Abstract

IDENTIFICATION OF A POTENTIAL RNAZYME IN *BRUCELLA ABORTUS* WITH HEME-DEPENDENT PEROXIDASE ACTIVITY

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December, 2020

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Bovine brucellosis is of significant concern to the U.S. cattle industry and has not been controlled by vaccination in some areas of the world. The inability to control this pathogen is currently a serious public health concern because infected animals or animal products can readily transmit brucellosis to humans with an extremely low infectious dose of 10-100 organisms (Neta et al., 2010, Franz, D.R. 1997). The characteristics of *Brucella* such as the extreme variability of symptoms it causes, as well as its durability and small size, deem it an attractive bioweapon and highlight the necessity for understanding its virulence mechanisms.

Iron (Fe) is an essential micronutrient for almost all microorganisms for a variety of cellular processes. Interestingly, heme is a biologically relevant source of iron for *Brucella* during infection (Roop, 2011), and the operon *bhuTUV* works in conjunction with the outer membrane heme transporter BhuA to bring heme into *B. abortus* (Ojeda, 2012). Interesting, a putative open reading frame (ORF) with an unknown function lies upstream of *bhuTUV*.

Catalase activity is also important to *Brucella,* and the sole catalase produced by *B. abortus* 2308 plays a minimal role in detoxifying endogenous H_2O_2 (Steele et al., 2010). Interestingly, there is strong evidence that DNA and RNA can fold to create cofactor-binding

sites capable of binding heme at the submicromolar level by folding into a guanine-quadruplex (Kong et al., 2010, Travascio et al., 1998, Travascio et al., 1999, Poon et al., 2011). Characteristics of oligonucleotides folding into G-quadruplexes also have peroxidase activity. However, this activity has only been shown *in vitro* and not in any living organism. This study aimed to determine if the hypothetical ORF upstream of the *bhuTUV* operon (named KAPF) possesses heme-dependent peroxidase activity by also folding into a guanine-quadruplex. Qualitative and quantitative *in vitro* and *in vivo* analyses were performed in this study to determine if KAPF acts in this way to rid of endogenous H_2O_2 in the cell. Furthermore, this work contributed to filling in the knowledge gap regarding the various ways *Brucella* responds to heme.

RNAseq data suggested that an RNA product of KAPF was produced, regardless of iron concentration. It was predicted that this segment is not only a transcriptional start site but is also turned on continuously to act as an RNAzyme with peroxidase activity by folding into a G-quadruplex and interacting with heme. This study found that KAPF binds heme *in vitro* and significantly more than nucleotides previously known to bind heme. However, no peroxidase activity was detected *in vitro* or *in vivo*.

This led to an alternative hypothesis as to why the *bhuTUV* operon is not transcribed when an RNA product of KAPF is produced. A riboswitch may be causing the termination of downstream genes in response to heme. This introduces a never-before described heme riboswitch and potentially fills the knowledge gap surrounding this segment of interest's response to heme. It is imperative to fill any knowledge gaps associated with *Brucella* virulence mechanisms in order to relieve any concerns regarding the impact brucellosis has on the overall health of agriculture and humans.

IDENTIFICATION OF A POTENTIAL RNAZYME IN *BRUCELLA ABORTUS* WITH HEME-DEPENDENT PEROXIDASE ACTIVITY

A Thesis Presented to

The Faculty of the Department of Biology

East Carolina University

In Partial Fulfillment

of the Requirements for the Degree

Master of Science in Molecular Biology and Biotechnology

By

Dalton L Chapman

December, 2020

IDENTIFICATION OF A POTENTIAL RNAZYME IN *BRUCELLA ABORTUS* THAT HAS HEME-DEPENDENT PEROXIDASE AND/OR KAPFSWITCH ACTIVITY

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Acknowledgements

I would first like to thank the department of Biology at East Carolina University for allowing me to pursue my Master of Science degree in Molecular Biology and Biotechnology. I would also like to thank Dr. Eric Anderson for providing me the opportunity to be involved in an interesting microbiology lab as an undergraduate and for helping me continue my research as a graduate student through globally unprecedented circumstances. I would also like to recognize the undergraduate students that provided a fun and friendly research environment and making the lab seem like a family. Lastly, I would like to thank my fiancé Kyra Price for always supporting me throughout my entire educational career, I couldn't have done any of this without you.

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Chapter I: Investigating a potential DNAzyme or RNAzyme with Heme-dependent Peroxidase Activity

Review of Literature

Brucella abortus

Brucellosis is a chronic disease of wild and domestic animals and humans, caused by an intracellular bacterium, *Brucella abortus* (Poester, Samartino, Santos, 2013). *B. abortus* is a facultative, Gram-negative bacterium that infects cattle resulting in infertility due to abortion in pregnant females, male sterility from epididymitis, and undulant fever in humans (Payne, 1959). Bovine brucellosis is of significant concern to the U.S. cattle industry and has not been controlled by vaccination in some areas of the world, creating the need for surveillance and quarantine programs (Neta et al., 2010). The inability to control this pathogen is currently a serious public health concern because infected animals or animal products can readily transmit brucellosis to humans with an extremely low infectious dose of 10-100 organisms. There are currently no vaccinations available for humans. This, along with *Brucella*'s extreme variability of symptoms, durability, and small size deem it an attractive bioweapon and highlight the necessity for understanding the virulence mechanisms of *B. abortus* (Franz, D.R. 1997).

Iron Importance and Regulation

Iron (Fe) is an essential micronutrient for almost all microorganisms for a variety of cellular processes, such as carbon metabolism, amino acid and nucleotide synthesis, nitrogen fixation, electron transport, and oxidative phosphorylation (Ferreira, G.C. 1999, Andrews, S.C.

2003). The ability of a bacterium to acquire sufficient iron is usually linked with its ability to survive in its biological niche (Neta et al., 2010). Mammals have Fe-withholding defenses as both innate and acquired immune responses to protect themselves from bacterial infections (Nairz et al., 2010). These defenses have not been well studied in cattle. Fe-withholding defenses cause bacterial pathogens to require high affinity Fe transport systems to overcome the Fe-withholding defenses and produce disease (Cassat and Skaar, 2013). These transport systems involve competing with pathogens for iron by producing compounds with higher affinity to Fe than compounds produced by pathogens that also bind Fe. These are known as siderophores, which chelate Fe and are responsible for bringing it back to the pathogen for its own purposes. Mammalian Fe transport systems try to outcompete siderophores using ferric iron binding proteins transferrin and lactoferrin that allow the amount of available ferric iron for bacteria to be approximately 10^{-18} M (Bullen J. J. 1999, Griffiths E. 1999), because the pathogenesis of many microorganisms is significantly enhanced when the host is unable to maintain an iron restrictive environment.

In many prokaryotes, gene transcription involving iron acquisition is regulated by the ferric iron uptake regulator (Fur) (Byers, B.R. 1998). Fur acts as a repressor in the regulation of iron-responsive genes by blocking transcription upon interaction with its co-repressor, Fe^{2+}. The Fe^{2+}-Fur complex binds -35 to -10 base pairs upstream of the Fur-repressed genes to a palindromic sequence known as the 'Fur box' to carry out this function (Andrews, S.C. 2003). Within the Fur family of transcriptional regulators, one of the iron responsive regulators, Irr, contributes to heme-dependent iron regulation in alphaproteobacteria (Anderson et al., 2011). Heme, or a heme prosthetic group (iron-protoporphyrin IX), is a protoporphyrin ring consisting of four pyrrole rings linked by methene bridges, and a central Fe atom. Heme performs a wide

variety of functions in nature such as electron transfer, catalysis, oxygen transport, and even gene regulation (Bowman and Bren, 2008).

To compensate for iron starvation, Irr has the ability to fill both roles of decreasing the cell's iron demands and inducing iron transport systems. Specifically, Irr senses intracellular concentrations of iron by directly interacting with heme molecules, such as newly synthesized heme and ferrochelatase to impart self-degradation and inhibit heme biosynthesis respectively (Anderson et al., 2011). Irr degrades in the presence of heme via oxidation but responds to protoporphyrin IX (heme without Fe), which allows Irr to resume its function as a repressor (Martinez, M. 2005). During iron-limited conditions, Irr also increases the expression of heme and siderophore uptake genes. For instance, in *Brucella,* Irr transcriptionally activates the outer membrane heme transporter BhuA. This activity was observed in an *Irr* mutant strain that had no *BhuA* transcription in low iron conditions and was unable to use heme as an iron source (Anderson et al., 2011).

The RirA protein also carries out Fur-like functions in the regulation of iron-responsive genes. RirA is a Fe-S protein that represses many iron-responsive genes in iron rich conditions and has been well characterized in organisms such as *Sinorhizobium meliloti, Agrobacterium tumefaciens,* and *Rhizobium leguminosarum* (Chao et al., 2005, Ngok-Ngam P, et al. 2009). *B. abortus* encodes a gene (BAB2_0678) that produces a product with ~70% ortholog identity in its amino acids with RirA in *R. leguminsarum*, suggesting it plays a similar role (Rodionov et al., 2006). A *rirA* mutation in *Agrobacterium tumefaciens* causes overproduction of siderophores and iron-independent expression of genes involved in siderophore uptake (*fhuA, irp6A,* and *fbpA*) and heme uptake (*hmuPSTUV)* compared to the wild-type strain. Also, transcription of *RirA* is

repressed during iron-limiting conditions and loss of RirA led to the expression of 17 gene products to be greatly reduced (Ngok-Ngam et al., 2009).

Managing Oxidative stress in Brucella

One possible reason for the presence of multiple iron-responsive regulators in *Brucella* is that excess iron can sometimes result in the production of toxic hydroxyl radicals, if not carefully regulated. Other sources of toxicity come from reactive oxygen species resulting from host immune systems or aerobic metabolism. Catalase has been well documented as an enzyme that pathogens use as a defensive tool to protect from oxidative stress (Kim et al., 2000). Oxidative metabolism produces superoxide and hydrogen peroxide as inevitable products (Chance at al., 1979), which increases the likelihood that catalase will be produced during this type of respiration. Phagocytes increase the consumption of oxygen and produce oxygen intermediates such as superoxide, hydrogen peroxide, HOCl, hydroxyl radicals, and singlet oxygen (Hassett and Cohen, 1989). Catalase activity is important to *B. abortus,* considering that they localize in phagocytic cells within hours of infection, but persist and multiply there in chronic disease (Kim et al., 2000, Braude, 1951). The cells surviving inside the phagosome are thought to alter their protein expression patterns in response to the new environment (Kim et al., 2000).

Brucella secretes metalloenzymes that catalyze the dismutation of superoxide (O_2^-) to hydrogen peroxide (H_2O_2) called superoxide dismutases (SODs), specifically Mn-SOD (SodA) and Cu-Zn SOD (SodC). These are active in the cytoplasm and periplasm respectively (Kim et al., 2000). Superoxide does not easily cross cellular membranes; thus, it is almost exclusively detoxified near the source of generation. Catalase activity is restricted to the periplasm and, in combination with SodC, protects *Brucella* from external sources of oxidative compounds,

whereas endogenous superoxide resulting from cellular metabolism is detoxified by SodA (Sha et al., 1994, Gee et al., 2005). The H_2O_2 byproduct can be further broken down by catalase and other sources of peroxidase activity. A *B. abortus* SodC mutant MEK2 was significantly more sensitive to extracellular superoxide than *B. abortus* 2308, but equally resistant to H_2O_2 in disk diffusion assays (Gee et al., 2005). This suggests that SodC and SodA are important for detoxifying superoxide resulting from oxidative burst.

