNA was collected from cultures in mid-exponential phase, grown in nutrient-rich broth. Each strain was prepared in triplicate and the threshold for dysregulation was 2-fold with a P-value <0.01, using Benjamini-Hochberg correction.

Chapter 2 Figures

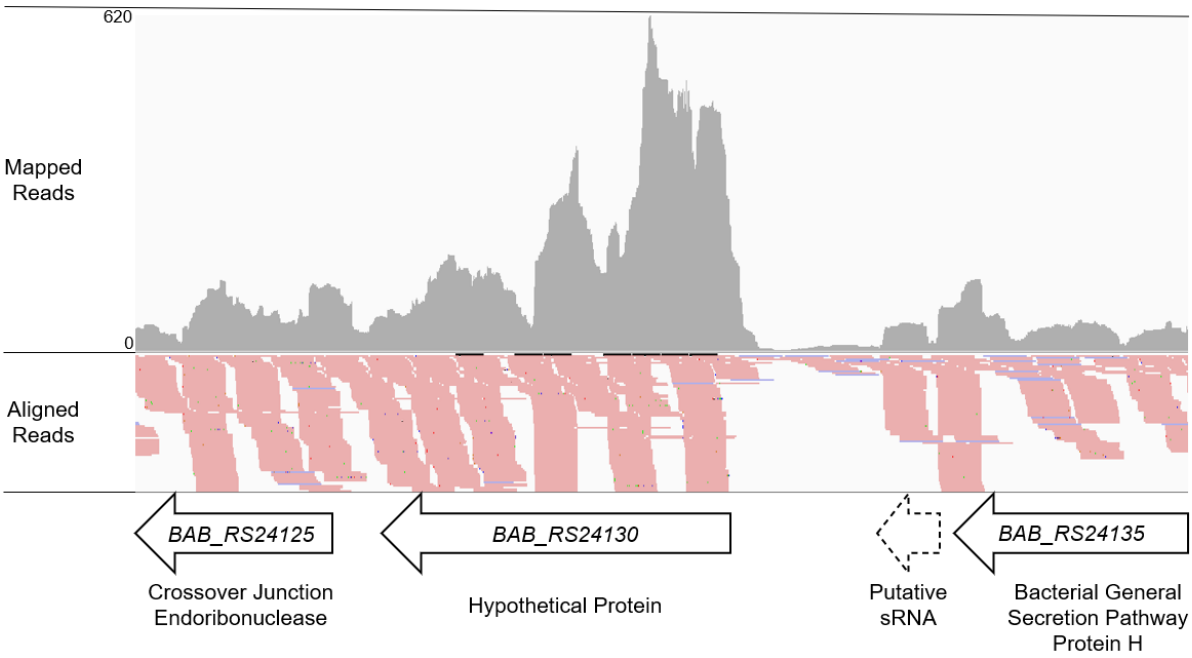

Figure 1. A putative sRNA is located in an intergenic region downstream of *BAB_RS24135.* RNA sequencing detected a transcript that did not align to an annotated gene in the *B. abortus* genome, indicating a putative sRNA. Each colored line represents a sequence read that aligns to the genome, and the resulting grey peaks represent the number of mapped reads at each nucleotide.

