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Small RNA MgrR Regulates Sensitivity of *Escherichia fergusonii* to Oxidative Stress

by

Austin Paul Wright

A thesis submitted in partial fulfillment of the
requirements for the degree of

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in
Biology

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ABSTRACT

Non-coding small RNAs (sRNAs) are integral to post-transcriptional gene regulation in bacteria. The function of an sRNA is dependent on both secondary structure and the sequence of its unstructured seed region. The sRNA seed region typically base-pairs with target mRNAs to down-regulate the expression of target genes by blocking the ribosome-binding site or by promoting RNase-mediated degradation of the sRNA-mRNA complex. sRNAs have also been shown to increase expression of target genes by releasing RNA secondary structures that block ribosome-binding sites. Selective pressure to maintain sRNA function conserves the sequence of the sRNA seed region, but mutations in mRNA sequences to match sRNA seed regions lead to the accumulation of new targets by an sRNA. In this study I identified a unique scenario where a 53-nucleotide insertion event occurred in the seed region of the sRNA MgrR in *Escherichia fergusonii*. This PhoP/PhoQ-regulated sRNA is conserved in most enteric bacteria and is known to increase bacterial resistance to the antimicrobial peptide polymyxin B by controlling the expression of the phosphoethanolamine transferase gene *eptB*. My analyses show that MgrR does not regulate the expression of *eptB* in *E. fergusonii*, as observed in *E. coli*. Instead, MgrR likely regulates the glycerol utilization pathway *glpABCFKTQ-frdABCD*, and the pentose phosphate sugar pathway *ulaAB-tktC* that produces NADPH. Cell sensitivity to oxidative stress is affected by the production of NADPH, and an *mgrR*-deletion strain of *E. fergusonii* was highly sensitive to hydrogen peroxide (H₂O₂) and deoxycholic acid *in vitro* and displayed a severe loss of fitness within murine gut. The *ulaAB-tktC* pathway is not present in *E. coli* MG1655 and deletion of MgrR did not cause

any sensitivity to H_2O_2 . This work demonstrates that sRNAs could evolve divergent functions in closely related bacteria.

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INTRODUCTION

Bacterial small RNAs

Small non-coding RNAs (sRNAs) regulate gene expression in bacteria by modulating transcription, translation or mRNA stability (Waters & Storz 2009). Typically, 50-200 nucleotides in length, sRNAs provide organisms a rapid and energetically efficient regulatory mechanism to respond to environmental stress. The first sRNA, which was reported in 1981, was a 104-nucleotide RNA that blocked the replication of the ColEI plasmid (Stougaard et al. 1981). The first chromosomal sRNA, MicF, was reported in 1984 to inhibit translation of the outer membrane porin OmpF in *Escherichia coli* (Mizuno et al. 1984). Extensive research, and advancement in sequencing technologies that allow deep interrogation of entire transcriptomes, have resulted in the discovery of hundreds of sRNAs in most bacteria (Klein & Raina 2015; Raghavan et al. 2015).

There are two types of sRNAs: *cis*-encoded and *trans*-encoded. *Cis*-encoded (also called antisense) sRNAs are coded in the same region of the target RNA but on the antisense strand (Waters & Storz 2009). The target mRNA are transcribed in the opposite direction of the *cis*-encoded RNA and can have extensive regions of complete complementary (Han et al. 2010). Almost all chromosomal *cis*-encoded sRNAs inhibit toxic proteins (Han et al. 2010). For example five Sib antitoxin sRNAs (i.e. SibA, SibB, SibC, SibD, and SibE) are coded on the opposite strand of five different toxic Ibs proteins (Han et al. 2010). The Sib sRNAs down regulate the Ibs proteins which are toxic when overexpressed (Han et al. 2010). Genes for *trans*-encoded sRNAs are typically not located close to their target genes on the genome, and the sRNA-mRNA interaction

region is only partially complementary (Waters & Storz 2009). Most *trans*-encoded sRNAs down-regulate the expression of the target RNA by enhancing RNase activity or inhibiting the ribosome from binding to the mRNA (Waters & Storz 2009). However, trans-encoded sRNAs can also up-regulate the expression of target genes by removing inhibitory mRNA secondary structures (Hammer & Bassler 2007). Many sRNAs require the help of the “chaperone” protein Hfq, which guides and stabilizes the sRNA-mRNA duplex (Vogel & Luisi 2011).

The region on the sRNA that binds to the target mRNA is called the seed region. A recent study showed that the seed regions of sRNAs were older than the corresponding binding sites on most mRNAs, suggesting that once an sRNA emerges and the first sRNA-mRNA interaction is established, selective pressure conserves the sRNA sequence (Peer & Margalit 2014). As the bacterium evolves, additional genes begin to evolve binding sites that interact with the sRNA (Peer & Margalit 2014), resulting in each sRNA being able to regulate the expression of a large number of genes. Hence, mutations in the seed region of an sRNA have the potential to greatly impact the physiology of a bacterium (Fröhlich & Gottesman 2018). What happens when an established sRNA sequence is dramatically changed? In this study I investigated the consequences of a relatively large insertion in the seed region of the sRNA MgrR in the enteric bacterium *Escherichia fergusonii*.

MgrR

In *E.coli*, MgrR is an sRNA that is 93 bp in length and contains a highly conserved region (**Figure 1**) that most likely base-pairs with the target mRNA (Moon & Gottesman

2009). MgrR negatively regulates the expression of the phosphoethanolamine transferase EptB (**Figure 2**) (Acuña et al. 2016; Moon et al. 2013; Moon & Gottesman 2009). Deletion mutants of *mgrR* are resistant to polymyxin B as a result of the overexpression of EptB (Moon & Gottesman 2009; Acuña et al. 2016). The *eptB* gene encodes an enzyme that transfers phosphoethanolamine to the outer Kdo (3-deoxy-D-manno-octulosonic acid) residue of LPS reducing the net negative charges of the LPS resulting in increased resistance to antimicrobial peptide Polymyxin B (**Figure 2**) (Needham & Trent 2013; Olaitan et al. 2014). *Salmonella* exhibits a feed-forward loop where the sponge RNA SroC decreases RNA levels of MgrR through direct binding leading to an increase of EptB expression and polymyxin B resistance (**Figure 2**) (Acuña et al. 2016). Recently, MgrR was shown to regulate the genes *grlR* and *grlA* on the pathogenicity island locus of enterocyte effacement (LEE) in enteropathogenic *Escherichia coli* (EPEC) (**Figure 2**) (Bhatt et al. 2017). MgrR directly binds to 5' untranslated regions of *grlR* and *grlA* leading to an increase in *grlA* and a decrease in *grlR* expression (Bhatt et al. 2017). Microarray analysis suggest that MgrR may play a role in the preferred nutrient resource as many genes that respond to carbon resources and various nutrients were positively impacted by the presence of MgrR (Moon & Gottesman 2009). The expression of MgrR is inversely related to Mg^{2+} concentrations in all enteric bacteria, including *E. coli*, *Shigella*, *Salmonella enterica*, *Citrobacter koseri* etc. and is regulated by the PhoP/PhoQ two-component system (**Figure 2**) (Moon & Gottesman 2009).

PhoP/PhoQ two-component system

The PhoP/PhoQ two-component system is comprised of a sensor histidine kinase (PhoQ) and a transcriptional activator (PhoP) (Groisman 2001). In low concentrations of Mg^{2+} and/or Ca^{2+} , PhoP becomes activated after being phosphorylated by PhoQ and then binds to a partially conserved sequence, (T/G)GTTTA-5nt-(T/G)GTTTA, found -35 bp upstream of the promoter (**Figure 3**) and activates transcription (Moon & Gottesman 2009). Proper function of the PhoP/PhoQ two-component system is essential for virulence (Miller & Mekalanos 1990). The regulon includes genes involved in LPS modifications, magnesium transport, and iron homeostasis (Hu et al. 2016; Soncini et al. 1996; Ernst et al. 2001). As Mg^{2+} concentrations get lower, the import of iron is down regulated by activation of the PhoP/PhoQ two-component system most likely to prevent the formation of toxic hydroxyl radicals during host infection (Hu et al. 2016). In fact, PhoP mutants of *Salmonella* are sensitive to H_2O_2 when grown in low Mg^{2+} (Tu et al. 2006).

Escherichia fergusonii

E. fergusonii is a potentially pathogenic bacterium that infects both animals and humans and is resistant to many antibiotics (Savini et al. 2008; Forgetta et al. 2012). Formerly known as Enteric group 10, *E. fergusonii* was defined as a new species in 1984 (Farmer et al. 1985). *E. fergusonii* was isolated from a 69 year old patient with pancreatic carcinoma and cholangiosepsis, a 52 year old patient with acute cystitis that could not be treated with beta-lactam, and has also been the cause of bacteremia in a diabetic patient with pancreatic cancer (Savini et al. 2008; Funke et al. 1993; Lai et al. 2011). *E. fergusonii* is considered a potential emergent pathogen but acts more as an opportunistic pathogen and is not only found in the gut but is also found in human vagina (Gao et al.