Toxic levels of H_2O_2 are produced from aerobic metabolism and the respiratory burst of host phagocytes, along with being a byproduct of superoxide dismutase. AhpC is an NADPH-dependent peroxidase that is the primary detoxifier of endogenous H_2O_2, whereas *Brucella*'s sole catalase, KatE, mainly scavenges exogenous and supraphysiologic H_2O_2, unless AhpC is absent (Steele et al., 2010). H_2O_2 may be the only substrate for AhpC, with its only role in nature being to detoxify H_2O_2 (Seaver and Imlay, 2001). Experimental evidence regarding *in vitro* peroxidase activity via genetic elements suggests that *Brucella* may possess unidentified methods of detoxifying H_2O_2 of endogenous origin.

Genetic Elements Binding Heme for Peroxidative Purposes

There is strong evidence that RNA can fold to create cofactor-binding sites with strong peroxidative activity capable of binding heme at the submicromolar level (Kong et al., 2010, Travascio et al., 1998, Travascio et al., 1999, Poon et al., 2011). In many cases synthetic DNAzymes and their RNA counterparts have been shown to bind Fe(III) heme by folding to a guanine quadraplex. These genetic elements also exhibited peroxidase activity as an apoenzyme for the heme cofactor (Travascio et al., 1999, Travascio et al., 1998, Poon et al., 2011). The peroxidative activity that RNA oligonucleotides exhibited had substantially higher catalytic

activity than other heme proteins such as catalase and Fe(III)-myoglobin, but was 30-fold weaker than that of its DNA counterpart (Travascio et al., 1999). In another study, RNA G-quadraplexes, when complexed with heme, showed catalytic activity greater than those of heme proteins by performing 2-electron oxidations (Poon et al., 2011).

Guanine Quadruplexes

G-quadruplexes are high-order structures of DNA or RNA with G-rich sequences and have been studied since the1960's (Gellert et al., 1962). They are formed by four adjacent Guanines that bind to one another to form stacked G-quartets with Hoogsteen-type base pairing and high structural stability (see Figure 1). When these stacked G-quartets bind together, a G-quadraplex is formed. G-quadruplexes have recently shown folding characteristics *in vitro* and have been found in high abundance in telomeres.

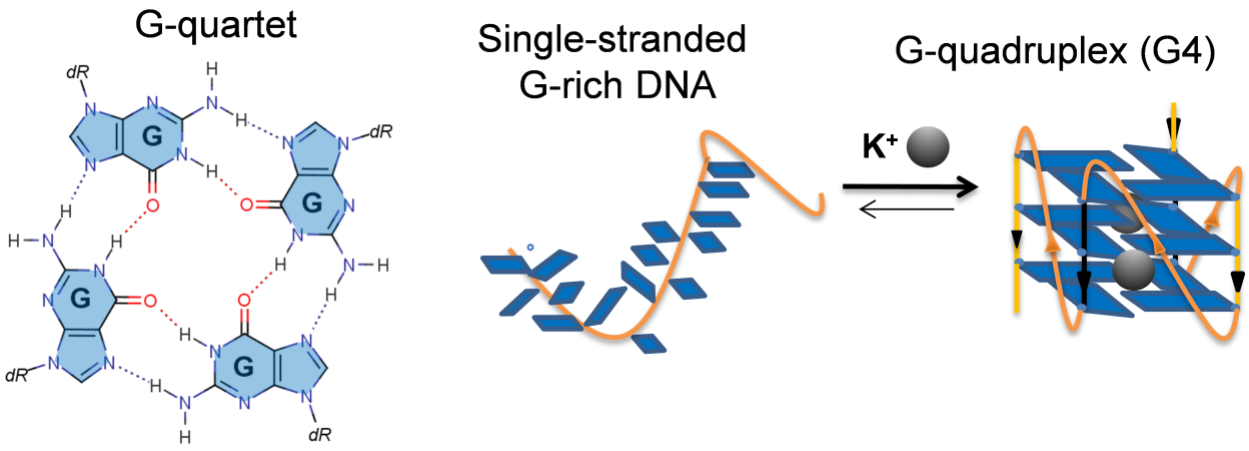


Figure 1. Mechanism of G-quadruplex formation. A guanine quartet formed between 4 guanines via Hoogsteen-type base pairing. Three of these quartets bind together to form a G-quadruplex.

Heme has the possibility of binding multiple sites on a G-quadruplex such as the connecting loops of DNA or RNA that wrap around each corner of the G-qudaruplex, as well as the flat outer faces of the two terminal G-quartets, or the four nonidentical grooves (see Figure 2)

(Travascio et al., 1999). Interestingly, there is a segment upstream of the *BhuTUV* operon has five regions with four consecutive Guanines (see Figure 4), which introduces its potential for folding into a G-quadruplex. For the purpose of this study, the segment has been named after the fiancée of the researcher (KAPF), with 'F' standing for full-length product. G-quadruplexes also share oxidative characteristics, whether DNA or RNA (Kong et al., 2010). When taking these things into consideration, it is possible that KAPF upstream of the *bhuTUV* operon (see Figure 3) is continuously transcribed to act as an apoenzyme by folding into a G-quadruplex while using heme as a cofactor.

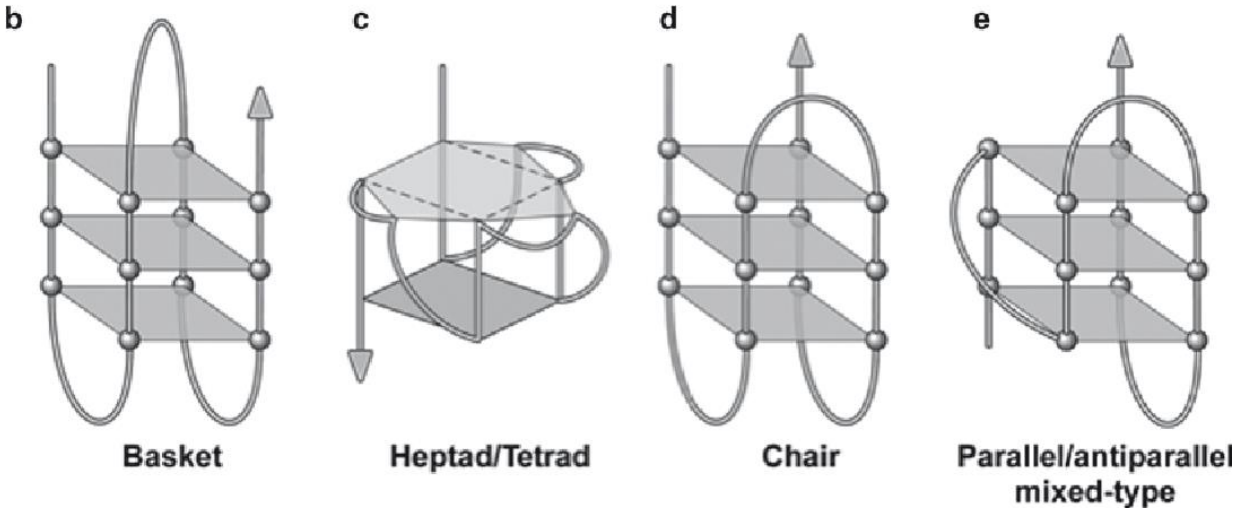


Figure 2. Diagram of different G-quadruplex structures. B-E represent four different folding orientations of a G-quadruplex. The variability in structure increases the potential affinity to heme by exposing many binding sites such as the various connecting loops, the flat outer faces of the two terminal G-quartets, or the four nonidentical grooves.

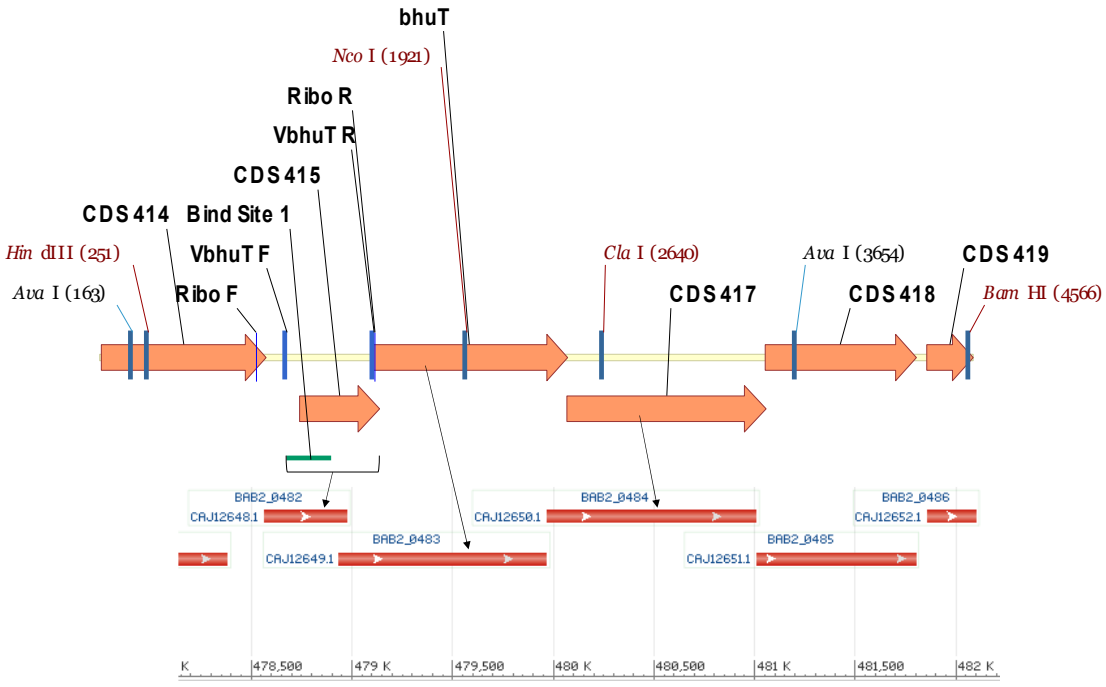


Figure 3. The *bhuTUV* operon map. Arrows represent a gene with homology to a para-hydroxybenzoic acid efflux protein (CDS 414), a hypothetical ORF (CDS 415), *bhuT*, *bhuU* (CDS417), and the two components of *bhuV* (CDS 418 and CDS 419). KAPF consists of the green segment below CDS 415 plus 169 bp upstream and 235 bp downstream (annotated by the bracket encompassing 'Ribo F' and 'Ribo R').

Figure 4. Sequence of KAPF. The italics regions indicate where the primers KAPF-F and KAPF-R bound. Guanine quartets are indicated by the highlighted regions The smaller regions designed to elicit G-quadruplex formation are notated by the brackets labeled KAP1, KAP2, KAP3, and KAP4. The +1 site for *bhuT* is indicated by the nucleotide highlighted in green.