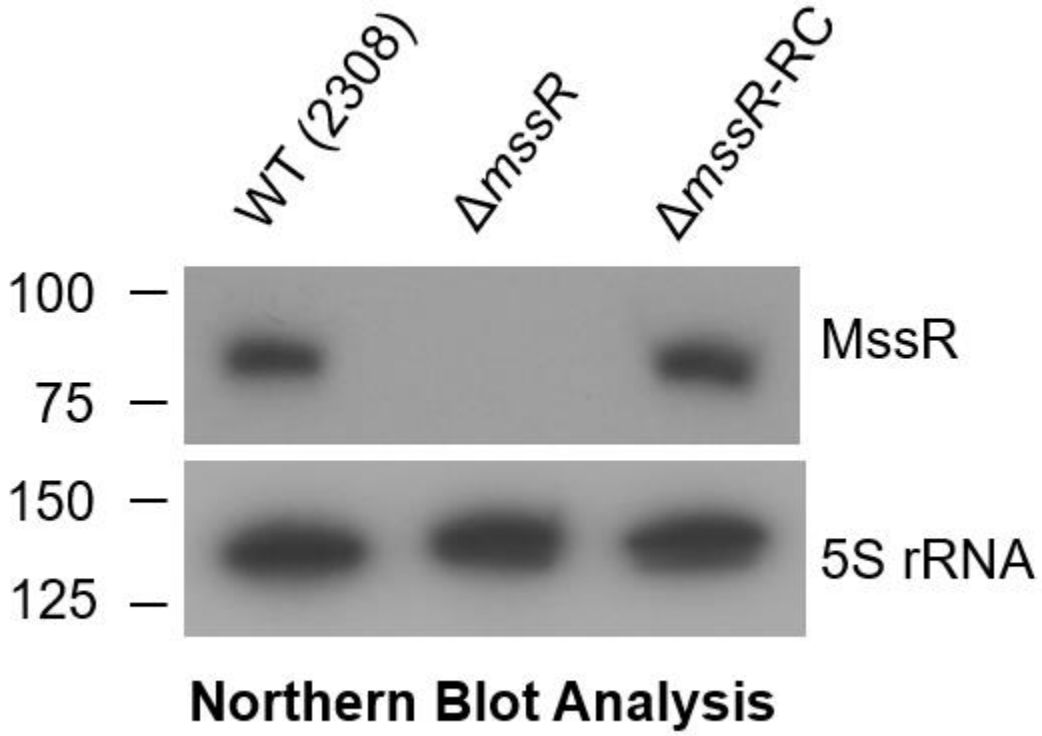

Figure 2. The putative sRNA locus encodes a discreet transcript. Northern blot analysis, probing specifically for the predicted sRNA using the RNA sequencing data, confirms that a discreet transcript is produced at the locus. This sRNA, now designated as MssR, is approximately 90 nucleotides in length. Additionally, it is validated that the Δ*mssR* mutant does not produce MssR and expression is restored when the *mssR* locus is chromosomally reconstructed.

Predicted *mssR* Genomic Sequence

	5' … 3'
B. abortus	GTAGGTCAAGCCGACCTAAGAATGGCTCACCAAATCGGTGAGACAAACTGCGGAGGGACAATTTGAGGACTCTCGGCGGGAACGCCGAGAAAAAAA
B. melitensis	GTAGGTCAAGCCGACCTAAGAATGGCTCACCAAATCGGTGAGACAAACTGCGGAGGGACAATTTGAGGACTCTCGGCGGGAACGCCGAGAAAAAAA
B. suis	GTAGGTCAAGCCGACCTAAGAATGGCTCACCAAATCGGTGAGACAAACTGCGGAGGGACAATTTGAGGACTCTCGGCGGGAACGCCGAGAAAAAAA
B. ovis	GTAGGTCAAGCCGACCTAAGAATGGCTCACCAAATCGGTGAGACAAACTGCGGAGGGACAATTTGAGGACTCTCGGCGGGAACGCCGAGAAAAAAA
B. canis	GTAGGTCAAGCCGACCTAAGAATGGCTCACCAAATCGGTGAGACAAACTGCGGAGGGACAATTTGAGGACTCTCGGCGGGAACGCCGAGAAAAAAA
B. pinnipedialis	GTAGGTCAAGCCGACCTAAGAATGGCTCACCAAATCGGTGAGACAAACTGCGGAGGGACAATTTGAGGACTCTCGGCGGGAACGCCGAGAAAAAAA
B. intermedia	GTAGGTCAAGCCGACCTAAGAATGGCTCACCAAATCGGTGAGACAAACTGCGGAGGGACAATTTGAGGACTCTCGGCGGGAACGCCGAGAAAAAAA
Ochrobactrum spp.	GTAGGTCAAGCCGACCTAAGAATGGCTCACCAAATCGGTGAGACAAACTGCGGAGGGACAATTTGAGGACTCTCGGCGGAAACGCCGAGAAAAAAA
Mesorhizobium spp.	GTAGGTCAAAAAGACCTAAGAATGGCTCACCAACCTCGGTGAAACAAACTGCGGAGGGACAATTTGAGGACTCTCGGCGGAATAGCGCGCCGAGAAAAAAA
Aminobacter spp.	GTAGGTCTAGTAGACCTAAGAATGGCTCACCAACCTCGGTGAAACAAACTGCGGAGGGACAATTTGAGGACTCTCGGCGCGAAAAGGCGCCGAGAAAAAAA