2013). High-fat diets and iron depletion enhance the gut population of *E. fergusonii* (Pereira et al. 2014; Chang et al. 2015). *E. fergusonii* modulates choline bioavailability by metabolizing choline into trimethylamine (TMA) which is metabolized by the liver into trimethylamine-N-oxide and causes atherosclerosis (Romano et al. 2015; Wang et al. 2015). The enhancement of *E. fergusonii* growth by a high-fat diet and the production of TMA could potentially link *E. fergusonii* to heart disease associated with high-fat diets around the world. Mistaken for a new multidrug resistant strain of *E. coli*, *E. fergusonii* was sequenced and tested against a large array of antibiotics and was observed to be resistant to almost every antibiotic (Forgetta et al. 2012). Though several studies have found *E. fergusonii* present in both infections and as part of human microbiome, no genetic studies have yet been carried out on this enteric bacterium that could have a significant impact on human health.

The PhoP/PhoQ two-component system, MgrR, and EptB are conserved in *E. fergusonii*. However, MgrR contains a 53 bp insert within the proposed seed region, which causes a major change in the secondary structure of the sRNA (**Figure 4**). The insertion appears to be a repetitive extragenic palindromic (REP) sequence (Stern et al. 1984). REP inserted downstream of protein-coding genes form hair-pin-like secondary structures and have been shown to terminate translation (Liang et al. 2015), but this is the first known instance of REP insertion in an sRNA, and its consequences on sRNA function are unknown. In silico RNA binding analysis indicates that the 53-nt insertion likely disrupts the mgrR-eptB binding site.

Oxidative stress

To survive and flourish in mammalian gut, bacteria have to withstand oxidative stress, bile, pH changes, and avoid the immune system. Bacteria have evolved specific genes that give them an edge over the competition in terms of stress survival and nutrient procurement. Cholic acid ($3\alpha,7\alpha,12\alpha$ -trihydroxy- 5β -cholan-24-oic acid) is primary bile acid found in several species of mammals, reptiles and fish (Lee R. Hagey et al. 2010; Lee R Hagey et al. 2010). Cholic acid is modified by gut bacteria into deoxycholic acid (Begley et al. 2005; Hill & Drasar 1968) Not only does DOC cause membrane stress but it also causes oxidative damage of DNA (Gaimster & Summers 2015). Efflux pumps like the ArcABZ-TolC efflux pump are utilized by gram-negative bacteria to get rid of DOC, and the oxidative stress response pathway is upregulated in the presence of DOC (Merritt & Donaldson 2009; Thanassi et al. 1997). Constantly being exposed to DOC and hydrogen peroxide (H_2O_2) produced by competing bacteria such as *E. coli* in the mammalian gut might have resulted in the evolution of genes that deal simultaneously with membrane stress and oxidative stress in *E. fergusonii*.

Reactive oxygen species (ROS) such as peroxides, free radicals, and hypochlorous acid cause oxidative damage (Small et al. 2007). H_2O_2 , which is highly abundant in the gut, is a strong oxidizer (Henle & Linn 1997). H_2O_2 freely passes through bacterial membranes and reacts with iron-sulfur clusters inside the cell. H_2O_2 reduces Fe^{2+} to Fe^{3+} inside the cell through the Fenton reaction and results in the production of hydroxyl radicals which are much more damaging than H_2O_2 (Henle & Linn 1997). Oxidative stress stimulates the expression of three transcription factors OxyR, SoxR, and SoxS, which together regulate the expression of a large number of genes involved in ROS detoxification, DNA damage repair, amino acid synthesis, metal ion transport and cell

wall synthesis in *E. coli* (Seo et al. 2015). In *E. coli*, MgrR was recently shown to down regulate the expression of SoxS (Lee & Gottesman 2016). SoxS is stimulated by the presence of superoxide anion (O_2^-), which up-regulates the expression of the sRNA MicF in *E. coli* (Fröhlich & Gottesman 2018). The outer membrane porin OmpC, which facilitates the influx of H_2O_2 is negatively regulated by MicF (Fröhlich & Gottesman 2018). OxyR expression increases in response to H_2O_2 and induces the expression of another sRNA, OxyS, which in turn down-regulates the transcription termination factor NusG (Fröhlich & Gottesman 2018; Barshishat et al. 2018). The decrease in NusG causes an increase in the prophage-encoded *kilR* gene that results in cell division arrest when KilR interferes with the formation of FtsZ rings that separate cells during division (Barshishat et al. 2018).

Metabolic pathways like the citric acid cycle also play an important role in the scavenging of ROS (Shimizu 2013a). NADH increases the toxicity of H_2O_2 and increases DNA damage but transitioning to NADPH production mediates this effect (Cabiscol et al. 2000). *soxS* and *soxR* mutants produce less nicotinamide adenine dinucleotide phosphate hydrogen (NADPH) because of a decrease in 6-phosphogluconate dehydrogenase (6PGDH) and Glucose-6-phosphate dehydrogenase (G6PDH) activity and are very sensitive to ROS (Shimizu 2013b). NADPH participates in the reduction of oxidized disulfide bonds in proteins that were oxidized by ROS and is a powerful antioxidant (Cabiscol et al. 2000). NADPH is produced in the citric acid cycle (TCA) indicating that the TCA may play an important role in ROS homeostasis.

Not all genes expressed under oxidative stress are regulated by OxyR, SoxS or SoxR. For example the *tnaA* gene is induced under oxidative stress independent of OxyR (Zheng et al. 2001). The importance of *tnaA* expression under oxidative stress is not understood. *tnaA* encodes the enzyme tryptophanase which breaks down tryptophan into pyruvate, NH_4^+ , and indole (Li & Kevin D. Young 2013). One possible explanation for *tnaA* expression under oxidative stress is the production of pyruvate, which has been shown to protect neurons against H_2O_2 (Desagher et al. 1997). Another possible explanation is that indole stalls cell division allowing for DNA repair similar to what was observed with KilR (Barshishat et al. 2018). In this study I investigated the function of the sRNA MgrR and show that it plays an important role in hydrogen peroxide and bile acid resistance in *E. fergusonii*.

MATERIALS AND METHODS

Bacterial strains and plasmids

Bacterial strains used in this study are listed in **Table 1**. All bacteria were either grown in Lysogeny Broth (LB) or modified N-minimal medium containing 5 mM KCl, 7.5 mM (NH₄)₂SO₄, 0.5 mM K₂SO₄, 1 mM KH₂PO₄, 0.1 mM Tris-HCl pH 7.4, 10 μM or 10 mM MgCl₂, 0.2% glucose, and 0.1% Casamino acids (Groisman et al. 1997). Kanamycin (KAN) was used at a final concentration of 50 μg/ml, ampicillin (AMP) was used at 100 μg/ml, and chloramphenicol (CAM) was used at 25 μg/ml. The wild type (WT) strain used in this study was *Escherichia fergusonii* type strain CDC 0568-73 [DSM 13698], ATCC 35469, which was obtained from American Type Culture Collection (ATCC) (Farmer et al. 1985). The *mgrR*-deletion strain **RL7** ($\Delta mgrR::kn$) was constructed using the λ Red recombination system (Datsenko & Wanner 2000). Briefly, a PCR fragment was obtained by amplifying the kanamycin cassette (KANr) from the pKD4 vector (Datsenko & Wanner 2000). To amplify the cassette, forward and reverse primers listed in **Table 2** were used with the PCR Master Mix (2x) system (Thermo Fisher Scientific). The resulting PCR product was recombined into the chromosome of wild-type *E. fergusonii* carrying the pKD78 vector encoding the λ Red function. The transformed cells were selected on LB plates containing 25 μg/ml kanamycin at 37°C. Recombinant products were verified by PCR and DNA sequencing was done at OHSU DNA Services Core.

KANr, which was inserted as part of the gene-deletion protocol was eliminated from the chromosome using thermal induction of FLP synthase on the pCP20 plasmid to

generate the strain **AW28** (Datsenko & Wanner 2000). To this end, RL7 cells were transformed via electroporation (see below for details) with pPCP20, recovered in SOC medium at 30°C, plated on LB agar plates containing chloramphenicol (CAM) and incubated at 30°C overnight. Colonies were streaked for isolation on LB-CAM and incubated at 30°C. Fresh LB was inoculated from LB-CAM plates that had no growth on corresponding LB-KAN plates and incubated at 43°C overnight to induce FLP recombinase and select against pCP20. The overnight culture was diluted and plated on LB agar and incubated at 30°C. Colonies were checked for the loss of pCP20 and KANr in LB-CAM and LB-KAN. Colonies that were sensitive to both antibiotics were checked for loss of KANr from the chromosome with PCR and confirmed with sequencing.