Peroxidase Activity in Brucella

When cultured under nutrient limited conditions, ridding of metabolic H_2O_2 is important to *B. aborus* 2308 for maintenance of stationary-phase viability. However, the sole catalase produced by *B. abortus* 2308 plays a minimal role in detoxifying endogenous H_2O_2 (Steele et al., 2010). Interestingly, this phenomenon is observed in other bacteria, where catalase and AhpC play overlapping compensatory roles in detoxifying cytoplasmic H_2O_2 (Cosgrove et al., 2007, Seaver and Imlay, 2001).

A defect in growth during routine aerobic cultivation was not observed in the *B. abortus ahpCD, katE* double mutant. This suggests there are other antioxidants produced by the bacterium that are capable of detoxifying H_2O_2 of metabolic origin. The potential peroxiredoxins encoded by BAB1_0941 and BAB1_0504 could possibly protect *B. abortus* from H_2O_2 damage, as seen in PrxV-type peroxiredoxins that mammalian mitochondria, but that remains unknown (Banmeyer et al., 2005, Steele et al., 2010).

Interestingly, the *E. coli ahpCF katG KatE* triple mutant exhibited no H_2O_2 scavenging activity (Seaver and Imlay, 2001). However, the *B. abortus ahpCD katE* double mutant still exhibited H_2O_2 scavenging (Steele et al., 2010). This suggests that the segment of interest in *Brucella abortus* upstream of *bhuTUV* (KAPF) may play a role in the peroxidation of endogenous H_2O_2. This also shines light on the importance of identifying any potential peroxidase activity in *Brucella* that aids in the removal of endogenous H_2O_2. Considering the G-rich components of KAPF, along with the necessity for *Brucella* to remove endogenous H_2O_2 when thriving in host macrophages, it is hypothesized that KAPF acts as a heme-responsive peroxidase by folding into a G-quadruplex.

Research Objectives

If *B. abortus* uses the KAPF region of the *bhuTUV* operon as a DNAzyme or RNAzyme with heme-dependent peroxidative activity, then the following properties should be observed: the segment binds heme *in vitro,* the segment exhibits peroxidase activity *in vitro,* and cloning the segment into an *E. coli* mutant lacking catalase activity returns its H_2O_2 tolerance. The outline of this research can be summarized by the objectives below.

Detecting an DNAzyme or RNAzyme in Brucella abortus that binds heme

The objective of this experiment was to determine if KAPF binds heme *in vitro* using a UV-Vis spectrophotometer. Results indicating heme binding would be indicated by an increase in the Soret band at 400 nm in solutions incubated with KAPF and heme, compared to heme alone. Smaller segments (~50-60 base pairs) of the KAPF with G-rich regions designed and synthesized in the form of DNA (KAP1-KAP4, see Table 2), a single stranded DNA product of the segment of interest, and its RNA counterpart should exhibit this increase in the Soret band when compared to heme alone. The lack of heme binding is indicated by similar absorbances in the Soret band between solutions incubated with the both segment of interest and heme, and heme alone.

Kinetics of Peroxidation

The goal of this assay was to determine if the heme-DNA or heme-RNA complexes exhibited peroxidase activity *in vitro* using a UV-Vis spectrophotometer. Classic enzymatic peroxidase activity is determined by monitoring the appearance of the 2,2'-azinobis(3-ethylbenzothiozoline)-6-sulfonic acid (ABTS) radical cation at 414 nm when reacted with the KAPF, heme, and hydrogen peroxide (H_2O_2). If the DNA and RNA forms of the segment of

interest are indeed enzymes, they would exhibit a color change in their reaction with ABTS and H_2O_2, indicating the appearance of the $ABTS^+$ radical cation in response to the enzyme oxidizing it using hydrogen peroxide. If no steady increase in the absorbance at 414 nm over time is observed, then no enzymatic activity is present.

E. coli ΔkatE/ΔkatG Cloning and H_2O_2 Tolerance

The goal of this experiment was to determine if transforming KAPF into an *E. coli* mutant lacking catalase activity, EC484 UM255 ΔKatE/ΔkatG (EC484), would return its H_2O_2 tolerance. This should increase H_2O_2 tolerance of the transformant when compared to the EC484. Conducting a disc diffusion assay with 1% H_2O_2 would show the difference in the zones of inhibition (ZOIs) between the strains with and without KAPF. IPTG and heme on discs surrounding the H_2O_2 disc should induce the plasmid containing KAPF and allow proper folding respectively. A decrease in the zone of inhibition in the transformant compared to EC484 would indicate a positive result. The positive controls PS5.M and PS2.M are known to possess peroxidase activity once bound to heme (Kong et al., 2010, Travascio et al., 1998, Travascio et al., 1999, Poon et al., 2011) and transformants possessing these segments should also show a decrease in the ZOIs when compared to EC484. Observing similar ZOIs between the transformants and that of the positive controls would also further indicate peroxidase activity. Observing similar ZOIs between the transformants and the mutants would indicate a negative result and imply no peroxidase activity.

Predicted Outcomes

The DNAzyme and its RNA counterpart will bind heme to fold into a G-quadruplex

Due to its 5 regions with four consecutive guanines (see Figure 4), KAPF will bind heme by folding into a guanine quadruplex. DNA and RNA quadruplexes are known to spontaneously form in sequences that have multiple guanine quartets. There is also strong evidence that genetic elements can fold to create cofactor-binding sites with strong peroxidative activity capable of binding heme at the submicromolar level *in vitro* (Kong et al., 2010, Travascio et al., 1998, Travascio et al., 1999, Poon et al., 2011). KAPF is expected to bind heme as ssDNA and RNA at levels comparable to the positive controls PS2.M and PS5.M, which would indicate its potential for peroxidase activity.

The DNAzyme and its RNA counterpart will have peroxidase activity once bound to heme as a G-quadruplex

The segment KAPF will act as an apoenzyme that has peroxidative characteristics by folding into a G-quadruplex while using heme as a cofactor. G-quadruplexes share oxidative characteristics, whether DNA or RNA and, when complexed with heme, showed catalytic activity greater than those of heme proteins (Poon et al., 2011). Thus, it is probable the segment of interest in *B. abortus,* as either DNA or RNA, will exhibit catalytic peroxidase activity *in vitro.* KAPF is expected to react with ABTS and produce the radical cation responsible for the increase in absorbance at 414 nm at levels comparable to that of the positive controls PS2.M and PS5.M.

EC484 UM255ΔKatE/ΔkatG Will Obtain H_2O_2 Tolerance once Cloned

Transforming a plasmid ligated to KAPF into EC484 UM255 ΔKatE/ΔkatG will restore H_2O_2 tolerance. A defect in growth during aerobic cultivation was not observed in a *B. abortus ahpCD, katE* double mutant, a trait not observed in the same mutant in *E. coli*. This suggests there are other antioxidants produced by the bacterium that are capable of detoxifying H_2O_2 of metabolic origin (Banmeyer et al., 2005, Steele et al., 2010). Once KAPF has been successfully taken up by *E. coli*, a return of function regarding the tolerance of H_2O_2 will occur as the segment has peroxidase activity as either DNA or RNA once induced by IPTG and heme. This will result in a decrease in the zones of inhibition for EC484-KAPF compared to EC484 UM255 ΔKatE/ΔkatG.

Methods

Amplification of Segment of Interest

All primers were ordered from Integrated DNA Technologies Inc. The primers KAPF-R and KAPF-F were designed to target KAPF, a 637 bp segment consisting of the potential DNAzyme, plus the hypothetical protein downstream in case its G-rich properties allow G-quadruplex formation. The PCR reactions were ran using a BIO-RAD C1000 Thermo Cycler. These products were ligated to the plasmid pBBR-1MCS-2 for transformation into EC484 UM255 ΔKatE/ΔkatG.

Primer Name	Primer Sequence
KAPF-F	5'-AGGCATCCATCTGGTG-3'
KAPF-R	5'-GCAGCAAGGAATGGC-3'
Biotin-Ribo-R	5'-/5Biosg/AATGGACTACGTAAGACGGCA-3'
M13-F	5'-GTAAAACGACGGCCAGT-3'
M13-R	5'-CAGGAAACAGCTATGAC-3'
Lac promo-F	5'-CAATTAATGTGAGTTAGC-3'

Table 1. Oligonucleotide primers. These primers were used to: amplify KAPF for the purpose of ligating it to the plasmid pBBR-1MCS-2 (KAP-F, KAPF-R), amplify and extract single stranded KAPF using Streptavidin beads (KAPF-BIO), determining the orientation in the plasmid (M13-F, M13-R), and amplify the pBBR1MCS-2 lac promoter plus KAPF (Lac promo-F, Ribo-R).

Amplification and Extraction of Single Stranded Segment of Interest

The forward primer KAPF-F and a biotinylated reverse primer Biotin-Ribo-R were used to amplify KAPF for the purpose of denaturing and extracting one single strand (ssKAPF). The biotinylated primer allowed the attachment of Streptavidin magnetic beads to one strand after

PCR by shaking 100 µl PCR product in 1 mL TE Buffer, pH 8.0, and 100 µl Streptavidin beads for one hour. A magnet was used to pull the beads to one side of the tube and allow the supernatant to be removed. The beads were suspended in 100 µl distilled water and heated at 90 °C for 2 minutes. A magnet was used again to pull the beads to one side and the supernatant was transferred to a tube containing 1 µl 100X TE Buffer. Stocks of ssKAPF were immediately used in binding or peroxidase assays.

Amplification of RNA Counterpart of Segment of Interest using T3 Polymerase

The plasmid pBBR1MCS-2 (17µl) was digested with the restriction endonuclease EcoRV (1 µl) with 2 µl of 10X Fast Digest Buffer at 37 °C for 8 minutes. This reaction was then heat inactivated at 85 °C for 10 minutes using a BIO-RAD C1000 Thermo Cycler for accurate temperature control. The PCR product of KAPF was ligated to the linear pBBR1MCS-2, using a TAKARA Ligation kit with 5µl of cut pBBR1MCS-2, 5 µl of a 1/100 dilution of KAPF in TE Buffer, and 10µl of TAKARA ligation mix.

This plasmid was then linearized by digesting it with the restriction endonuclease SMAI using 1 µl of SmaI, 19.6 µl of pBBR-1MCS-2-KAPF, 3 µl 10X Fast Digest Buffer, and 6.4 µl dH_2O. The linearized pBBR1MCS-2-KAPF was precipitated by incubating in ethanol at -80 °C for 1 hour, rinsed with 70% ethanol, and inverted to dry. The plasmid was resuspended in 31.5 µl dH_2O and the concentration was read immediately using a Thermo Fisher Scientific Nanodrop 2000 and used as the template for RNA synthesis in its entirety. The T3 RNA polymerase was used to synthesize KAPF that was adjacent to the T7 promoter in the plasmid pBBR1MCS-2. The reaction consisted of 6 µl dTT, 6 µl T3 polymerase, 24 µl 5X reaction buffer, 6 µl RNAse inhibitor, 15 µl ribonucleotides and the suspended, linearized pBBR-1MCS-2-KAPF. This

incubated in a 37 °C water bath for 2.5 hours. The RNA concentration was immediately read in the nanodrop and used for both heme-binding and peroxidase assays in its entirety.