Figure 3. The *mssR* locus is highly conserved between many *Brucella spp.* and other alphaproteobacteria. Bioinformatics analysis shows that the *mssR* locus is highly conserved across many of the sequenced *Brucella* species and three other *alphaproteobacteria* genera. The predicted sRNA sequence is shown, with the highlighted regions signifying differences, relative to *B. abortus* MssR.

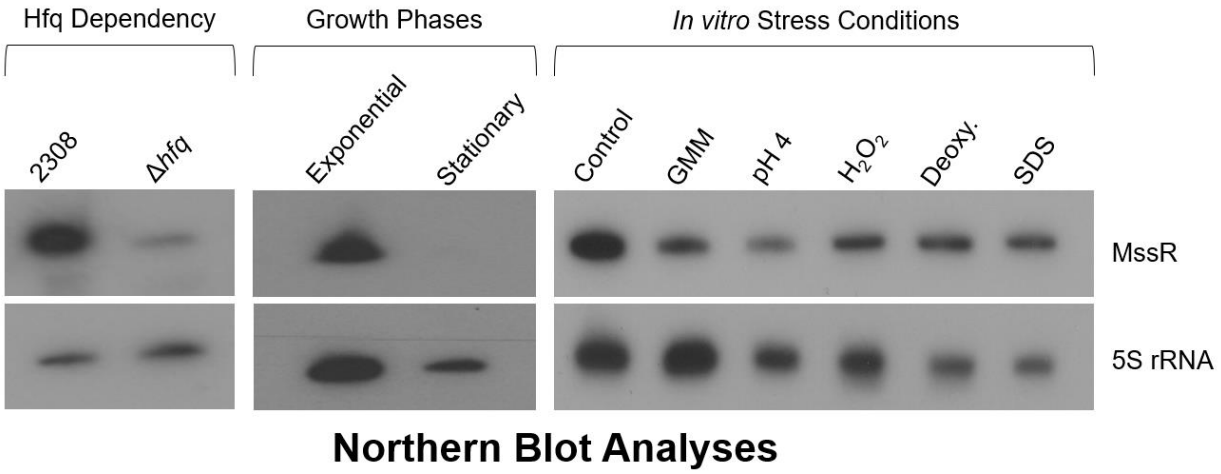

Figure 4. MssR is Hfq-dependent and is primarily expressed during exponential phase of growth. sRNAs often rely on the protein chaperone Hfq for stability and to facilitate the binding to their mRNA targets. In the absence of Hfq, there is a significant decrease in the amount of the MssR transcript, indicating the sRNA may depend on Hfq for stability. Additionally, *mssR* is expressed significantly more during mid-exponential phase growth, compared to late stationary phase in strain 2308. Expression of *mssR* was also measured during other biologically relevant conditions, such as acidic stress, exposure to reactive oxygen species, nutrient limitation (GMM), and exposure to a bile salt mimic (0.1% deoxycholate). A detergent stress (0.01% SDS) was also included due to preliminary findings showing that the *mssR* mutant was more sensitive to this stress. Densitometry using FIJI indicates a potential decrease in expression after growth in minimal medium (GMM) or exposure to a pH of 4.

