ESTERASES IroE and Fes PARTICIPATE IN THE PROTECTION OF *SALMONELLA* AGAINST OXIDATIVE STRESS.

An Undergraduate Research Scholars Thesis

by

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TABLE OF CONTENTS

ABSTRACT

Esterases IroE and Fes Participate in the Protection of *Salmonella* Against Oxidative Stress

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Salmonella enterica serotype Typhimurium is the leading cause of foodborne illnesses in the United States causing nearly 1.4 million cases and causes hundreds of millions of cases worldwide each year. A multidrug efflux pump called MacAB is essential for the survival of *S.* Typhimurium in the presence of reactive oxygen species, including hydrogen peroxide (H_2O_2), *in vitro.* We know that the expression of *macAB* is induced upon exposure to H_2O_2 and is necessary for the survival of *S.* Typhimurium in the presence of H_2O_2.

Two esterases, Fes and IroE, produced by *Salmonella*, degrade enterobactin to linear products including 2,3-dihydroxybenzoylserine trimer ($DHBS_3$), a linear trimer of enterobactin. We found that removing the genes encoding the pump (MacAB) or the *iroE* and *fes* esterases, reduces survival of the mutant strains when reactive oxygen species are present. Surprisingly, inserting a functional *iroE* gene back into the mutant strain lacking both esterases (*ΔfesΔiroE*) allows *S.* Typhimurium to survive oxidative stress. From this data we conclude that IroE can restore peroxide resistance to a double mutant lacking both IroE and Fes. In the future, we will

determine if Fes can also rescue this double mutant from oxidative stress. These genes and the processes in which they participate are potential targets for the development of novel therapeutics to combat this increasingly antibiotic resistant pathogen.

DEDICATION

This work is dedicated to Helene, Lydia, the HAP lab, and my Amma.

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I would like to thank my faculty advisor, Dr. Helene Andrews-Polymenis, for showing me my innate worth and helping me realize anything can be done if I have the courage to ask for it.

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And finally, to the most important woman in my life, I would like to thank Dr. Latha Ravichandran, my Amma, for raising me to know that I can accomplish anything with a little bit of hard work.

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NOMENCLATURE

Carb	Carbenicillin antibiotic
LB	Lysogeny Broth
H_2O_2	3mM Hydrogen Peroxide
ROS	Reactive Oxygen Species
CM	Chloramphenicol
DHBS3	2,3-dihydroxybenzoylserine linear trimer
Nal	Nalidixic acid

CHAPTER I

INTRODUCTION

Salmonella enterica subspecies Enterica organisms are rod-shaped, facultative anaerobic, gram-negative bacteria and it is also the leading bacterial cause of foodborne illnesses in the United States. These organisms cause approximately 1.4 million cases of disease each year in the United States, and hundreds of millions of cases worldwide[7]. S. Typhimurium, one serotype in this group, colonizes the intestine, where it is exposed to harsh conditions including reactive oxygen species produced by neutrophils and intestinal epithelial cells[11]. Symptoms of this common bacterial infection include, abdominal cramps, nausea, and diarrhea that occur 12-72 hours after ingesting a contaminated substance, usually either food or water[1]. The infection of the intestinal tract during human salmonellosis is a classic inflammatory diarrhea, specifically characterized by substantial neutrophilic inflammation and ROS production in the gut[6]. In otherwise healthy individuals, this bacterial infection runs its course in 5-7 days after initial infection[3,4]. Unlike other bacterial infections, antibiotics given to humans with salmonellosis do not shorten the length of the infection. In fact, such treatment may result in prolonged fecal shedding and an increased chance of relapse[2,5]. Therefore, antibiotics are usually not prescribed to treat salmonellosis. However, antibiotic use is appropriate for individuals at high risk for complications, including children under the age of three, elderly people, and those with compromised immune systems usually from diabetes or HIV[12].

In general, bacterial resistance to antibiotics is not uncommon and can be caused by a large variety of mechanisms that include drug and target modification, and inhibition of uptake. A

reduction in expression of porins protected against β-lactams and mutations in the genes *gryA* and *gyrB* can also result in resistance to antibiotics

A common bacterial defense is to actively efflux antimicrobial compounds[1]. Drug efflux pumps are integral membrane proteins that actively pump antibiotics from the cell and are recognized as a necessary mechanism for protection of the cell against the toxic action of antibiotics and other drugs[10]. Drug efflux pumps are usually chromosomally encoded and belong to an ancient family of proteins[1].

The antibiotic typically used in cases of *Salmonella* infections is ciprofloxacin. However, *Salmonella* appears to be resistant to this antibiotic *in vivo* even though the drug is present in extremely high doses in the intestinal tract and was known to successfully penetrate the mammalian cells after oral administration[1].