A chromosomal-complement strain (**AW51**) was constructed by introducing the WT *mgrR* gene along with its natural promoter in to the chromosome of AW28. A novel approach was developed to generate this strain because it is difficult to genetically modify *E. fergusonii*. As a first step, a potential site for gene insertions was located by mapping RNA-seq reads to the intergenic regions (IGRs) of *E. fergusonii* (NC_011740.1) using CLC Workbench 9 ($\geq 95\%$ read alignment length, $\geq 95\%$ identity) and IGRs with expression values less than 10 were earmarked as potential locations. Nonfunctional Insertion Sequences (IS) within these IGRs were detected using ISFinder (Siguier 2006), filtering for alignments $>30\text{bp}$ in length, $\geq 90\%$ identity, e-values ≤ 0.01 , and belonged to IS families previously characterized in *E. coli*. The IGR (3963595:3963677) between the coding sequences for rhamnulose-1-phosphate aldolase (EFER_RS19330) and

lactaldehyde reductase (EFER_RS19335) was selected as the preferred site for further experiments because it was 82 bp in length, had an expression value of 0, and contained a defunct partial IS621. PCR was used to produce a fragment containing *mgrR* along with upstream and downstream sequences (126 bp and 85 bp, respectively), two fragments of 1000 bp of homology flanking the insertion site, and a 1,477 bp fragment containing KANr flanked with FRT sites. The three PCR fragments along the linearized vector pKD78 (using BstXI) were cloned into pKD78 using the IN-Fusion kit (Clontech) and transformed into Stellar cells (Clontech) by following the manufacturer's protocol. Transformed cells were plated on LB-CAM plates, incubated at 30°C overnight and streaked onto LB-KAN plates and incubated at 30°C. The complement construct was isolated using GeneJet miniprep and confirmed with PCR. The complement construct was ethanol precipitated to remove any salts and electroporated into AW28 electrocompetent cells as described below and cells were recovered at 30°C. After recovery, cells were plated on LB-KAN and incubated overnight at 43°C. Colony-PCR and sequencing were used to confirm integration of the WT *mgrR* gene along with its natural promoter in to the chromosome of AW28 to generate the strain AW51.

Electrocompetent cells and electroporation

Fresh LB was inoculated 1:400 with overnight growth of the appropriate strain of bacteria and grown to an OD₆₀₀ of ~0.5 and then incubated on ice for 30 min. 50 ml aliquots of culture were centrifuged at 4°C, 21000 x g for 3 min and the pellet was resuspended in 50 ml of nano-filtered water. The cells were washed three times using nano-filtered water and twice with 10% glycerol. The final pellet was resuspended in the

liquid that remained after aspiration of the supernatant. The cells were either flash frozen on dry ice and stored at -80°C or used immediately for electroporation. 50 µl of electrocompetent cells were mixed with 2 µl of DNA and immediately placed in an ice-cold cuvette. Electroporation was done using a BioRad MicroPulser set to Ec2 (2.5 kV and no time constant). Cells were recovered in 1 ml SOC at 37°C for 2 hrs with gentle agitation. Recovered cells were plated on LB agar containing appropriate antibiotics and incubated at 37°C overnight.

RNA extraction

Fresh N-minimal medium with 10 mM MgCl₂ was inoculated with bacteria from glycerol stocks and incubated overnight (37°C, 250 RPM). Overnight culture was diluted 1:1000 in N-minimal medium with either low magnesium (10 µM) or high magnesium (10 mM) concentrations. 20 ml cultures were grown to mid log phase (~0.5 OD₆₀₀) and 2.5 ml RNA stop solution (19 ml 100% EtOH and 1 ml phenol) was added, cells were pelleted at 4°C, 21000 x g for 3 min. Total RNA was isolated using TRI reagent (Thermo Fisher Scientific).

Quantitative PCR (qPCR)

5 µg of RNA was treated with DNase I (Thermo Fisher Scientific) at 37°C for 30 min, and the reaction was stopped with the addition of 0.1 mM EDTA and incubated at 65°C for 10 min. RNA samples were cleaned with ethanol precipitation and checked for DNA

contamination with PCR using universal 16S rDNA primers. 500 µg of RNA was used to generate cDNA with the RevertAid RT transcription Kit (Thermo Fisher Scientific) using random hexamer primers provided with the kit. All cDNA samples were diluted 1:100 for 16S rRNA quantification, which was used as the house keeping gene for normalization. qPCR reactions were set up using 10 µl SYBR Green master mix (Thermo Fisher Scientific), 1 µl forward primer (10 mM), 1 µl reverse primer (10mM), 2 µl cDNA, and 6 µl water. The thermo cycler was set to 95 °C for 3 min, followed by 40 cycles of 95°C for 3s and 60°C for 30s. Fold change (fc) was calculated using the formula below where Ct is the cycle threshold value:

$$fc = 2^{-[(Ct \text{ gene of interest treated} - Ct \text{ 16S treated}) - (Ct \text{ gene of interest control} - Ct \text{ 16S control})]}$$

All primers used in qPCR are listed in **Table 2**.

Construction of expression vectors

Overexpression of MgrR was accomplished using the arabinose inducing promotor on the pBAD33 plasmid (Guzman et al. 1995). PCR was used to produce the wild type (WT) *mgrR* fragment using primers *mgrR* XbaI Forward and *mgrR* PstI Reverse (**Table 2**), which included restriction enzymes sites for XbaI and PstI, respectively at the 5' end of each primer. To determine if the insert sequence had any impact on MgrR function, a construct (*mgrR-NI*) expressing MgrR without the insert sequence (nucleotides 54-106) was generated using PCR as described below. Two oligos with overlapping regions were extended with PCR: The oligo mgrR-NI1 contained nucleotides 1-53 and 107-113, and oligo mgrR-NI2 contained nucleotides 39-53 and 107-150 of *E. fergusonii*'s *mgrR* (**Table 2**). 12.5 µl of PCR master mix, 8.5 µl water, 2 µl of mgrR-NI1 (10mM), and 2 µl of

mgrR-NI-2 (10mM) were mixed and extended with touchdown PCR. The thermocycler settings were denaturing (94°C, 2 min), overlap extension (94°C for 30s, 70°C-55°C for 45s, and 72°C for 1 min) — the annealing temperature went down 1°C per cycle starting at 70°C for the first fifteen cycles and maintained at 55°C for the last fifteen cycles, the final extension was 72°C for two minutes. The fragment was cleaned using GENEJet PCR cleanup kit and used as the template in a PCR reaction using primers XbaI Forward and *mgrR* PstI Reverse (**Table 2**) to amplify and add restriction sites to the *mgrR*-NI fragment. Fragments containing *mgrR*, *mgrR*-NI, and pBAD33 were double-digested with XbaI and PstI following the manufacturer's protocol (Thermo Fischer Scientific). WT *mgrR* (6.3 nM) and *mgrR*-NI (6.3 nM) fragments were ligated into pBAD33 (36.5 nM) overnight at 4°C with T4 DNA ligase. Chemically competent *E. coli* Stellar cells (Clontech) were transformed with the constructs the next day by following the manufacturer's protocol. Transformants were selected by plating on LB-AMP plates and incubated overnight at 37°C. PCR and sequencing were used to confirm each over-expression construct. GENEJet minipreps were used to purify the new constructs and were electroporated into the RL7 strain (*ΔmgrR::kn*) of *E. fergusonii* (**Table 1**) as described above, and appropriate cells were selected by plating on LB-AMP plates and incubated overnight at 37°C. Transformants were checked using PCR. Empty pBAD33 were electroporated into both WT and RL7 to be used as controls.

Overexpression of MgrR

LB-CAM was inoculated with WT_{pBAD33}, RL7_{pBAD33}, RL7_{pBAD33-*mgrR*}, and RL7_{pBAD33-*mgrR*-NI} (**Table 1**) from -80°C glycerol stocks and grown overnight at 37°C. The overnight

cultures were diluted 1:1000 in fresh LB-CAM containing 0.2% L-arabinose and grown to an OD₆₀₀ of ~0.5. 2.5 ml RNA stop solution (19 ml 100% EtOH and 1 ml phenol) was added to 20 ml of culture, and cells were pelleted at 4°C, 21,000 x g for 3 min. Total RNA was isolated using TRI reagent and qPCR was done as described above.

Rapid amplification of 3' cDNA ends (3' RACE)

Fresh N-minimal medium with 10 mM Mg⁺² was inoculated with wild-type *E. fergusonii* from glycerol stocks and grown overnight at 37°C. Overnight cultures were diluted 1:400 in fresh N-minimal medium with 10 µM Mg⁺² and grown to an OD₆₀₀ of ~0.5 when rifampicin 300 µg/ml was added. RNA was isolated using TRI reagent (Thermo Fisher Scientific). After DNase treatment, a poly-A tail was added to the 3' end of all RNA transcripts by mixing 10 µl of total RNA (100 ng/µl), 2 µl of 10 mM ATP, 2 µl 10x buffer, 1 µl *E. coli* poly(A) polymerase, and 5 µl water. The 20 µl reaction was incubated at 37°C for 30 min, and 5 µl of 0.1 mM EDTA was added to terminate the reaction. Poly(A) RNA was ethanol precipitated and resuspended in 11 µl of water. The 11 µl of RNA was mixed with 1 µl of the adapter primer provided in the kit and incubated at 70°C for 10 min, chilled on ice and then mixed with 2 µl 10x PCR Buffer, 2 µl 25 mM MgCl₂, 1 µl 10 mM dNTP, and 2 µl 0.1M DTT. The reaction was heated to 42°C and 1 µl reverse transcriptase was added and incubated at 42°C for 50 min followed by 70°C for 15 min, and 1 µl RNase H was added and incubated at 37°C for 20 min. PCR was used to amplify the MgrR cDNA using primer EF-MgrR-F-XbaI and the antisense primer was the abridged universal amplification primer (**Table 2**). 57 ng of the amplified product was cloned into pGEM following the pGEM-T Easy Vector System manufacturer's protocol

(Promega). Ten colonies were selected for forward and reverse DNA sequencing using M13 primers. Sequencing was performed at the Oregon Health and Science University DNA Services Core.