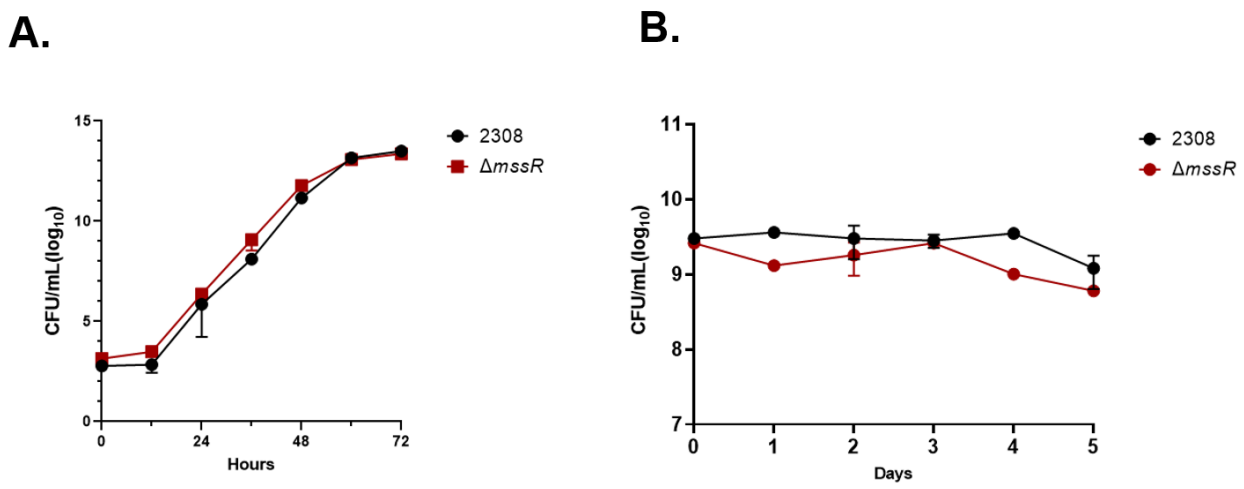

Figure 5. The Δ*mssR* mutant has no growth defect in nutrient-rich broth or nutrient-limited medium. Wild-type *B. abortus* (2308) and the Δ*mssR* strains were first grown on agar before being resuspended in PBS for inoculation. Fifty milliliter cultures were seeded at a density of $1x10^3$ CFU/mL and incubated shaking at 37°C for 72 hours. Every 12 hours, a portion of each culture was serially diluted and plated on agar to enumerate CFUs (A). The experiment was repeated using Gerhardt's Minimal Medium (B), where cultures were seeded at $5x10^9$ CFU/mL and survival was assessed every 24 hours by serially diluting the cultures and plating on agar.