DNA or RNA Binding to Heme

Oligonucleotides KAP1, KAP2, KAP3, KAP4, PS2.M and PS5.M (see Table 2) were ordered from Integrated DNA Technologies Inc and were designed to possess enough guanines to elicit G-quadruplex formation (see Table 2). 100 μM DNA stocks were heated at 90-95 °C for 5 mins in TE buffer (10 mM Tris, pH 7.5; 0.1 mM EDTA) to ensure single stranded products. These stocks, and prepared ssDNA and RNA were immediately made up to appropriate concentrations, as described in Figure 6, in 40KT Buffer (50 mM MES, pH 6.2; 100 mM Tris acetate, 40 mM potassium acetate, 1% DMSO, 0.05% Triton X-100). Heme was added to a total of 0.5 μM and incubated with the DNA or RNA for 30 minutes at room temperature to allow proper binding. Spectra from 320-680 nm for each sample were obtained using a UV-Vis spectrophotometer and the peaks at 400 nm were recorded.

Segment name	Segment sequence
KAP1	5’-GGGCATGCTACCAGAATAGCTCCGGTTAAAACGGTATCG CCTCTCTTATCTTGGGGG-3’
KAP2	5’-GCCGTAATACCGTCATGACGGTTCCCCGACCGAGAGCGAA GGGGATTAATAGGG-3’
KAP3	5’-GGGGATTAATAGGGAACACGGTGAGGACGACCCATCAAGGGG-3’
KAP4	5’-GACCTGCCGTCTTACGTAGTCCATTGTCACGGGCGGGG-3’
PS2.M	5′-GTGGGTAGGGCGGGTTGG-3′
PS5.M	5’-GTGGGTCATTGTGGGTGGGTGTGG-3’

Table 2. Smaller segments of interest. KAP1, KAP2, KAP3, and KAP4 encompass regions within KAPF and were designed to elicit G-quadruplex formation similarly to the positive controls PS2.M and PS5.M.

Peroxidase Activity of the putative DNAzyme or Ribozyme

100 μM DNA stocks were heated to 90-95 °C for 5 mins in TE buffer to ensure single stranded products in order to mimic single stranded mRNA binding to heme in the cell. These stocks, along with prepared ssKAPF and its RNA counterpart, were immediately made up to appropriate concentrations in 20 KH buffer (50 mM MES ((2-(N-morpholino) ethanesulfonic acid)), 100 mM Tris-acetate [pH 6.2], 40 mM KOAc, 0.05% Triton X-100, 1%DMSO). Heme was added to a total of 0.5 μM and incubated with the DNA or RNA for 30 minutes at room temperature to allow proper binding. ABTS was added to a total of 1.5 mM and the appearance of the radical cation was monitored at 414 nm for one minute using a UV-Vis spectrophotometer. Reactions were initiated by adding H_2O_2 to a total of 5 mM, inverting the cuvette, then reading.

Transformation of pBBR-1MCS-2-KAPF into EC484 UM255 ΔKatE/ΔkatG

The plasmid pBBR-1MCS-2 was digested with the restriction endonuclease EcoRV and heat inactivated using a thermocycler for accurate temperature control. The PCR product of KAPF as well as the positive controls PS2.M, and PS5.M, were all separately ligated to the linear pBBR-1MCS-2 using the TAKARA Ligation kit, as previously mentioned (see *Amplification of RNA Counterpart of Segment of Interest using T3 Polymerase)*. Overnight cultures of *E. coli DH5α* were diluted using Luria Bertani broth to an OD_{600} of 0.5, read using a UV-Vis spectrophotometer. These were diluted by half and allowed to incubate while shaking at 37 °C for 1 hour to get the OD_{600} back up to 0.5, ensuring they were in an exponential phase of growth. 1.5 ml of these competent cells were centrifuged for 1 minute and the supernatant discarded. The pellet was then suspended in ice cold 1M $CaCl_2$ (1 ml) and incubated on ice for 10 minutes. 10 μl of chilled ligated products were added to 250 μl cell suspension and heat shocked at 42 °C for

2 minutes. The recovering transformants incubated for an hour in 250 μl Luria Bertani broth and were plated on KIA plates consisting of 0.045 mg/ml Kanamycin, 2mM IPTG, and 0.08 mg/ml X-GAL for blue-white screening. White colonies were cultured overnight in Luria Bertani broth and plasmid minipreps were performed to obtain the plasmid with the appropriate insert using a Qiagen Plasmid Miniprep kit. The prepared plasmids pBBR1MCS-2-KAPF, pBBR1MCS-2-PS5.M, and pBBR1MCS-2-PS2.M were then transformed into EC484 UM255 ΔKatE/ΔkatG in the same way and were plated on LB agar with Tetracycline and Kanamycin.

Determining orientation of fragment in pBBR-1MCS-2 for RNA expression

The white colonies that formed on KIA plates after transforming into *E. coli DH5α* and the colonies formed on agar plates with tetracycline and kanamycin were confirmation that transformation of the plasmid with the insert occurred. However, these data do not suggest the directionality of the KAPF in pBBR1MCS-2. In order to determine if *in vivo* data is directly related to KAPF being transcribed by the cell, the orientation of the fragment needs to be verified. Two PCR reactions were ran using primers associated with the insert (KAPF-F and KAPF-R) and the plasmid (M13-F and M13-R). One reaction ran with 1 μl M13-F, 1 μl KAPF-R, 1 μl of a 1/50 dilution of pBBR-1MCS-2-KAPF, 10 μl 2X My Taq Master Mix, and 7 μl dH_2O. The other reaction only differed in the primers used: M13-R and KAPF-F. One product was predicted to form and suggest the orientation of the insert.

Comparing H_2O_2 Tolerance in ΔKatE/ΔkatG Mutant With and Without Segment of Interest

Overnight cultures of EC484 UM255 ΔKatE/ΔkatG, EC484-KAPF, EC484-PS2.M, and EC484-PS5.M were spread plated onto LB agar plates. Three discs were placed 1 cm apart in the form of a triangle with 8 μl of each respective solution pipetted onto a single disc: 50 μM heme, 1M IPTG, and 1% H_2O_2. Discs sizes and shapes were measured and reported.

Results

A truncated RNA product upstream of the bhuTUV operon is produced regardless of Fe concentration

According to RNAseq data obtained by Dr. Martin Roop's laboratory at East Carolina University Brody School of Medicine, an RNA product of KAPF was produced, regardless of iron concentration (see Figure 5). This may suggest that KAPF is being transcribed in all growth conditions tested, due to *Brucella's* constant need for it. Also, the amount of iron was monitored when collecting this data, but heme was not. This introduces the possibility of it being regulated by heme, not iron. The extreme oxidative stress *Brucella* experiences when residing the host macrophage cells, shines light on the necessity for the constitutive expression of KAPF seen in all conditions tested. This would allow KAPF to act as a heme-dependent apoenzyme with peroxidase activity in any growth conditions.

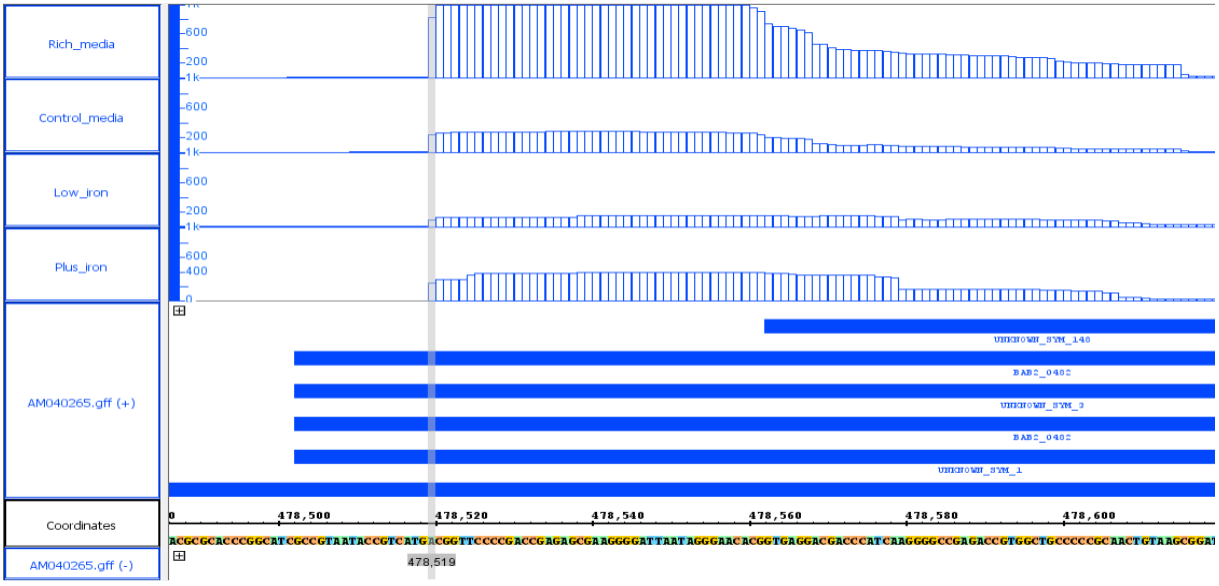


Figure 5. RNAseq data of the *bhuTUV* operon and upstream regions. This shows that KAPF RNA is produced upstream of the *bhuTUV* operon.

KAPF to heme in vitro

The KAPF in the form of single stranded DNA (0.1 μM) exhibited a Soret band at 400 nm significantly higher than heme alone ($p \leq 0.01$) (see Figure 6). The positive controls PS2.M and PS5.M, which have been found to bind Fe(III) heme by folding to a guanine quadraplex and functioning as an apoenzyme for the heme cofactor (Travascio et al., 1999, Travascio et al., 1998, Poon et al., 2011), bound heme less effectively than KAPF. The average absorbances at 400 nm for ssKAPF and the positive controls PS2.M and PS5.M were 0.108, 0.045 and 0.050 respectively. When comparing these absorbances to that of heme alone (0.035), KAPF bound heme significantly more than the positive controls with previously determined heme-binding abilities ($p \leq 0.05$). This was deduced from the differences in the Soret bands, representing the proportional differences in their ability to bind heme. The smaller segments of interest (KAP1-KAP4), as well as KAPF's RNA counterpart, did not show any increase in the sorbet band at 400 nm when complexed with heme, and exhibited binding comparable to the negative control CALC2. This suggests they do not possess heme-binding abilities *in vitro.* The overall conclusions regarding the fact that the full-length segment KAPF, binds heme significantly more than that of the positive controls (PS2.M and PS5.M), are that this interaction must be important to *Brucella.* This leads to the possibility that the entirety of the segment is necessary for G-quadruplex formation, considering the G-rich regions within.