Deoxycholic acid sensitivity assay

Overnight cultures of WT, AW28, and AW51 strains of *E. fergusonii* were diluted 1:400 in fresh LB next day and grown to an OD₆₀₀ of ~0.5 and 100 µl was plated onto LB agar containing 0.1% w/v deoxycholic acid and incubated at 37°C overnight. Colonies were counted, and percent survival values for AW28, and AW51 was determined by comparing their CFUs (colony forming units) to WT CFUs. Each experiment was replicated at least three times.

Hydrogen peroxide sensitivity assay

Overnight cultures of WT, AW28, and AW51 strains of *E. fergusonii* were divided into two tubes containing 10 ml of culture. One tube was immediately serially diluted (1:10 to 10e-5) in PBS and 100 µl from each dilution was plated on LB agar. 100 µl of 19 mM H₂O₂ was added to the other 10 ml of culture and incubated at 37°C, 250 rpm, for 15 min. Catalase (10 mg/ml) was made fresh each day in catalase buffer (50 µM KH₂PO₄, pH7) and filter sterilized. After the 15-min incubation with H₂O₂, 150 µl of catalase (10 mg/ml) was added into each tube and incubated for 1 min at room temperature. Cultures were serially diluted (1:10 in PBS up to 10e-3) and 100 µl was plated on LB agar in triplicate. Percent survival was determined by comparing CFU/ml before and after H₂O₂ treatment. Unpaired student t-tests were performed to determine statistical significance.

Hydrogen peroxide disk assay

100 μ l of overnight culture of WT, AW28, and AW51 strains of *E. fergusonii*, NM 22540 (wild-type *E. coli*), and KM128 (*Δ mgrR::kn* *E. coli*) were spread on plates that contained exactly 25 ml of LB agarose (1.5% w/v). 10 mm paper disks were placed in the center of the LB plates, loaded with 10 μ l of 9.7 M H₂O₂ and incubated at 37°C overnight. The next day photographs were taken of each plate and the area of clearing was measured with ImageJ software.

High-throughput RNA sequencing (RNA-seq)

RNA samples were collected from WT and AW28 before and after exposure to H₂O₂ using TRIzol, as described above. H₂O₂ exposure was the same as outlined above except that rifampicin was added to a final concentration of 300 ng/ml before RNA isolation. RNA samples were treated with TurboDNase (Thermo Fisher Scientific) following manufacturer's protocol to get rid of contaminating DNA. RNA-seq was performed on six samples: one WT untreated (control), one AW28 untreated (control), two WT after H₂O₂ treatment, and two AW28 after H₂O₂ treatment. Sequencing reads were trimmed using Trimmomatic (Bolger et al. 2014), mapped to *E. fergusonii* genome (NC_011740) using CLC Genomic Workbench, and log-2 fold change was calculated using DESeq2 (Love et al. 2014). All genes that had a fold-change greater than 2x and an adjusted p-value less than 0.05 were considered significant. Expression of a subset of genes were confirmed using qPCR as described above.

Murine infection model

Six 5-week-old C57BL/6 mice were fed ampicillin (1 mg/ml) in their drinking water for seven days to clear bacteria from their guts. The drinking water was then replaced with

water without antibiotic and the mice were not given any further antibiotic treatment. The Mice were orally inoculated with $\sim 10^9$ bacteria in 100 μ l phosphate-buffered saline (PBS) as outlined in **Table 3**. The bacteria were resuspended from cultures grown to mid-log phase as described below. Overnight cultures of WT and AW28 were diluted 1:400 in 25 ml of fresh LB and grown at 37°C and 250 rpm to an OD₆₀₀ of ~ 0.5 . Each culture was divided into 14 ml aliquots and centrifuged at 4°C 10,000x g for 10 min. For the competition assay (mice 4-6, Table 3), bacterial pellets were resuspended in PBS and AW28 and WT cell suspensions were mixed 1:1 and brought to a final volume of 100 μ l with PBS. 100 μ l suspensions of WT and AW28 in PBS were used as controls (mice 2 and 3, respectively). A portion of the remaining cell suspension was then serially diluted and plated on LB agar to determine the input CFU. All mice were raised on standard laboratory rodent diet and maintained in pathogen-free barrier facilities at Oregon Health & Science University. Animal protocols were approved by the Institutional animal care and use committee at Oregon Health & Science University (protocol # IP00000228) according to standards set forth in the *Animal Welfare Act*.

Fresh fecal samples were collected once daily for seven days, weighed and suspended in PBS (10% feces w/v) and left overnight at 4°C. The next day 1 volume of 50% glycerol was added to the fecal slurry and stored at -80°C. *E. fergusonii* is naturally resistant to ampicillin allowing us to use this antibiotic to selectively grow *E. fergusonii* without contamination from the rest of the mice microbiota. Serial dilutions of each fecal sample were plated in triplicate on LB agar, LB-AMP (selects both WT and AW28), and LB-AMP + KAN (selects only AW28). Plates were incubated at 37°C overnight. The %

population of AW28 was calculated by dividing the average CFU on the LB-AMP+KAN plates with the average CFUs on the LB-AMP plates.

Electrophoretic mobility shift assay (EMSA)

RNA fragments used for EMSA were produced using T7 MAXIscript as outlined by the manufacturer's protocol (Ambion). Template for each *in vitro* RNA reaction were made by PCR with forward primers that contained the T7 promoter (TAATACGACTCACTATAGG). After transcription the RNA fragments were purified either with TRIzol or Ambion NucAway Clean Up columns. 5x Hybridization buffer (100 mM HEPES pH 8, 500 mM KCL₂, 5 mM MgCl₂, and 5 mM DTT) was made immediately before each experiment. Hybridization was done in 10 µl reactions of 1x hybridization buffer with 0.2 pmol of mRNA and either 0 pmol, 0.4 pmol or 4 pmol MgrR RNA. Each reaction was incubated at 37°C for 20 min and 2.5 µl of 5x loading buffer (Bio Rad) was added to each reaction and immediately analyzed on a 5% native polyacrylamide gel at 100 V for 1 hr. Each gel was also contained a well with the RiboRuler Low Range RNA Ladder (Thermo Fisher Scientific). Gels were stained with 1x Syber Green II (Thermo Fisher Scientific) for 20 min before being imaged with UV 254 nm.

RESULTS

Full-length MgrR is produced in *E. fergusonii*

The 53 bp insertion found in the *mgrR* is very similar in sequence to REP elements that forms a stem-loop, which has been shown to terminate transcription (Johnson et al. 2014). To determine if the 3' end of MgrR was truncated due the stem-loop insertion, we performed a 3' RACE assay to determine the 3' end of MgrR. Our results showed that the MgrR transcript terminated at the Rho-dependent terminator located at positions 54-106, producing an sRNA of 151 nt in length (**Figure 5**). Thus, although a REP-like sequence is found within the *mgrR* gene, this insertion does not interfere with sRNA transcription.

MgrR is regulated by PhoP/PhoQ in *E. fergusonii*

Expression of MgrR is controlled by PhoP/PhoQ, in *E. coli*, which is a two-component system that regulates gene expression in response to periplasmic concentrations of Mg^{2+} (Moon & Gottesman 2009). It was previously shown that MgrR expression was inversely correlated with Mg^{2+} concentrations (Moon & Gottesman 2009). To determine if MgrR is regulated by the PhoP/PhoQ system in *E. fergusonii*, the bacterium was grown in N-minimal medium with low (10 μ M) and high concentrations (10 mM) of Mg^{2+} . MgrR expression was 27-fold higher in low concentrations of Mg^{2+} (10 μ M) than in high concentrations of Mg^{+2} (10 mM) (**Figure 6**). PmrD, which showed an 11-fold increase, was used as a control because it is known to be directly regulated by the PhoP/PhoQ system (Lejona et al. 2003). The PhoP/PhoQ two component system most likely regulates MgrR in *E. fergusonii* as the expression pattern in response to Mg^{2+} concentrations are consistent with MgrR expression in *E. coli*.

MgrR does not regulate the expression of *eptB* gene in *E. fergusonii*

Phosphoethanolamine transferase, which adds a phosphoethanolamine to the outer KDO molecule of the LPS is encoded by the *eptB* gene in *E. coli* (**Figure 2**) (Reynolds et al. 2005). EptB was the only validated target of MgrR in *E. coli*, where a microarray analysis showed that over-expression of MgrR resulted in a 8-9 fold decrease of EptB mRNA (Moon & Gottesman 2009). In *E. coli*, MgrR is hypothesized to bind to the 5' end of the EptB mRNA via a highly conserved region (**Figure 1**), (Moon & Gottesman 2009). In *E. fergusonii*, a 53 bp insertion disrupts this highly conserved region (**Figure 1**). Hence, to determine whether the insertion has an impact on the regulation of EptB by MgrR in *E. fergusonii*, we measured *eptB* mRNA levels with RT-qPCR in *E. fergusonii* strains over-expressing either full-length MgrR or a modified version of MgrR that lacked the insert (MgrR-NI) (**Table 1**) and compared it to expression in WT and RL7 ($\Delta mgrR$) strains (**Figure 7**). As shown in **Figure 7**, over-expression of WT MgrR and MgrR without the insert (MgrR-NI) showed no significant effect on EptB expression levels. This data indicates that unlike in *E. coli*, MgrR does not regulate EptB expression in *E. fergusonii*, and that the insertion of the 53 bp REP-like sequence did not have any discernable effect on *eptB* regulation.