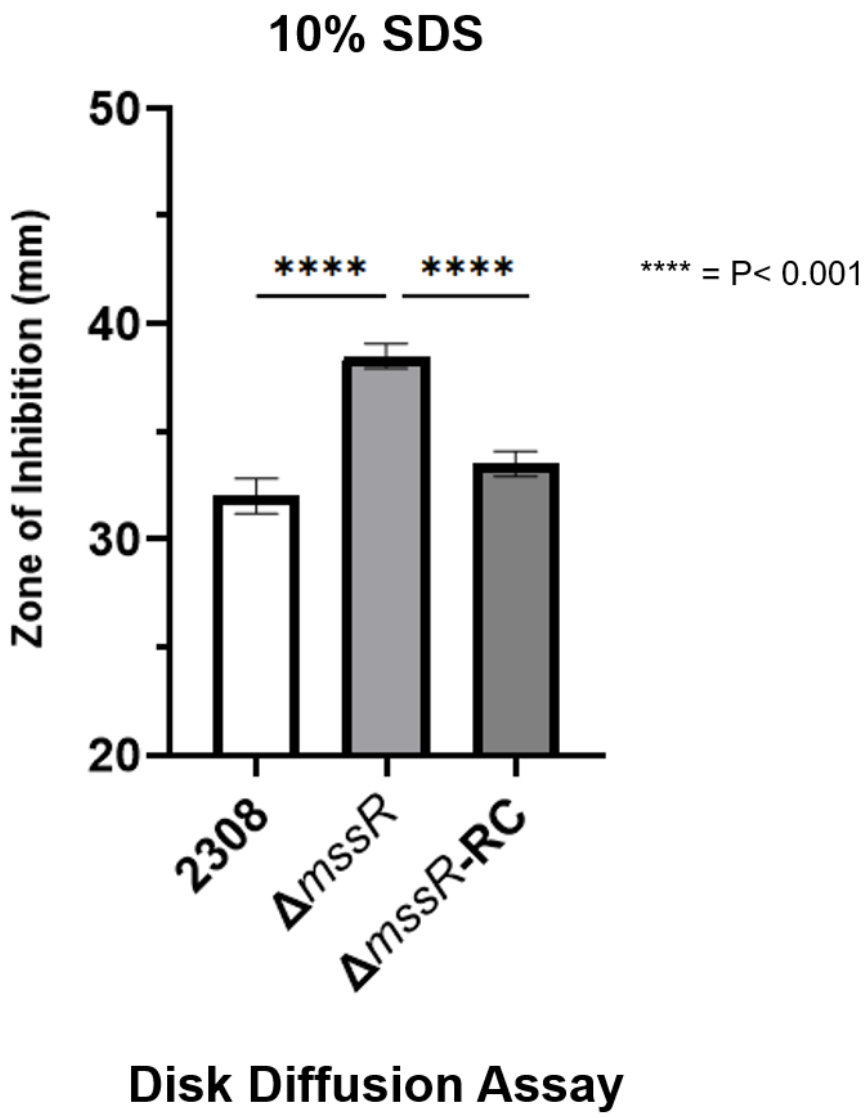

Figure 6. The Δ*mssR* mutant is sensitive to the detergent SDS. Soft agar, inoculated with either wild type *B. abortus* 2308 or the Δ*mssR* strain, was overlaid on TSA plates. A small circle of Wattman paper was placed in the center and 10% SDS was spotted onto the paper and allowed to diffuse out into the soft agar, forming a gradient. The bacteria were allowed to grow, and the zone of inhibited growth was measured. A larger zone, or growth further from the Wattman disk, indicates increased sensitivity to the agent, as the concentration gradient increases the closer to the disk you are. The Δ*mssR* strain is significantly more sensitive to SDS and this sensitivity is rescued when the *mssR* locus is chromosomally reconstructed.