A multidrug efflux pump called MacAB sits in the bacterial inner membrane and periplasm of *Salmonella* and protects the bacteria from macrolide antibiotics. MacAB pump is essential for the survival of S. Typhimurium in the presence of reactive oxygen species *in vitro*, for survival environments where reactive oxygen species are a prominent innate immune defense, including in the intestines, and in macrophages. This pump plays a role in the detoxification of various ROS compounds that Salmonella are exposed to during the different stages of infection. We know that MacAB is induced upon exposure to H_2O_2, is necessary the survival of S. Typhimurium in the presence of H_2O_2 and is required for detoxifying peroxide on the outside of the bacterial cell. Our previous work suggests that MacAB is important for the secretion of a product that protects the cell from oxidative stress. This product appears to be related to an important iron chelating metabolite called enterobactin that is produced by S. Typhimurium by

non-ribosomal peptide synthesis (NRPS). The role of ferric ion chelating molecules, known as siderophores, is to scavenge iron in the environment and deliver it to the cell.

Two esterases, Fes and IroE, produced by *Salmonella* degrade enterobactin to linear products including 2,3-dihydroxybenzoylserine trimer, a linear trimer of enterobactin ($DHBS_3$). We found that removing the genes encoding the pump (MacAB) or the IroE and Fes esterases, reduces survivability of the mutant strains when reactive oxygen species are present. Our data shows that wild type *S.* Typhimurium needs both esterases to ensure the inactivation of peroxide in the media. Surprisingly, inserting a functional *iroE* gene back into the double mutant strain lacking both esterases allows the *S.* Typhimurium to survive oxidative stress. From this data we conclude that IroE can rescue the peroxide sensitivity of a double mutant lacking both *iroE* and *fes*. In the future, we will determine if *fes* can also rescue this double mutant from oxidative stress. These genes and the processes in which they participate are potential targets for the development of novel therapeutics to combat this increasingly antibiotic resistant pathogen.

CHAPTER II

METHODS

Strains and Growth Conditions

All strains used in this study are derivatives of *Salmonella* enterica. Typhimurium 14028S. HA420 is a spontaneously Nalidixic acid resistant mutant of *Salmonella* enterica. Typhimurium 14028[1]. pWSK29 is a low copy plasmid[13]. A mutant lacking genes *iroE* and *fes* was made by homologous recombination using lambda red technology[13]. pWSK29:*iroE* plasmid contains the coding region of the *iroE* gene as well as approximately 500 base pairs upstream. Construction of this plasmid will be discussed elsewhere.

In all the experiments, bacteria were grown in LB broth supplemented with 100mg/L of carb. The strains used in this study are labeled in Table 1.

Table 1: Strain References

Strain #	Strain Name	Notes
Strain 1	HA420 pWSK29	Wild type strain.
Strain 2	HA420 *ΔiroEΔfes*+pWSK29	*ΔiroEΔfes*. A strain lacking the esterases IroE and Fes.
Strain 3	HA420 *ΔiroEΔfes*+pWSK29::*iroE*	Double deletion strain (Strain 2) complemented with IroE
Strain 4	*ΔmacAB pWSK29*	Strain lacking MacAB efflux pump, previously shown to be sensitive to hydrogen peroxide. (Used as a positive control)

Growth Curves

To prepare the overnight cultures 5 mL of LB with Carb was added into four different 15mL conical tubes along with a single colony of each of the four strains listed in Table 1. The colonies were added into the tubes by taking a 200 µL pipette tip and gently picking up a colony from the streaked plates using the bottom of the tip. The pipette tip was then gently dropped into the tubes marked for each strain. These tubes were placed in the shaker with aeration at a speed of 200 rpm and a temperature of 37 ºC for 16-24 hours. The next day, the tubes were removed from the shaker and the process of inoculation began.

5 mL of LB were added into each of 8 different 15 mL conical tubes. The first 4 tubes were filled with only LB labeled for strains 1 through 4 while the second 4 tubes were filled with both LB and hydrogen peroxide to a final concentration of 3 mM and labeled with the strain numbers 1 through 4. Then 50 µL of each of the corresponding overnight cultures were added into both an LB tube and an LB tube with hydrogen peroxide (1:100 dilution). The cultures were grown for 5 hours at 37ºC with aeration and were sampled at hourly intervals for 200 µL which was serially diluted and plated (hours 0-5). Each time point bacterial suspensions were serially diluted by taking 20 µL of the previous dilution, plus 180 µL of 1x PBS solution. Resulting dilutions were plated on plates containing carbenicillin. 50 µL of each dilution was plated and placed in the incubator and left at 37° C overnight. The following morning, plates were removed from the incubator and the colony forming units from each time point of each strain were counted, recorded, and graphed using a pre-developed template in GraphPad PRISM. The overview of the growth curve procedure can be seen in Figure 1 below.