Potential gene targets of MgrR in *E. fergusonii*

To find potential targets of MgrR, RNA-seq was performed using RNA from WT and AW28 ($\Delta mgrR$ with KANr removed) before and after H₂O₂ exposure. Genes that showed a significant increase in expression (log2 fold change greater than 1, $p < 0.05$) in the

presence of MgrR were considered to be positively regulated (**Table 4**) by the sRNA. MgrR expression under oxidative stress led to the most dramatic increases in expression for *tnaA* (EFER_RS20020) gene (6.5-fold), and *speF* (EFER_RS12190) gene (6.09-fold). The *tnaA* gene encodes a tryptophanase/ L-cysteine desulfhydrase, which breaks down tryptophan into indole, pyruvate and NH_4^+ and breaks down L-cysteine into pyruvate, H_2S and NH_4^+ (Newton & Snell 1964), whereas the *speF* gene encodes an ornithine decarboxylase which breaks down L-ornithine into CO_2 and putrescine. Additionally, *glpABC* operon, *glpTQ* operon, *frdABCD* operon, *preAT* operon, and the *nanATEK-yhcH* operon were positively impacted by the presence of MgrR (**Table 4**). Furthermore, **Table 5** lists 16 genes that showed a significant decrease in expression (log2 fold change lower than -1, $p < 0.05$) in the presence of MgrR under oxidative stress. *ygdQ* (EFER_RS13950) was impacted the most by MgrR with a 3.94-fold decrease. Similar to our data in *E. fergusonii*, *ygdQ* is also the gene most impacted by MgrR expression in *E. coli* (Moon & Gottesman 2009).

Deletion of *mgrR* increases *E. fergusonii*'s sensitivity to deoxycholic acid

Cholic acid is the primary bile salt produced by humans and mice (Hill & Drasar 1968). Certain gut bacteria, including *E. coli*, metabolizes cholic acid into deoxycholic acid (DOC), which is highly toxic to other bacteria because it causes extensive membrane, protein and DNA damage (Merritt & Donaldson 2009). Since DOC is abundant the gut, where *E. fergusonii* is naturally found, genes having a role in DOC survival would be very important to this enteric pathogen. Deletion of *mgrR* caused a significant decrease ($p = 0.0005$, paired two-tailed student t-test) in survival of *E. fergusonii* when exposed to

0.1% DOC. DOC survival was restored to the WT levels in AW51, the complementation strain (**Figure 8**).

Deletion of *mgrR* increases *E. fergusonii*'s sensitivity to H₂O₂

Pathogenic bacteria and enteric bacteria are constantly being exposed to extracellular H₂O₂. To survive in the gut, bacteria must evolve mechanisms to deal with extracellular H₂O₂, which freely passes through the cellular membrane. To determine if MgrR plays a role in oxidative stress survival, we exposed WT, AW28, and AW51 strains of *E. fergusonii* to 19 mM H₂O₂ for 15 min and calculated the percent survival. The MgrR deletion strain AW28 was significantly more sensitive to H₂O₂ exposure than the WT or complement strain (AW51) (**Figure 9**). Additionally, to compare the H₂O₂ sensitivity between *E. coli* and *E. fergusonii*, overnight culture of wild-type *E. fergusonii*, AW28, AW51, NM 22540 (wild-type *E. coli*), and KM128 (*E. coli* $\Delta mgrR::kn$) was spread on plates that contained exactly 25 ml of LB agarose (1.5% w/v). 10 mm paper disks loaded with 10 μ l of 9.7 M H₂O₂ were placed in the center of the LB plates and incubated at 37°C overnight. *E. fergusonii* is naturally more resistant to H₂O₂ than *E. coli* (**Figure 10**) and deletion of *mgrR* did not impact H₂O₂ sensitivity in *E. coli* but significantly increased ($p < 0.0001$) the sensitivity of *E. fergusonii* to H₂O₂ (**Figure 10**).

MgrR likely stabilizes TnaA mRNA in *E. fergusonii*

A previous study showed that *tnaA* is induced independently of OxyR in response to H₂O₂ exposure in *E. coli* (Zheng et al. 2001). In accordance with these studies, our RNA-seq data (**Table 4**) indicated that *tnaA* was a potential target of MgrR under oxidative stress. To confirm this, WT, AW28, and AW51 were grown in LB to mid-log phase

(OD₆₀₀ ~0.5) and exposed to 19 mM H₂O₂ for 15 min. RNA samples were taken before and after H₂O₂ exposure and qPCR was conducted. The presence of MgrR had no significant impact on the mRNA levels of the control gene *katG* (catalase), but MgrR levels in WT and AW51 decreased about two-fold after H₂O₂ exposure (**Figure 11**). Interestingly, while *tnaA* expression did not increase in response to H₂O₂ in WT and AW51, we did observe a two-fold decrease of *tnaA* mRNA levels in AW28 ($\Delta mgrR$) after exposure to H₂O₂ (**Figure 11**). This data indicates that the loss of MgrR could lead to an increase in TnaA mRNA degradation. *In vitro* non-radioactive EMSA was performed to determine if MgrR could directly bind to TnaA mRNA. As shown in **Figure 12**, there was no observable shift in TnaA mRNA in the presence of MgrR, indicating that MgrR does not directly bind to TnaA mRNA under the conditions tested.

Deletion of *mgrR* decreases *E. fergusonii*'s fitness within murine gut

To determine if there was any fitness loss in *E. fergusonii* caused by the deletion of *mgrR*, we conducted a competition assay between WT and RL7 ($\Delta mgrR::kn$) strains in the mouse gut. Mice were inoculated with 10⁹ CFUs of WT and RL7 at a 1:1 ratio. Fecal samples were taken once a day for one week and plated on LB-AMP plates (both WT and RL7 grow on this medium) and on LB-AMP+KAN plates (only RL7 grows on this medium). The population of RL7 was determined by dividing the CFUs on the LB-AMP+KAN plates by the CFUs on the LB-AMP plates. The relative population of RL7 began to decrease by the second day and continued to decrease each subsequent day (**Figure 13**). The mice contained 100% WT by the sixth day of the competition assay. Control mice inoculated individually with either WT or RL7 maintained relatively

equivalent populations of each strain throughout the experiment (**Figure 14**), indicating that the loss of MgrR has significantly reduced the ability *E. fergusonii* to colonize murine gut.

DISCUSSION

Non-coding RNAs that regulate gene expression have been identified in all three domains of life: Archaea, Bacteria and Eukarya. The function of a bacterial sRNA is dependent on the sequence of the seed region that base-pairs with the target mRNA. Artificial mutations to the seed region of a sRNA are utilized as a common genetics technique for studying how sRNAs interact with the target mRNAs. For example, the sRNA EcsrR2 in *E. coli* down regulates AnsB expression (Kacharia et al. 2017). The seed region of EcsrR2 lays between nucleotides +51 and +80 (Kacharia et al. 2017). When nucleotides +51 to +80 are deleted from EcsrR2 no longer negatively impacts the AnsB mRNA levels (Kacharia et al. 2017). Most sRNAs are evolutionarily older than many of the known binding sites on the target mRNAs, indicating that new mRNA targets emerge as they evolve binding sites that fit the seed region of the sRNA (Peer & Margalit 2014). Despite the selection pressure acting to conserve the sRNA seed regions, sRNAs have been shown to evolve rapidly and even closely related sRNAs can have different target genes (Dutcher & Raghavan 2018). It is possible that the rapid evolution of sRNAs is a result of the emergence of new target genes, but the current understanding of sRNA evolution is limited, and further research is needed to determine the exact mechanism of sRNA evolution.

The sRNA MgrR is known to be regulated by the PhoP/PhoQ two-component system and negatively regulates the phosphethanolamine transferase EptB in *E. coli* and *Salmonella enterica* serovar Typhimurium (Moon & Gottesman 2009; Acuña et al. 2016). Strains of *E. coli* and *S. Typhimurium* with *mgrR* deleted are resistant to polymyxin B because of the LPS modification caused by unchecked EptB expression

(Moon & Gottesman 2009; Acuña et al. 2016). The *mgrR* gene in *E. fergusonii* contains a 53 bp insertion sequence that lies two nucleotides into the seed region of MgrR (**Figure 1**). The dramatic change in the *mgrR* sequence in *E. fergusonii* is a very interesting scenario that has the potential for great insights into how sRNAs evolve. The inserted sequence is very similar to a REP sequence, which have been shown to impact gene expression, mRNA stability, and transcription attenuation (Stern et al. 1984; Liang et al. 2015; Messing et al. 2012). REP sequences are characterized by inverted repeats that form stable stem-loop secondary structures (Stern et al. 1984). There are many possible consequences the inserted REP sequence could have on the function of MgrR in *E. fergusonii*, including changes in expression patterns, target genes, and transcription length. My analyses show that MgrR is regulated by the PhoP/PhoQ two-component system and has an inverse relationship to Mg^{2+} concentrations (**Figure 6**). The inserted REP sequence does not terminate transcription and likely plays a role in MgrR function (**Figure 5**). We showed that unlike in *E. coli*, MgrR does not regulate the expression EptB in *E. fergusonii* (**Figure 7**). The inserted REP sequence was most likely not the reason that MgrR does not regulate EptB in *E. fergusonii* because deletion of the insertion sequence from MgrR did not impact mRNA levels of *eptB* (**Figure 7**). It is possible that *eptB* is an evolutionarily younger target of MgrR that evolved after *E. coli* and *E. fergusonii* diverged. Future experiments to determine when *eptB* became a target of MgrR as well as further research on how *eptB* is regulated in *E. fergusonii* would allow us to make a more informed analysis of why MgrR does not impact *eptB* mRNA in *E. fergusonii*.