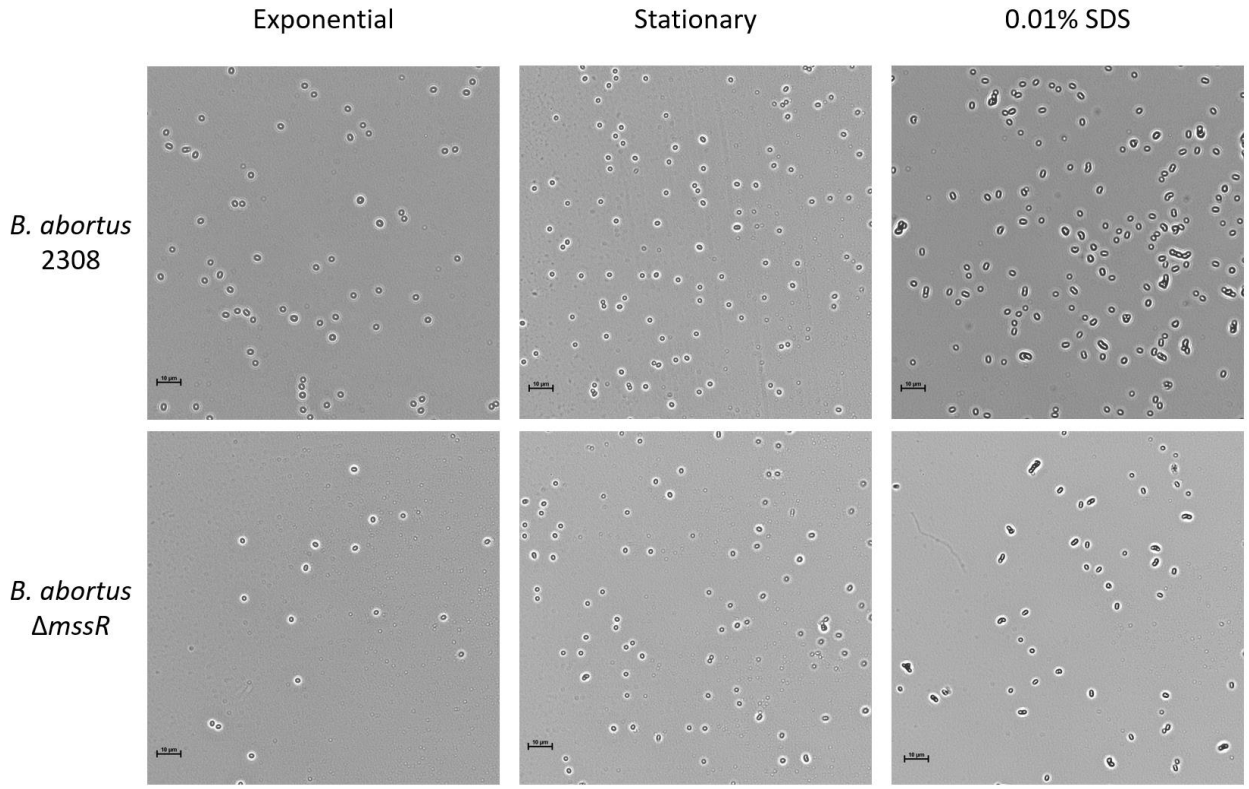

Figure 7. DIC microscopy does not reveal any major morphological differences in the Δ*mssR* strain. After a potential chaining phenotype was observed in the Δ*mssR* strain via cryo-electron microscopy, *B. abortus* cultures were stained and viewed by DIC microscopy. In these larger fields of view, there does not appear to be any gross morphological differences between the two strains after being stressed with SDS.