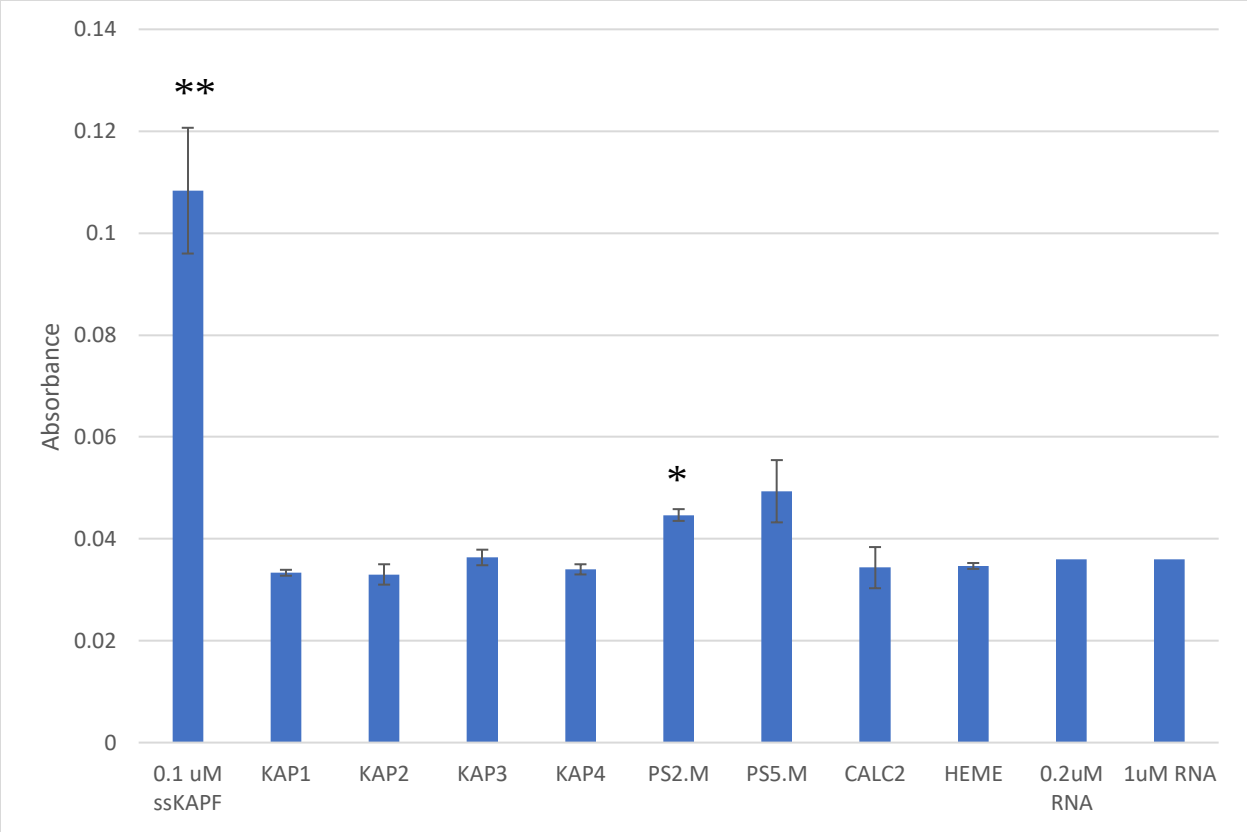


Figure 6. Absorbance of oligonucleotides complexed with heme. This chart represents the differences in the Soret band at 400 nm of each sequence complexed with heme compared to heme alone. All reactions consisted of a heme concentration of 0.5 μM. KAPF as RNA, KAP1, KAP2, KAP3, KAP4, positive controls PS2.M and PS5.M, negative control CALC2 were all 1 μM. The concentration of ssKAPF was 0.1 μM. A significant increase in the Soret band at 400nm occurred for KAPF ($p \leq 0.01$) when compared to heme alone. Also, KAPF bound heme significantly more than the positive controls ($p \leq 0.05$).

The Segments of Interest Do Not Exhibit Peroxidase Activity

Neither DNA nor RNA versions of KAPF showed peroxidase activity *in vitro.* There were no increases in the absorbances at 414 nm observed for KAPF, or the smaller potential G-quadruplex forming segments KAP1-KAP4, once complexed with heme and reacted with ABTS (see Figure 7). The kinetics of these samples resembled that of the negative control CALC2, suggesting the lack of peroxidase activity. However, there was a strong increase in absorbance at

414 nm over time when testing the positive controls PS2.M, PS5.M complexed with heme, as well as for HRP alone. This suggests that these segments do possess peroxidase activity *in vitro* once complexed with heme. However, it cannot be deduced from this assay that peroxidase activity is carried out by genetic elements *in vivo.*

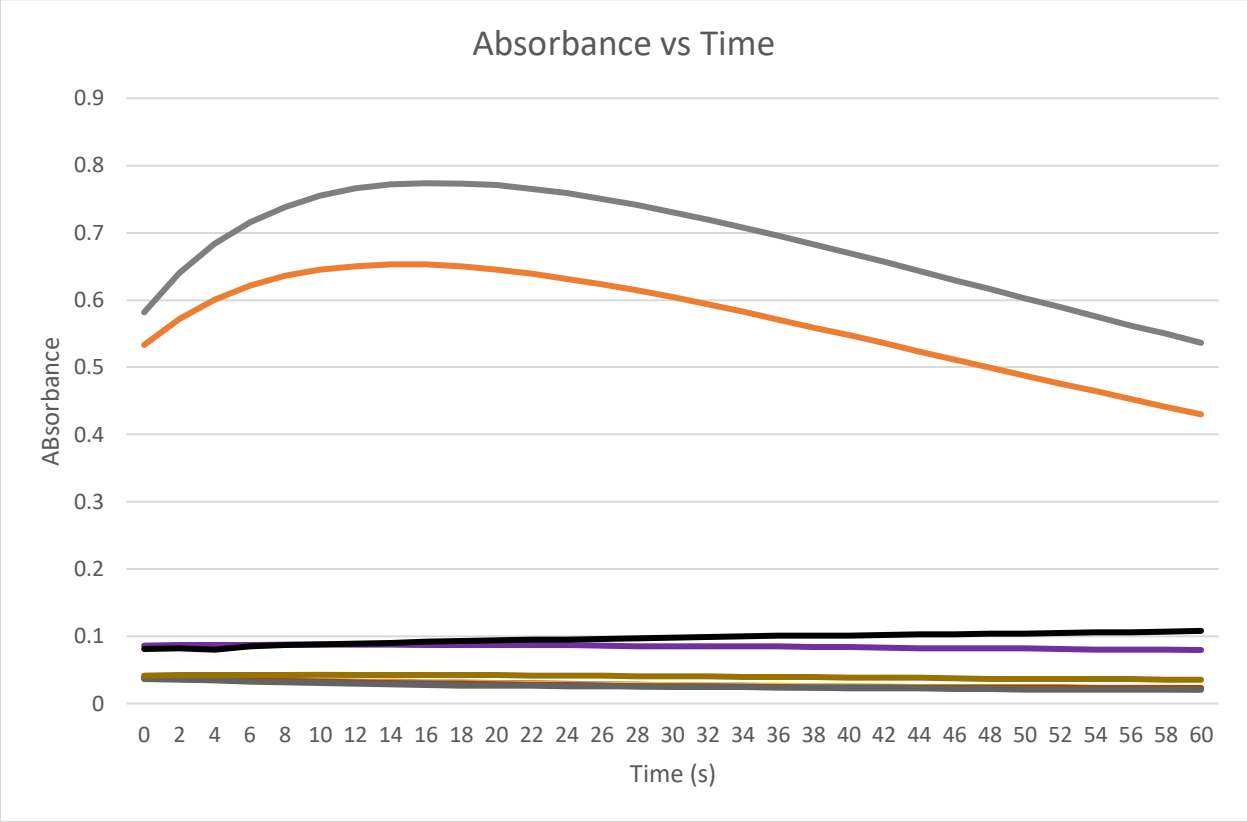


Figure 7. Peroxidase activity of multiple segments of interest. Positive controls PS2.M (orange), PS5.M (grey) and horse radish peroxidase (black); KAPF as RNA (gold) and single stranded DNA (purple); heme (light blue); and negative control CALC2 (yellow). All reactions consisted of 1.5 mM ABTS, 0.5 µM heme, 0.4 µM DNA or RNA complexed with heme, with 0.1 µM ssKAPF.

KAPF does not restore peroxide resistance in EC484 UM255 ΔKatE/ΔkatG

The *E. coli* strain EC484 UM255 ΔKatE/ΔkatG transformed with KAPF (EC484-KAPF) showed no decrease in the zone of inhibition in the disc assays (see Figure 8). The zones of inhibition (ZOI) for EC484-KAPF and EC484 had the same diameter (28.33 mm), showing no

differences or signs of decrease in H_2O_2 tolerance in the transformant. However, a significant decrease in the ZOI diameters occurred for both cultures transformed with the positive controls (EC484-PS2.M and EC484-PS5.M), measuring at 22 mm and 21 mm respectively (both $p \leq 0.05$). This indicates a significant decrease in the ZOI for these strains when compared to that of EC484, suggesting these segments both possess peroxidase activity, as well as the ability to restore peroxide tolerance *in vivo.* This contributes to the knowledge gap surrounding the potential for enzymatic genetic elements to occur in nature.

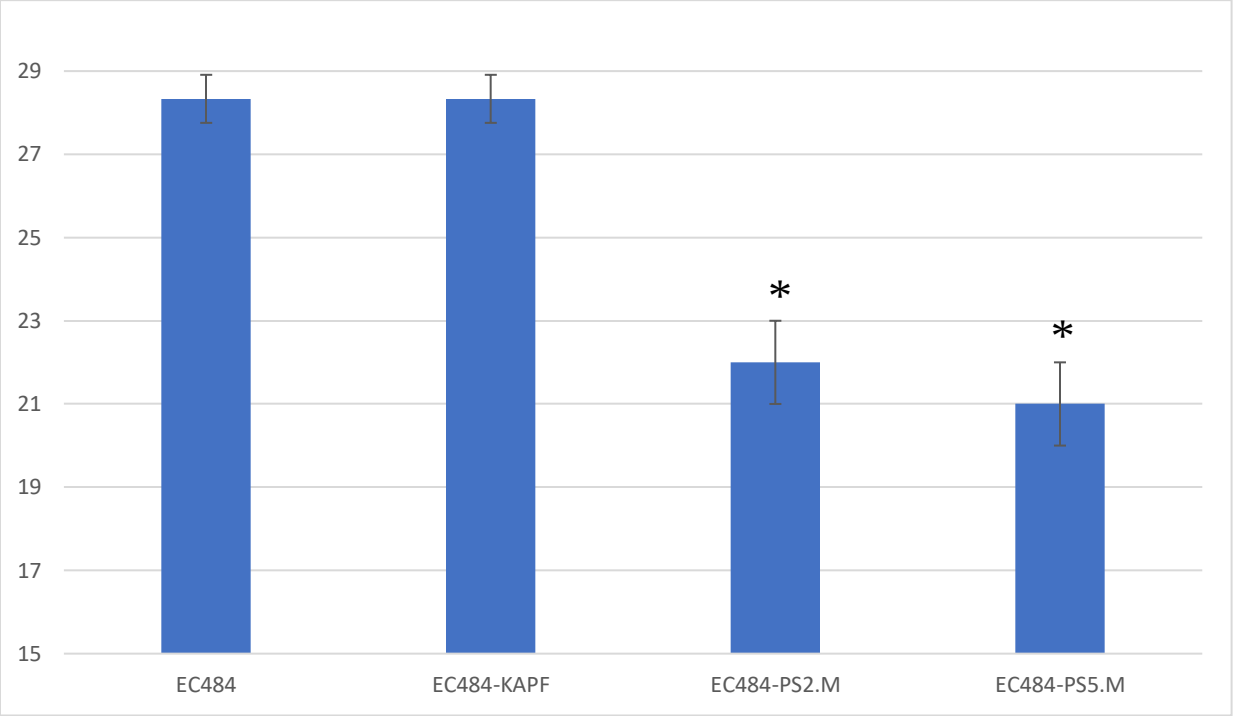


Figure 8. Zones of inhibition measurements in mm. ZOI's for EC484 UM255 ΔKatE/ΔkatG (EC484), EC484-KAPF, EC484-PS2.M, and EC484-PS5.M grown on LB agar with 8 µl 1% H_2O_2, 8 µl 50 µM Heme, and 8 µl 1M IPTG on three paper discs oriented in a triangle, 1 cm apart. There was a significant decrease in the ZOIs between the positive controls and the mutant strain (both $p \leq 0.05$).