Reactive oxygen species such as H_2O_2 , O_2^- , and hydroxyl radicals are natural byproducts of aerobic respiration, but oxidative stress from exposure to ROS can lead to DNA, lipid and protein damage (Ling & Soll 2010; Fridovich 1978; Goerlich et al. 1989; Cabiscol et al. 2000). Cells have evolved specialized enzymes such as superoxide dismutase that catalyzes O_2^- into H_2O_2 , and catalase, which decomposes H_2O_2 into H_2O to defend against the buildup of ROS (Fridovich 1978; Cabiscol et al. 2000). H_2O_2 reacts with Fe(II) found inside the cell through the Fenton reaction to produce Fe(III) and hydroxyl radicle (Cabiscol et al. 2000). Most of the DNA damage from oxidative stress is caused by Fe(III) interacting with DNA (Henle & Linn 1997; Fridovich 1978). NADPH is an antioxidant and inhibits the DNA damage caused by H_2O_2 (Cabiscol et al. 2000; Henle & Linn 1997). No previous studies have been done on the oxidative stress response in *E. fergusonii*. Here I show that *E. fergusonii* is more resistant to H_2O_2 than *E. coli*, and the deletion of *mgrR* caused *E. fergusonii* to be as sensitive to H_2O_2 as *E. coli* (**Figure 10**). The exact function of MgrR under oxidative stress has not been confirmed, but the list of genes that were positively impacted by the presence of MgrR in **Table 4** provides new insights into possible explanations for the difference in H_2O_2 sensitivity between WT and $\Delta mgrR$ strains of *E. fergusonii*. The RNA-seq data revealed a pentose phosphate operon that exists in *E. fergusonii* but not in strains of *E. coli* used in this study. **Table 4** lists four genes that appear to be all part of the same operon *ulaB* (EFER_RS04450), *ulaA* (EFER_RS04455), *tktC₁* (EFER_RS04460), and *tktC₂* (EFER_RS04465). All four of these genes are up regulated by the presence of MgrR under oxidative stress and the operon encodes genes that homologous to known mechanism for importing L-ascorbate (*ulaAB*) and producing NADPH by a transketolase enzyme (*tktC*) (Shaw et al. 2018;

Zhang et al. 2003). The production of NADPH by the transketolase enzyme is vital for *Salmonella enterica* serovar Typhimurium host survival and virulence (Shaw et al. 2018). The increased production of NADPH would give *E. fergusonii* protection from oxidative stress, H₂O₂/iron-mediated DNA damage, and host defense mechanisms, which could explain why deletion of *mgrR* causes increased sensitivity to H₂O₂ (**Figures 9 and 10**), DOC (**Figure 8**) and does not compete with WT inside murine gut (**Figure 13**).

Tryptophanase (encoded by the *tnaA* gene) is responsible for producing indole from tryptophan, which has important roles in biofilm formation, cell invasion, and motility (Li & Kevin D Young 2013). Tryptophanase activity is dependent on formation of localized foci that are disrupted in the presence of glucose but not glycerol (Li & Young 2014). In this study I show that deletion of *mgrR* caused a decrease in *tnaA* mRNA levels under oxidative stress (**Figure 11**). However, we were not able to show that MgrR directly binds to *tnaA* mRNA (**Figure 12**). The positive impact that MgrR over-expression has on *tnaA* mRNA levels under oxidative stress is most likely an indirect result of MgrR expression promoting glycerol import and utilization. **Table 4** lists 11 gene (*glpABCFKTQ* and *frdABCD*) that together make up a complete mechanism for the import glycerol and utilization of glycerol as an energy source. The *glpF* gene encodes a glycerol facilitator that allows the transport of glycerol across the inner membrane (Braun et al. 2000). Once glycerol is inside the cell it is metabolized into *sn*-glycerol 3-phosphate by glycerol kinase, *glpK* (Law et al. 2007). The anaerobic glycerol-3-phosphate dehydrogenase (*glpABC*) and fumarate reductase (*frdABCD*) function together in a complex that transfers an electron from *sn*-glycerol 3-phosphate to fumarate and from fumarate to succinate (Miki & Lin 1973; HIRSCH et al. 1963). It is possible that the

transition from glucose to glycerol metabolism would allow for better localization of TnaA foci under oxidative stress.

In conclusion, enteric bacteria are in constant competition with other flora and host resistance mechanisms for survival within the mostly-hostile gut environment. The constant flux in environmental stress factors found in mammalian gut requires enteric bacteria to quickly reprogram their gene expression profiles to survive in that niche. sRNAs are a fast and energy efficient method for gene regulation that benefit bacteria in unstable environments (Gottesman et al. 2006). Hence, it is no wonder that most sRNAs play important regulatory roles in stress response systems. In this study, I show that the sRNA MgrR enhances *E. fergusonii*'s survival under oxidative and bile stress by up-regulating several metabolic pathways. To further understand the function of MgrR in *E. fergusonii*, the increased utilization of L-ascorbate and glycerol under oxidative stress need to be confirmed. Genetic studies that disrupt these metabolic pathways would be very beneficial for understanding their exact roles in oxidative stress. To accomplish these goals, I have laid the foundation for future genetic experiments in *E. fergusonii*, by engineering new vectors and by describing a new gene-insertion location in its genome that could be utilized for investigating the functions of sRNAs and proteins in this understudied enteric bacterium that significantly impacts human health.

TABLES

Table 1. Bacterial strains and plasmids used in this study.

Strains or Plasmids	Description	Source
Strains		
<i>Escherichia fergusonii</i> ATCC 35469	Wild-type (WT) <i>E. fergusonii</i>	ATCC; Farmer <i>et al.</i> (1985)(Farmer <i>et al.</i> 1985)
RL7	<i>E. fergusonii</i> $\Delta mgrR::KANr$	Raghavan lab
AW28	RL7 with KANr removed	This Study
AW51	Chromosomal insertion of MgrR in AW28	This Study
<i>E. coli</i> Stellar Cells	F ⁻ , endA1, supE44, thi-1, recA1, relA1, gyrA96, phoA, $\Phi 80d$ lacZ Δ M15, Δ (lacZYA - argF) U169, Δ (mrr - hsdRMS - mcrBC), $\Delta mcrA$, λ^-	ClonTech
RL7 _{pBAD33} ,	RL7 with empty pBAD33 vector	This Study
RL7 _{pBAD33-mgrR}	RL7 with pBAD33 _{mgrR} vector	This Study
RL7 _{pBAD33-mgrR-NI}	RL7 with pBAD33 _{mgrR-NI} vector	This Study
Plasmids		
pBAD33	AmpR and CmR; araBAD promotor-based expression vector having a pACYC origin	Guzman <i>et al.</i> (1995)(Guzman <i>et al.</i> 1995)
pBAD33 _{mgrR}	AmpR and CmR; <i>mgrR</i> gene cloned into pBAD33	This Study
pBAD33 _{mgrR-NI}	AmpR and CmR; <i>mgrR</i> gene without nucleotides 54-106 cloned into pBAD33	This Study
pPC20	AmpR and CmR; temperature-sensitive replication and thermal induction of FLP synthesis	Cherepanov and Wackernagel (1995)

Table 2. PCR Primers used in this study.