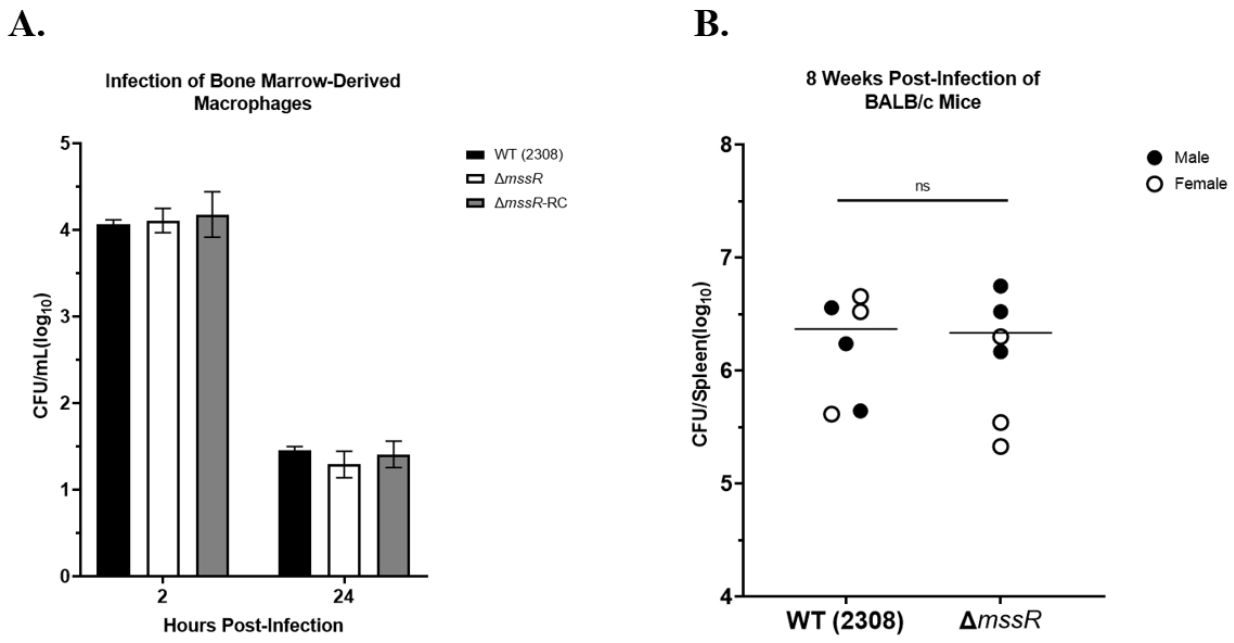

Figure 8. The Δ*mssR* mutant is not attenuated in a macrophage or mouse infection model. Wild-type *B. abortus* (2308) and the Δ*mssR* mutant strains were first grown on agar before being resuspended in PBS for OD adjustment. Primary macrophages, isolated from the femurs of BALB/c mice, were infected with $1x10^7$ CFU/mL. At each time point, the macrophages were lysed with 0.1% deoxycholate, the lysate was serially diluted and then plated on agar for enumeration (A). BALB/c mice were infected with $1x10^5$ CFU via intraperitoneal injection. Spleens were harvested from all animals at 8 weeks post-infection, the entire organ homogenized, serially diluted and plated on agar to enumerate CFUs (B). There were no statistical differences between 2308 and Δ*mssR*, nor were there any sex differences in our model.

Chapter 3
Discussion

Bacteria provide a unique opportunity to observe evolution unfolding in real-time. This is perhaps most often seen with the continued rise in antibiotic resistance of bacterial pathogens [48]. While this resistance is a pressing health concern, the focus of this study is aimed at the older adaptations that have withstood the test of time, specifically small regulatory RNAs. It is unclear when bacterial sRNAs were first used as regulators and if there was a selective pressure promoting their use, but they are a fast and energy-efficient method of regulating gene expression [17, 49]. In *Brucella*, it was hypothesized that the novel sRNAs found within *B. abortus* are important for virulence. This has proven to be partially incorrect, as multiple sRNAs are dispensable for *B. abortus* to infect macrophages or mice, suggesting that the regulatory roles of those sRNAs are subtler in nature. Because of the membrane irregularities in the Δ*mssR* strain, MssR is a unique and puzzling example of a regulator that would be expected to impact the fitness of *B. abortus* in a more dramatic manner. One possible explanation is that MssR has no major link to the overall fitness of *B. abortus*, but instead has a minor role in regulating aspects of the cellular envelope. Another hypothesis is that MssR has a more relevant role in a cow infection model, the natural host of *B. abortus*.

Taken together, sRNAs in *Brucella* are an intriguing area of research, one that could both impact the global economy and human health. Much of the current focus with *Brucella* research is centered on improving the vaccines for our domestic herds, and sRNA mutants have recently shown potential as vaccine strains. Developing a vaccine is beyond the scope of this project, but the methods used to characterize MssR can be applied to the remaining novel sRNAs in *B. abortus*, any of which could prove to be the sought-after vaccine strain. Even beyond the field of *Brucella*, we encourage other microbiologists to investigate sRNAs within their bacterial model systems . It is my opinion that these versatile transcripts have a much larger regulatory role than their name implies.

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