Orientation of the insert allowed for correct RNA amplification

The purpose of verifying the orientation of the insert was to ensure validity in the RNA binding and *in vivo* peroxidase correlations made. There was one product produced between the two PCR reactions ran using the primers M13-F/KAPF-R and M13-R/KAPF-F, suggesting the insert was indeed ligated to the plasmid prior to transformation (see Figure 9). The PCR product observed was ran with M13-F and KAPF-R primers, suggesting the segment was oriented in the direction that allowed for the correct RNA sequence to be obtained during transcription by T3 polymerase. This also suggested that the *in vivo* transcription of this product was indeed the same product produced by *B. abortus* observed in the RNAseq data (see Figure 5).

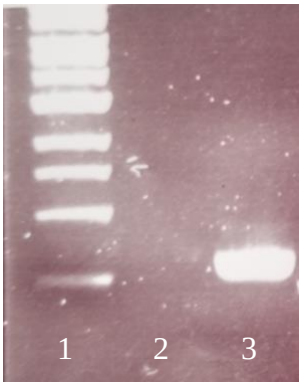


Figure 9. Gel electrophoresis reading of PCR product confirming proper orientation. This figure represents confirmation that KAPF was successfully ligated to the plasmid pBBR-1MCS-2 in the proper orientation for induction of RNA using the T3 polymerase as indicated by the absence of a product in well 2 resulting from primers KAPF-F, M13-R and a visible adjacent product resulting from the primers M13-F and KAPF-R in well 3.

Chapter 6: Discussion

This study investigated the potential for the segment upstream of the *bhuTUV* operon, KAPF, to bind heme and degrade peroxide *in vivo*. KAPF bound heme significantly more than the positive controls PS2.M and PS5.M known to have heme-binding abilities (Travascio et al., 1998 and Travascio et al., 1999) (see Figure 6). In our hands, these positive controls bound heme at a level consistent with these previous studies. Notably, for KAPF, we were not able to reach

the amount of ssDNA needed to at the higher concentrations used for the controls (1 μM), so we were limited to carrying out 0.1 μM reactions in triplicate. However, the results demonstrate that the full length KAPF bound heme twice as effectively as the positive controls, even though KAPF was ten times less concentrated in these assays. The smaller, G-rich sequences KAP1, KAP2, KAP3, KAP4 were designed to act in the same way as PS2.M and PS5.M by encompassing guanine quartets capable of forming G-quadruplexes, but these did not exhibit any heme-binding abilities considering the lack of an increase in the Soret band at 400 nm compared to heme alone (see Figure 6). This suggests that the full-length product is necessary for *Brucella*, which is further supported by the RNAseq data (see Figure 5).

There is strong evidence that both DNA and RNA can fold to create cofactor-binding sites with strong peroxidative activity capable of binding heme at the submicromolar level (Kong et al., 2010, Travascio et al., 1998, Travascio et al., 1999, Poon et al., 2011). However, neither the full segment of interest, nor the small G-rich sequences, KAP1, KAP2, KAP3, and KAP4, exhibited peroxidase activity *in vitro* or *in vivo.* There was no increase at 414 nm over time when the samples were complexed with heme and reacted with ABTS and H_2O_2. Thus, no enzymatic activity was observed. The positive controls PS2.M and PS5.M exhibited very strong peroxidase activity, showing a rapid increase in the radical cation $ABTS^+$, followed by a steady decrease within one minute (see Figure 7). This suggests that the oligonucleotides execute peroxidase activity very rapidly *in vitro*. However, this data alone does not lead to the assumption that either DNA or RNA bind heme with peroxidase activity *in vivo* in either eukaryotic or prokaryotic cells.

The sole catalase produced by *B. abortus* 2308 plays a minimal role in detoxifying endogenous H_2O_2 (Steele et al., 2010). However, this phenomenon is not observed in other

bacteria, where catalases play a compensatory role in detoxifying cytoplasmic H_2O_2 (Cosgrove et al., 2007). This study determined if transforming KAPF into an *E. coli* strain lacking catalase activity would restore its peroxide tolerance. There was no decrease in the zones of inhibition caused by the addition of 1% H_2O_2 in the EC484 UM255 ΔKatE/ΔkatG strain transformed with KAPF when compared the culture lacking the insert (EC484). This suggests that there was no return in function of catalase/peroxidase activity once the plasmid with the insert was taken up. Thus, the segment of interest does not possess catalytic abilities *in vivo* or *in vitro.* Interestingly, EC484-PS2.M, and EC484-PS5.M showed significantly smaller zones of inhibition than EC484. This suggests that there was indeed restoration of peroxide tolerance once EC484 ΔKatE/ΔkatG had taken up either of the positive controls, PS2.M or PS5.M. Interestingly, the peroxidase activity of PS2.M and PS5.M has only been observed *in vitro.* Thus, these data introduce the potential for strains possessing sequences such as these in nature to possess peroxide resistance by forming G-quadruplexes and represent the first demonstration of *in vivo* peroxidase activity for a G-quadruplex.

Part II: Investigating heme-dependent riboswitch activity upstream of the *bhuTUV Operon*

Review of Literature

Riboswitches

While the upstream segment in the *bhuTUV* operon (KAPF) does not appear to have peroxidase activity, an alternative hypothesis is brought to light regarding what exactly it is responsible for. There is a broad range of the catalytic possibilities of both RNA and DNA (Poon et al., 2011). In the idea of an "RNA world", it is possible that cells that incorporated RNAs capable of self-replication preceded protein and nucleic acid-based organisms (White, H.B. 1976). This hypothesis has caused an increase in studying catalytic activities for nucleic acids. The primordial ribozymes may have used cofactors such as heme, a ubiquitous metabolic cofactor, to compensate for the poor functionality compared to current proteins (Poon et al., 2011). RNA regulators have been widely used in molecular engineering due to the cost effectiveness. A large variety of small RNAs have been characterized as belonging to two main classes according to their mechanism of action: small noncoding RNA (sRNA) and riboswitches. (Khang et al., 2014).

Riboswitches reside in the 5' untranslated region (UTR) of bacterial mRNA (Carbino, K.A. 2005). The two functional domains of riboswitches are the aptamer and the expression platform with an overlapping region called the switching sequence (Garst A.D. 2011). The expression platform responds to the distinctive, ligand-induced secondary and tertiary folding at the aptamer region and toggles between two different secondary structures to activate or terminate downstream gene transcription in response to the changing structure of the aptamer (Abduljalil J.M. 2018). This acts as a regulatory response by interfering with downstream

transcription and translation processes. Considering their target specificity, riboswitches have been useful tools for controlled regulation of gene expression and in synthetic molecular biology (Kent and Dixon, 2019).

Riboswitches are clustered into 17 classes based on similar global architecture or by similar binding site architecture (Breaker R.R. 2012). Riboswitches that respond to thiamin pyrophosphate (TPP) are the most widespread class of riboswitches discovered (Mironov et al., 2002, Barrick and Breaker, 2007). Many riboswitch mechanisms have been reported such as transcription activation, translation initiation inhibition, ribozyme-like cleavage, with transcriptional termination as the most common (Serganov A., Patal D.J. 2012). For some bacteria, the aptamer upstream of a potential intrinsic transcription terminator stem binds a metabolite to control both transcription termination and translation initiation, only if there are any mRNAs transcription products that overlap the ORF. Before RNA polymerase passes the 3' boundary of the riboswitch during transcription, the riboswitch adopts the final and stable conformation of ligand-bound status to properly arrest the transcriptional machinery. To accomplish this, the tertiary structure plays a key role in pre-organizing the binding pocket prior to ligand binding (Abduljalil J.M. 2018).

A variety of riboswitch structural configurations like helices, loops, and bulges are driven by structural motifs such as tetraloops, kink-turns, kissing-loops, sarcin loops, T-loops, and pseudoknots (Hendrix et al., 2005). The high specificity of these is based on the ligand interactions with the nucleobases of the binding pocket. This allows for an extreme level of discrimination power against closely related compounds with ligand dissociation constants (K_D) values ranging from micromolar to nanomolar, detecting one compound per cell (Abduljalil J.M. 2018).

Cobalamin-dependent riboswitches (B12 riboswitch) are found to regulate many operons such as *nrdABS,* which is responsible for producing a cobalamin-dependent ribonucleotide reductase (RNR), as well those responsible for vitamin B12 biosynthesis and transport (*cob* and *btuB* respectively) in *E. coli* (Fang et al., 2017, Borovok et al., 2006). RNRs are the only known *de novo* pathway for deoxyribonucleotide biosynthesis and are highly regulated via B12 genetic control elements that fold and control downstream transcription upon binding B12 and removing the B12 box that blocks ribosome access (see Figure 10). These enzymes are classified as either class Ia, the oxygen dependent RNR in eukaryotes and prokaryotes, class Ib, which are confided to bacteria, class II, the oxygen-independent type which are dependent on binding coenzyme B12, and class III which are present in anaerobic bacteria (Borovok et al., 2006). All three initiate the reduction of ribonucleotides.

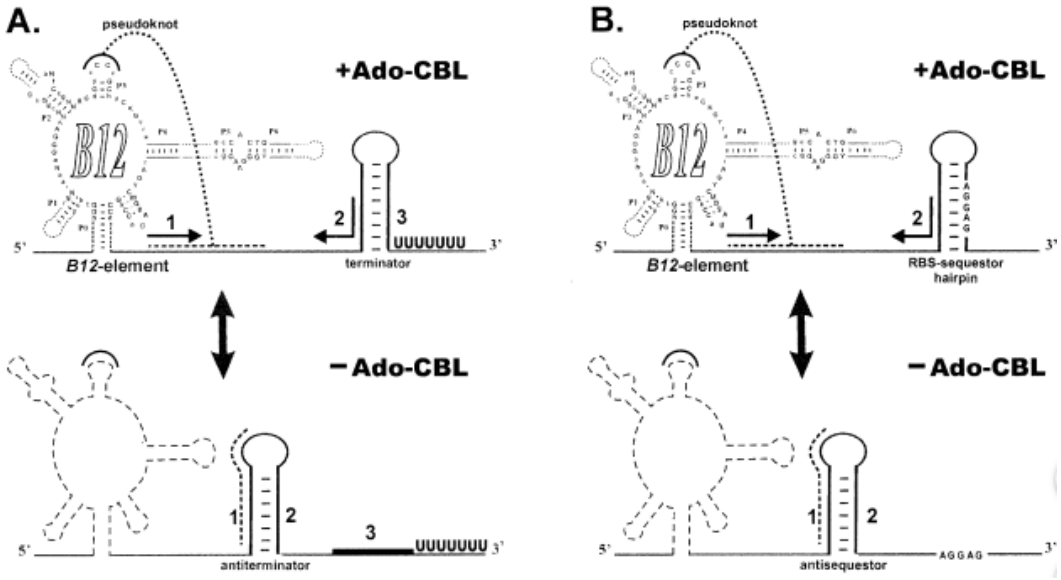


Figure 10. Mechanism of B12-mediated regulation of CBL genes. Inhibition of transcription (A) and translation initiation (B).