Name	Sequence
Abridged universal amplification primer	GGCCACGCGTCGACTAGTAC
Adapter primer	GGCCACGCGTCGACTAGTACTTTTTTTTTTTTTTTTTT
EF-mgrR-pstI_R	CTGCAGAAAAAAACCGCTGGAGTGA
EF-mgrR-xbaI_F	TCTAGAGAGTCGTTATCGGGTACAAA
katG qPCR F	TGCGTCAGTTTGAACCAAGC
katG qPCR R	TGACTTGACGCTGCGTTTTG
MgrR Comp Check Forward	TATCGGCCTGGATTTCTTTG
MgrR Comp Check Reverse	ATTTGCCAGTAAAGGCATGG
MgrR Comp pKOV LFF	TGACCGTTTGCTCATCCATCACATGCCATCTATCTGGA ATCAGAC
MgrR Comp pKOV RFR	CCGGTCGACTCTAGAGGATCGCACCATGCAGGCTAATT TTCGG
MgrR Compliment Kanamycin Resistance Forward	ACCTTTCAGCGTGTAGGCTGGAGCTGCT
MgrR Compliment Kanamycin Resistance Reverse	GATAAGGCGCCATATGAATATCCTCCTTAGTTCT
MgrR Compliment Left Flank Forward	GAAACAGACGAAGAATCCATACATGCCATCTATCTGG AATCAGAC
MgrR Compliment Left Flank Reverse	TACGTCCGGTCTTGCGCCGCATCCGGCAG
MgrR Compliment Right Flank Forward	TATTCATATGGCGCCTTATCCGGCCTACG
MgrR Compliment Right Flank Reverse	TCAAAGGGAAAACTGTCCATGCACCATGCAGGCTAAT TTTCGG
MgrR Natural Promoter Forward	GCGGCGCAAGACCGGACGTAATCGCTGTATG
MgrR Natural Promoter Reverse	CAGCCTACACGCTGAAAGGTATGCGGGTAAAACC
mgrR qPCR Forward	TCGGGTACAAAATGCCTGGT
mgrR qPCR Reverse	AGCGGAAAATGCTTGCATGG
mgrR_-NL-1	GAGTCGTTATCGGGTACAAAATGCCTGGTAGCGTAAA AAGCAAAACACATATCTATCCAT
mgrR_-NL-2	AAAAAAAACCGCTGGAGTGACCAGCGGAAAATGCTTG CATGGATAGATATGTGTTTTGCT
MgrR_T7	TAATACGACTCACTATAGGGGAGTCGTTATCGGGTACA AA

MgrR_T7_Anti	TAATACGACTCACTATAGGGAAAAAAAAACCGCTGGAG TGA
mgrR-KO-pKD4 _ R	CGGTGGTTTGCTGAAAGGTTTGCTGGCGAAAGCAGTCC TGGATGAGAGATCATATGAATATCCTCCTTAG
mgrR-KO-pKD4_F	ATAACCCTTGTTTAACTGTCATTTAGCTTAAAAGCAGT AAAATTCTTAATGTGTAGGCTGGAGCTGCTTC
pKD4 KAN Forward	GTGTAGGCTGGAGCTGCTTC
pKD4 KAN Reverse	CATATGAATATCCTCCTTAG
pmrD qPCR Forward	CGCCGAAGTGAAGAGTGACT
pmrD qPCR Forward	CGCCGAAGTGAAGAGTGACT
pmrD qPCR Reverse	TTCCCAGCCGATTGACACTG
pmrD qPCR Reverse	TTCCCAGCCGATTGACACTG
tnaA_qPCR_F	ATTCCGCGCGCAACATATAC
tnaA_qPCR_R	AGTTTTGCGGTGAAGTGACG
TnaA_T7	TAATACGACTCACTATAGGGATTTACCCTTCTGTAGCC ATC

Table 3. Mouse Inoculation.

	Sex	Infection
1	M	PBS only
2	F	WT <i>E. fergusonii</i> only
3	F	AW28 (Δ mgrR::kn) only
4	M	1:1 WT and AW28
5	F	1:1 WT and AW28
6	F	1:1 WT and AW28

Table 4. Potential positive targets of MgrR under oxidative stress in *E. fergusonii*.

Locus tag	Name and description	Fold change (log2) ^a	Adjusted p-value
EFER_RS 04450	<i>ulaB</i> , L-ascorbate specific PTS enzyme IIB component	3.6	2.60E-03
EFER_RS 04455	<i>ulaA</i> , PTS ascorbate transporter subunit IIC	4.0	4.19E-05
EFER_RS 04460	<i>tktC₁</i> , transketolase subunit 1	3.6	3.34E-05
EFER_RS 04465	<i>tktC₂</i> , transketolase subunit 2	3.3	3.78E-05
EFER_RS 04725	<i>glpC</i> , anaerobic glycerol-3-phosphate dehydrogenase subunit C	5.0	9.84E-11
EFER_RS 04730	<i>glpB</i> , anaerobic glycerol-3-phosphate dehydrogenase subunit B	4.8	7.62E-09
EFER_RS 04735	<i>glpA</i> , anaerobic glycerol-3-phosphate dehydrogenase subunit A	4.2	3.98E-07
EFER_RS 04740	<i>glpT</i> , glycerol-3-phosphate transporter	3.6	2.54E-06
EFER_RS 04745	<i>glpQ</i> , glycerophosphoryl diester phosphodiesterase	3.6	7.97E-06
EFER_RS 05205	S110 family transposase	2.2	4.64E-02
EFER_RS 05840	<i>agp</i> , glucose-1-phosphatase	2.6	4.55E-02
EFER_RS 06675	<i>astD</i> , aldehyde dehydrogenase	2.8	4.65E-02
EFER_RS 08530	<i>ompW</i> , outer membrane protein	5.5	7.34E-05
EFER_RS 08710	<i>narI</i> , DNA-binding transcriptional dual regulator	2.3	2.52E-02
EFER_RS 09540	<i>cadB</i> , cadaverine/lysine antiporter	5.0	2.58E-03
EFER_RS 10810	IS110 family transposase	2.3	3.07E-02
EFER_RS 11210	<i>preT</i> , NAD(P)-dependent oxidoreductase	5.0	3.08E-05
EFER_RS 11215	<i>preA</i> , NAD-dependent dihydropyrimidine dehydrogenase subunit PreA	3.6	3.10E-03
EFER_RS 11315	<i>psuG</i> , pseudouridine-5'-phosphate glycosidase	4.9	1.64E-05
EFER_RS 11320	<i>psuK</i> , putative pseudouridine kinase	3.7	4.32E-03
EFER_RS 12190	<i>speF</i> , ornithine decarboxylase	6.1	3.34E-05
EFER_RS 12195	<i>potE</i> , putrescine:H ⁺ symporter / putrescine:ornithine antiporter	4.8	7.20E-04

EFER_RS 14300	<i>gcvP</i> , glycine decarboxylase	3.3	1.59E-04
EFER_RS 14305	<i>gcvH</i> , glycine cleavage system H protein	2.5	6.65E-03
EFER_RS 14585	<i>ansB</i> , L-asparaginase 2	5.0	3.74E-04
EFER_RS 14770	<i>hybE</i> , hydrogenase-2 specific chaperone	3.3	1.78E-02
EFER_RS 14780	<i>hybC</i> , hydrogenase 2 large subunit	3.7	8.22E-03
EFER_RS 14785	<i>hybB</i> , hydrogenase 2 membrane subunit	4.2	2.69E-03
EFER_RS 14790	<i>hybA</i> , hydrogenase 2 iron-sulfur protein	4.2	6.25E-03
EFER_RS 14795	<i>hybO</i> , hydrogenase 2 iron-sulfur protein	4.1	9.60E-03
EFER_RS 14810	<i>gpr</i> , L-glyceraldehyde 3-phosphate reductase	3.0	3.08E-02
EFER_RS 15490	<i>btsT</i> , pyruvate:H ⁺ symporter	2.4	2.57E-02
EFER_RS 15500	<i>yjiA</i> , P-loop guanosine triphosphatase	2.3	2.50E-02
EFER_RS 15550	conserved hypothetical protein, putative DEAD-box helicase-related protein	2.2	4.08E-02
EFER_RS 16080	<i>yhcH</i> , DUF386 domain-containing protein	2.2	3.44E-02
EFER_RS 16085	<i>nanK</i> , N-acetylmannosamine kinase	3.7	6.02E-06
EFER_RS 16090	<i>nanE</i> , putative N-acetylmannosamine-6-phosphate epimerase	4.0	5.34E-06
EFER_RS 16095	<i>nanT</i> , N-acetylneuraminate:H ⁺ symporter	4.4	5.34E-06
EFER_RS 16100	<i>nanA</i> , N-acetyl- β -neuraminate lyase / N-acetylneuraminate lyase	4.2	1.64E-05
EFER_RS 17100	<i>glgP</i> , glycogen phosphorylase	2.0	3.96E-02
EFER_RS 17105	<i>glgA</i> , glycogen synthase	2.4	1.09E-02
EFER_RS 17765	<i>yhjX</i> , putative pyruvate transporter	5.5	4.91E-06
EFER_RS 19215	<i>glpF</i> , glycerol facilitator	3.4	8.42E-05
EFER_RS 19220	<i>glpK</i> , glycerol kinase	4.0	6.70E-08
EFER_RS 19860	<i>dsdA</i> , D-serine ammonia-lyase	5.3	3.65E-12
EFER_RS 20020	<i>tmaA</i> , tryptophanase / L-cysteine desulfhydrase	6.5	3.65E-12

EFER_RS 20025	<i>tnaB</i> , tryptophan:H ⁺ symporter TnaB	3.1	1.52E-03
EFER_RS 20565	<i>malG</i> , maltose ABC transporter membrane subunit MalG	2.2	3.60E-02
EFER_RS 20590	<i>lamB</i> , maltose outer membrane porin / phage lambda receptor protein	2.4	2.25E-02
EFER_RS 20595	<i>malM</i> , maltose regulon periplasmic protein	2.2	2.37E-02
EFER_RS 20830	<i>fumB</i> , fumarase B	4.0	6.66E-03
EFER_RS 20835	<i>dcuB</i> , anaerobic C4-dicarboxylate transporter	5.8	4.79E-05
EFER_RS 20850	<i>dmsA</i> , dimethyl sulfoxide reductase subunit A	4.4	3.93E-03
EFER_RS 20855	<i>dmsB</i> , dimethyl sulfoxide reductase subunit B	3.6	2.01E-02
EFER_RS 20975	<i>frdD</i> , fumarate reductase membrane protein	2.9	1.78E-02
EFER_RS 20980	<i>frdC</i> , fumarate reductase membrane protein	3.1	1.19E-02
EFER_RS 20985	<i>frdB</i> , fumarate reductase iron-sulfur protein	3.4	9.91E-03
EFER_RS 20990	<i>frdA</i> , fumarate reductase flavoprotein subunit	3.3	2.01E-02
EFER_RS 21425	<i>fdhF</i> , formate dehydrogenase-H	2.9	4.44E-02
EFER_RS 21860	<i>exuT</i> , hexuronate transporter	3.2	1.93E-02

^a Fold change in gene expression between growth under oxidative stress and normal growth.

Table 5. Potential negative targets of MgrR under oxidative stress in *E. fergusonii*.

Locus tag	Name and description	Fold change (log2) ^a	Adjusted p-value
EFER_RS 01690	<i>queD</i> , 6-carboxy-5,6,7,8-tetrahydropterin synthase	-2.6	4.44E-02
EFER_RS 02210	<i>stpA</i> , H-NS-like DNA-binding transcriptional repressor with RNA chaperone activity	-2.7	4.94E-02
EFER_RS 03305	<i>suhB</i> , inositol-phosphate phosphatase	-3.1	3.98E-03
EFER_RS 04985	<i>pdeI</i> , c-di-GMP phosphodiesterase	-3.2	2.46E-02
EFER_RS 04990	<i>dgcI</i> , putative diguanylate cyclase	-3.3	9.53E-03
EFER_RS 04995	<i>rimO</i> , ribosomal protein S12 methylthiotransferase RimO	-2.7	2.50E-02
EFER_RS 06790	<i>menI</i> , 1,4-dihydroxy-2-naphthoyl-CoA hydrolase	-2.9	8.85E-03
EFER_RS 07790	<i>ydeE</i> , dipeptide exporter	-2.5	5.41E-03
EFER_RS 08415	<i>yciH</i> , putative translation factor	-2.9	1.41E-02
EFER_RS 10540	<i>plaP</i> , putrescine:H ⁺ symporter	-2.8	5.93E-03
EFER_RS 10825	<i>udk</i> , uridine/cytidine kinase	-3.1	2.34E-02
EFER_RS 13595	<i>borD</i> , DLP12 prophage; prophage lipoprotein	-3.1	4.27E-03
EFER_RS 13950	<i>ygdQ</i> , UPF0053 inner membrane protein	-3.9	4.93E-06
EFER_RS 16305	<i>fis</i> , DNA-binding transcriptional dual regulator	-3.1	7.34E-03
EFER_RS 17425	<i>yibA</i> , putative lyase	-3.2	1.19E-02
EFER_RS 18115	<i>yihG</i> , putative acyltransferase	-2.8	3.43E-02

^a Fold change in gene expression between growth under oxidative stress and normal growth.

FIGURES

Figure 1. Alignment of *E. coli* and *E. fergusonii* *mgrR* sequences. The transcribed region of *mgrR* is shown. The yellow highlight represents the highly conserved seed region which is disrupted by the insertion of the underlined sequence in *E. fergusonii*. The nucleotides were aligned using LALIGN (Huang & Miller 1991).

Figure 2. The transcriptional regulation and known functions of MgrR. In low concentrations of Mg^{2+} expression of MgrR increases. MgrR down regulates EptB, GrlR and SoxS and up regulates GrlA. The RNA sponge SroC down regulates MgrR through direct base-pairing. EptB encodes a phosphoethanolamine transferase which modifies the KDO molecule of the LPS with the addition of a phosphoethanolamine (PETn). The addition of the PETn to the KDO molecule increases resistance to polymyxin B.

PhoP binding site -1

ACAGAACTG**GATAA**CCCTT**GTTTA**ACTGTCATTAGCTTAAAAGCAGTAAAATTCTTAAT

+1

GAGTCGTTATCGGGTACAAAATGCCTGGTAGCGTAAAAAGCAAAACACATATCGCGACT

CTCCATAGGTCGAGTTGATGCAAATGCAACTGAATTATGTCGAGTATATCCATGCAAGCATT

TTCCGCTGGTCACTCCAGCGGTTTTTTTT

Figure 3. The organization of *mgrR* in *E. fergusonii*. The GATAA-5nt-GTTTA sequence in bold italics is a possible PhoP binding site. The bolded G represents the transcriptional start site (+1) of *mgrR* which is determined from RNA-seq. The nucleotides highlighted in yellow represent the highly conserved seed region and the underlined region represents the 53 bp insertion sequence. The end of the sequence was determined from

Figure 4. Predicted RNA secondary structures of MgrR. Wild-type *E. coli* (left), wild-type *E. fergusonii* (center) and *E. fergusonii* MgrR lacking nucleotides +54 to +106 (right) are shown. Secondary structures were predicted using RNAstructure software (Reuter & Mathews 2010).

Figure 5. Full length MgrR is expressed in *E. fergusonii*. ~200 bp PCR fragment of MgrR from 3' RACE is shown (Lane 2) next to a DNA ladder (Thermo Fisher Scientific) (Lane 1). The PCR fragment is slightly larger than the original length of MgrR (151 nt) due to the addition of a poly-adenosine tail and adapter sequence during the 3' RACE. The 3' end of MgrR was confirmed with Sanger sequencing.

Figure 6. MgrR expression increases in low Mg²⁺ concentrations. MgrR and PmrD (used as a control) expression (mean +/- standard deviation) measured using qPCR when *E. fergusonii* was grown in low Mg²⁺ (10 μ M) condition compared to high Mg²⁺ (10 mM) condition is shown. The values were calculated from three independent growth experiments. Values were normalized with 16S mRNA values.

Figure 7. Over-expression of MgrR does not impact EptB mRNA levels. Fold difference of *mgrR* and *eptB* expression as measured by RT-qPCR in WT *E. fergusonii* during over-expression of either full-length MgrR (P_{BAD33-mgrR}), MgrR lacking the insertion sequence (nucleotides +54 to +106; P_{BAD33-mgrR-NI}) or empty vector (P_{BAD33}) in comparison to *eptB* expression in RL7 ($\Delta mgrR$ with empty P_{BAD33}) is shown. Data represents the mean values of three biological replicates $\pm$ 95% confidence intervals. All samples were normalized with 16S mRNA levels.

Figure 8. Deletion of *mgrR* causes sensitivity to DOC. Survival rates of AW28 ($\Delta mgrR$) and AW51 (AW28 complemented with WT *mgrR* gene) on LB agar plates containing 0.1% DOC (w/v) were normalized to WT (*E. fergusonii* ATCC 35469) rate considered as 100%. Data represents the means of three biological replicates $\pm$ 95% confidence intervals. *** represents $p=0.0005$ (between AW28 and WT).

A **B**

Figure 9. Deletion of MgrR causes sensitivity to H₂O₂. The indicated strains were exposed to 19 mM H₂O₂ for 15 min at 37°C and then were plated on LB agar to determine CFU. (A) 10⁻² dilution plates are shown. (B) H₂O₂ sensitivity is expressed in terms of percent survival. Data The represents the mean of three biological replicates ± 95% confidence intervals. Student t-tests were performed with GraphPad Prism software. ** represents p<0.005 and * represents p<0.05.

Figure 10. Impact of MgrR loss on H₂O sensitivity. Overnight growths of the indicated strains wild-type *E. fergusonii*, $\Delta mgrR$ complement *E. fergusonii* (AW51), $\Delta mgrR$ *E. fergusonii* (AW28), wild-type *E. coli* (NM22540), $\Delta mgrR$ *E. coli* (KM128) were plated on LB agar. (A) 10 mm paper disks were loaded with 10 μ l of 9.77 M H₂O₂. (B) The area of the clearing zone was measured with ImageJ. The error bars show 95% confidence intervals between three biological replicates. Student t-tests were performed with GraphPad Prism software. *** represents $p < 0.0001$ and ns represents $p > 0.05$.

Figure 11. mRNA levels of TnaA are likely maintained by MgrR under oxidative stress. Fold change of the mRNA levels of KatG, MgrR and TnaA in response to H_2O_2 as determined by RT-qPCR. The dotted lines represent a fold change of 1 or -1 which is equivalent to no change. Error bars represent variation between three biological replicates. * represents $p < 0.05$.

Figure 12. MgrR and TnaA mRNA do not base-pair. EMSA was performed using increasing amounts of MgrR RNA incubated with 0.2 pmol *tnaA* mRNA or DIG labelled anti-MgrR RNA and visualized with Syber II.

Figure 13. Deletion of MgrR decreases the fitness of *E. fergusonii*. Colony forming units of WT and RL7 were calculated from fecal samples and the relative % population of RL7 was determined for each day. Data represents the mean of three biological replicates $\pm$ standard deviation.

Figure 14. Mice maintain wild-type and RL7 at equivalent levels. Colony forming units (CFU) from fecal samples collected on indicated days from mice inoculated either with WT or RL7 strain of *E. fergusonii*. Data represents the mean + standard error from three technical replicates.

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