Many bacteria posess more than one kind of RNR. In bacteria that possess both B12-dependent and B12-independent isozymes, the B12-independent enzymes are still regulated by B12 riboswitches (Vitreschak et al., 2003). Interestingly, an RNR in *E. coli* was found to be an

ROS resistant variant and its function is thought to help protect from ROS produced by the host (Taga and Walker, 2010). Considering *Brucella* resides in the host macrophage cells, it is possible that cobalamin binds and regulates its multiple RNRs for a similar function.

Heme Transport in Brucella

Heme is a biologically relevant source of iron for *Brucella* during infection (Roop, 2011). Thus, heme-containing proteins represent an important source of iron for *B. abortus.* Transporting compounds bound to heme into Gram-negative organisms involves transporting the compound into the periplasmic space prior to the translocation into the inner membrane space (Noinaj N. 2010). Gated outer membrane porins, known as TonB-dependent transporters (TBDTs), shuttle these large compounds across the outer membrane. The TonB-dependent outer membrane heme transporter, BhuA, has been shown to be of importance to *B. abortus* by shuttling heme across the outer membrane (Paulley et al., 2007). TBDTs transport siderophores, heme, vitamin B(12), nickel complexes and carbohydrates using a proton motive force and a system of intermembrane proteins consisting of TonB, and the cytoplasmic membrane proteins ExbB, and ExbD (Postle K., 2007, González-Carreró, 2002). TonB directly contacts and transduces energy to the outer membrane transporter (BhuA) at the outer membrane and releases the bound ligand (heme) into the periplasmic space, where its shuttled by binding proteins to the ABC transporter bhuTUV residing in the cytoplasmic membrane (Postle K., 2007). The protein Brucella heme utilization oxygenase Q (BhuQ) is then responsible for oxygenating heme to release iron once brought into the cytoplasm (Ojeda, 2012). BhuQ is homologous to heme oxygenases in *Bradyrhizobium japonicum, HmuD and HmuQ,* and has heme oxygenase activity

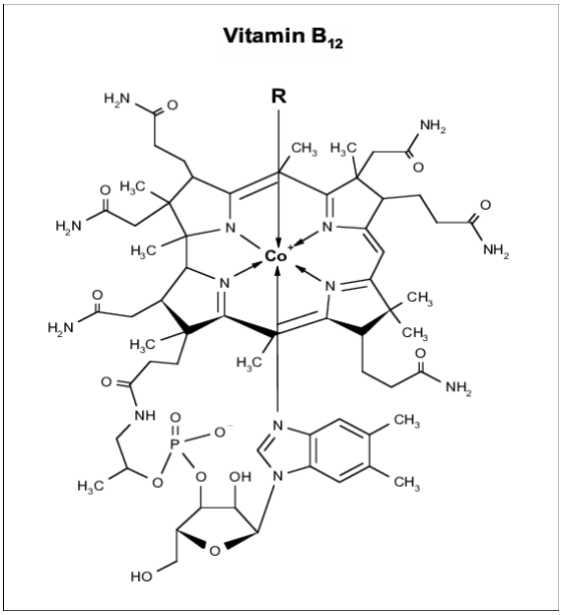
in vitro. Also, a recombinant *HmuQ* homolog from *Brucella melitensis* degraded heme in a dose dependent manner using NADPH-cytochrome P450 reductase and ascorbate as electron donors (Puri, 2006).

The locus that produces the bhuTUV gene products in the *Brucella* has high homology to the cytoplasmic heme uptake machinery in *Shigella shuTUV.* The ShuT periplasmic binding protein is necessary for heme binding and its Tyr-94 correlates to the Tyr-89 of BhuT in *Brucella* (Eakanunkul, 2005, Ojeda, 2012). BhuU contains two histidine residues thought to act in a similar fashion as the two histidines in ShuU that are required to bind ShuT and release heme. Also, there is a conserved ATP-binding subunit found in BhuV, matching ShuV. Together, BhuT, BhuU, and BhuV make up the TBDT needed to shuttle heme from the periplasm to the cytoplasm. An isogenic *bhuTUV* mutant lost its ability to use heme as an iron source in iron limited conditions, suggesting this operon works in conjunction with the outer membrane heme transporter BhuA to bring heme into *B. abortus* (Ojeda, 2012). The expression of these types of transport proteins is regulated in many ways such as metal-dependent regulators, σ/anti-σ factor systems, small RNAs, and even by riboswitches (Noinaj N. 2010). There does not seem to be a role cobalamin could play in the regulation of the *bhuTUV* operon, considering its aforementioned, potential ROS-resistant functions in regulating RNRs.

Heme vs Cobalamin

The structures of heme and cobalamin are quite similar when taking into consideration their protoporphyrin rings (see Figure 11). Their structures only vary in their central element and

side groups. Also, the folding potential of KAPF is strikingly similar to that of the cobalamin-dependent riboswitch controlling *nrdABS* (see Figure 12). The only variation between these structures are the size of the terminal stem loops, while the central ring structure is conserved. This led to the hypothesis that KAPF binds heme for the purpose of acting as a riboswitch to terminate the transcription of the downstream operon *bhuTUV*.



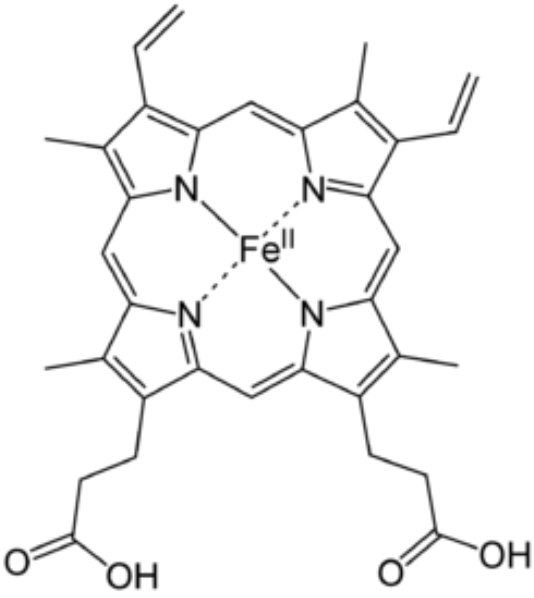


Figure 11. Structural comparison of cobalamin and heme. These two compounds are similar in structure, both having a protoporphyrin ring with varying central elements. This suggests the feasibility of a riboswitch reacting to heme instead of cobalamin, to control the heme-uptake operon *bhuTUV*.

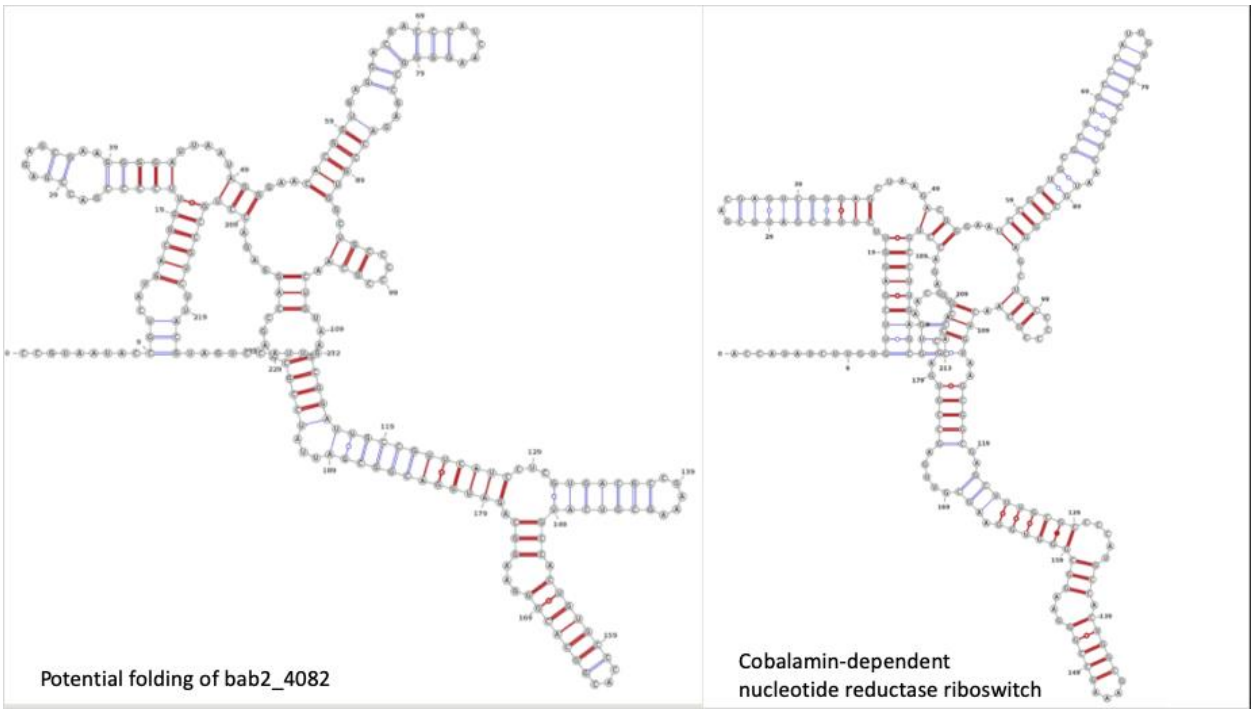


Figure 12. Secondary structures of riboswitches. The potential folding of the segment of interest (left) and a cobalamin-dependent nucleotide reductase riboswitch (right). The similarity in structures of these two RNA segments raises the question of whether they react to ligand binding similarly.

Experimental evidence indicates that KAPF has strong heme binding characteristics (see Figure 6). Interestingly, RNAseq data obtained from Dr. Martin Roop II's lab at East Carolina University Brody School of Medicine also shows an RNA product of KAPF is produced, regardless of iron concentration (see Figure 5). This further contributes to the question regarding whether or not KAPF a riboswitch-acting sRNA that is responds to heme binding to terminating the downstream operon *bhuTUV*.

If *B. abortus* does indeed possess a heme-dependent riboswitch, it would exhibit heme binding significantly stronger than that of other segments known to bind heme. This study found that when comparing the absorbances KAPF complexed with heme to that of PS2.M and PS5.M-heme complexes, a significant increase in the Soret band at 400 nm was observed, indicating strong binding. Also, if *B. abortus* does indeed possess a heme-dependent riboswitch, it should

terminate the transcription of downstream products in a riboswitch reporter system. Current work is being done to investigate the ability for this segment to terminate the transcription of the downstream *LacZ* in the plasmid pMR15.

Research Objectives

Riboswitch Reporter System in E. coli

The goal of this study was to identify a riboswitch in *B. abortus* using a riboswitch reporter system. If KAPF binds heme to act as a riboswitch that turns off downstream genes, then this will be observed *in vivo.* Attaching the lac promoter to KAPF, ligating that to pMR15, then transforming that in *E. coli,* should cause *LacZ* to be turned off in the presence of heme, and stay on in the absence of heme.

KAPF possesses heme-dependent riboswitch activity

If KAPF is a riboswitch for *B. abortus,* then it will exhibit termination of downstream genes *in vivo.* It is predicted that KAPF will cause the termination of the lac operon that lies downstream once ligated to pMR15. The expression platform of riboswitches responds to the distinctive, ligand-induced secondary and tertiary folding at the aptamer region and toggles between two different secondary structures to activate or terminate downstream gene transcription in response to the changing structure of the aptamer (Abduljalil J.M. 2018). This is expected to be observed *in vivo* via transformation and blue-white screening.

Methods

Riboswitch Reporter System Design

All primers were ordered from Integraded DNA Technologies Inc. The primers KAPF-R and Lac promo-F were designed to target the lac promoter in the plasmid pBBR1MCS-2-KAPF, as well as the 637 bp segment of interest consisting of the potential riboswitch plus the hypothetical protein downstream (KAPF). The plasmid pMR15 (17 μl) was linearized with the restriction endonuclease EcoR1(1μl) and 10x fast digest buffer (2 μl). This cut plasmid (5 μl) was then ligated to the PCR product consisting of the promoter plus the segment of interest (5 μl) using the TAKARA ligation mix (10 μl). This ligation product was transformed into *E. coli DH5α* and spread plated on KIA and KIA + 0.5 μM heme agar plates to observe blue-white screening. Also, a disc assay was used to observe the competition between cobalamin and heme for this riboswitch activity by placing 8 μl of 32 μM cobalamin onto discs with lawn growth of the transformants on KIA to also observe blue white screening.

Results

lacZ was not expressed in E. coli transformants possessing KAPF in the presence of heme

Cultures possessing the plasmid pMR15 grown on KIA exhibited white colonies prior to being transformed with the lac promoter and KAPF (data not shown). This ensured that the strain did not originally possess the ability to produce β-galactosidase. Transformants exhibited blue colonies when grown on KIA agar plates, suggesting the PCR product of KAPF with the lac promoter attached was successfully ligated into pMR15. Interestingly, transformants grown on KIA plates supplemented with 0.5 μM heme resulted in white colonies (see Figure 13). This suggested that no β-galactosidase was produced in the presence of heme. Thus, premature

termination of *lacZ* occurred upon the addition of heme to cultures that had been transformed with KAPF. This not only validates the reporter system, but potentially identifies a novel heme riboswitch.

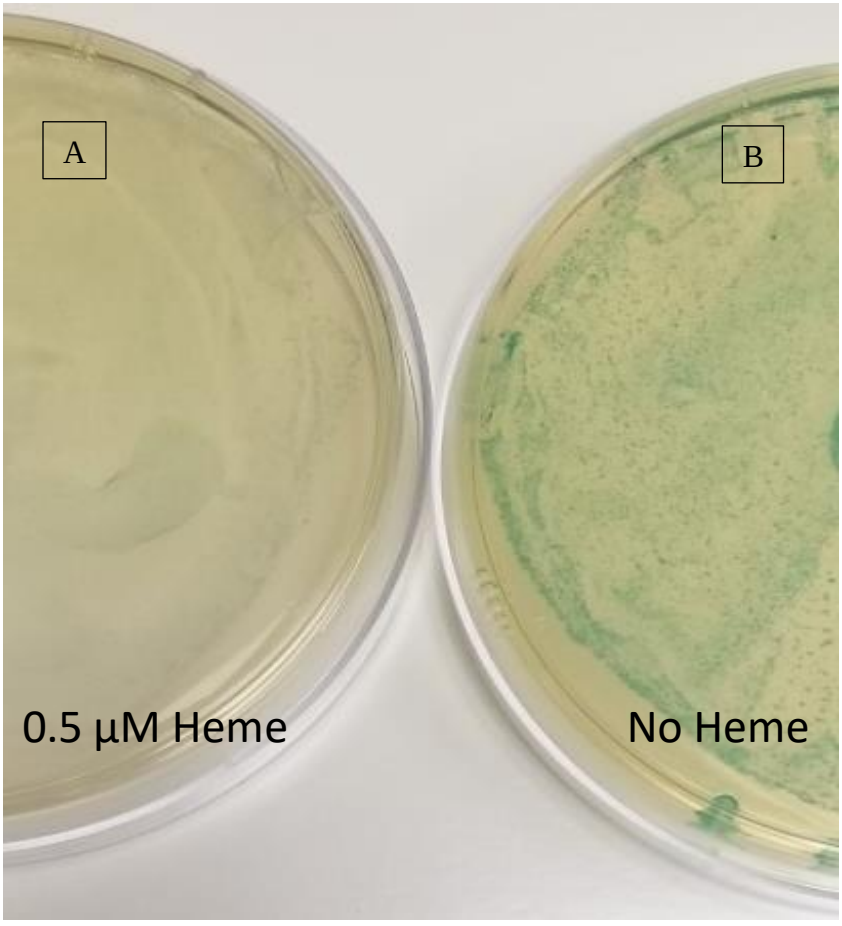


Figure 13. Blue-white screening of the heme riboswitch reporter system. The addition of heme to the KIA agar plates (A) resulted in white colonies, whereas blue colonies were formed in the absence of heme (B). This suggested that *lacZ* was prematurely terminated, in the presence of heme, due to the folding of an upstream, heme-responsive riboswitch.

lacZ was not expressed in E. coli transformants possessing KAPF in the presence of cobalamin

Transformants grown on KIA plates with discs consisting of 32 µM cobalamin resulted in white colonies (see Figure 14). This suggests that no β-galactosidase was produced under these conditions. Thus, premature termination of *lacZ* occurred upon the addition of cobalamin to

cultures that had been transformed with KAPF. This suggests that not only does KAPF respond to heme by terminating downstream genes, but to cobalamin as well.

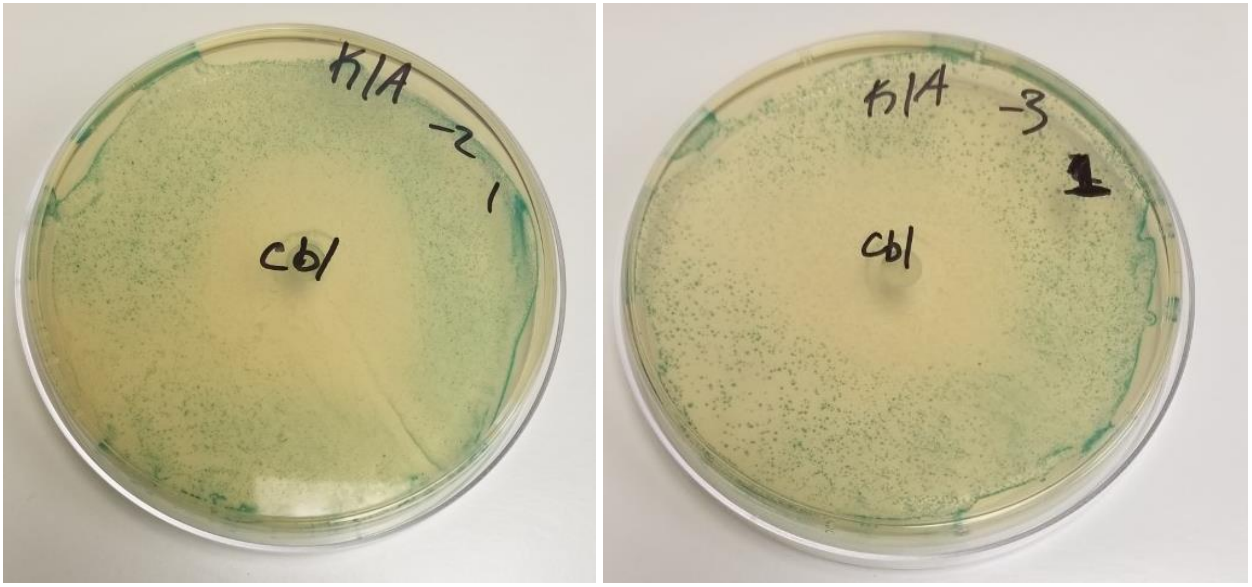


Figure 14. Blue white screening of the cobalamin riboswitch reporter system. The addition of cobalamin to the *E. coli-KAPF* transformants resulted in white colonies, whereas blue colonies were formed in the absence of cobalamin. This suggested that *lacZ* was prematurely terminated, in the presence of heme, due to the folding of an upstream, heme-responsive riboswitch.

Discussion

It was found that KAPF significantly bound to heme with high affinity *in vitro* when compared to the positive controls PS5.M and PS2.M ($p \leq 0.01$) (see Figure 6). However, it was also concluded that the segment of interest does not possess heme-dependent peroxidase activity. This introduces a knowledge gap regarding why heme to binds the segment of interest. Furthermore, this introduces an alternative hypothesis regarding the segment of interest acting as a novel heme-responsive riboswitch responsible for the termination of the downstream operon

bhuTUV via secondary structure formation in response to binding to its corresponding ligand: heme.

Data from the RNAseq analysis of B. abortus 2308 (figure 5) shows that the expression of an RNA product for the upstream KAPF segment is formed under both high and low iron conditions. RNAseq data has identified a plethora of *cis* and *trans*-acting small regulatory RNAs (sRNA) within the past two decades (Tomason and Storz, 2010). This suggests the possibility that KAPF acts a riboswitch, leading to premature termination of the full-length heme transport operon in response to a ligand, heme, that was not monitored when obtaining the RNAseq data. To further investigate this potential riboswitch activity, a riboswitch reporter system has been constructed using β-galactosidase activity as an output.

Standard blue/white screening was used to observe if the putative riboswitch segment has the ability to turn off *lacZ* expression in the presence of heme. The gene *lacZ* is responsible for the production of β-galactosidase, which cleaves off the indole in the galactose sugar X-GAL, causing a blue pigment to be produced in colonies when grown on X-gal supplemented agar. Placing the KAPF segment between the lac promoter and the *lacZ* gene in the plasmid pMR15 resulted in blue colonies when heme was not present, and white colonies in the presence of heme (see Figure 13). This suggests that premature termination of *lacZ* occurred when heme was present due to potential hairpin loop formation in the pMR15-KAPF.

This reporter system also tested the potential of KAPF responding to cobalamin. A disc assay was performed on *E. coli-KAPF* transformants using 32 μM cobalamin. The addition of cobalamin to these transformants resulted in a gradient of white colonies surround the disc. This suggested that KAPF not only responds to heme, but cobalamin as well. This may be due to how similar heme and cobalamin are in structure (see Figure 11). These data provide evidence

supporting the possibility that KAPF binds either heme or cobalamin to cause premature termination of the operon *bhuTUV* in *Brucella* in response to the change in the shape of the transcript. This work not only potentially identifies a novel heme riboswitch, or a cobalamin riboswitch upstream of *bhuTUV* in *Brucella.* This also potentially fills the knowledge gap surrounding the regulation of *bhuTUV*. Filling in any knowledge gaps associated with *Brucella* may alleviate concerns regarding the impact of brucellosis.

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