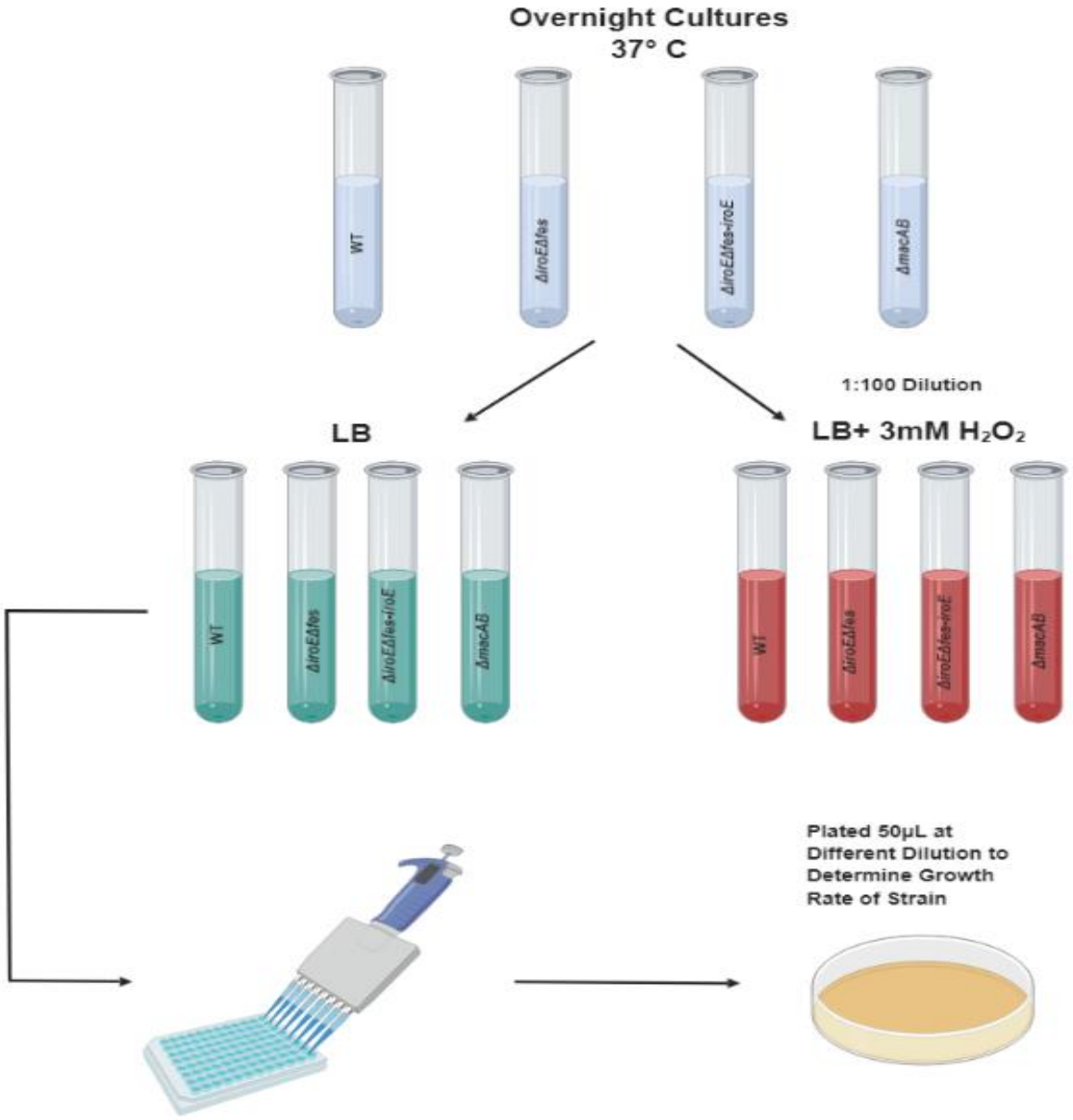


Figure 1: Outline of Growth Curve Procedure

Mouse Infections

All animal experiments used in this study were approved by conducted as approved by the TAMU IACUC, and followed the guidelines set by the Guide for the Care and Use of Laboratory Animals. To mimic human colitis animals were pretreated with 20 mg of streptomycin by gavage 24 hours prior to infection with Salmonella strains. The inocula were generated by growing strains HA420 ΔiroEΔfes+pWSK29 and HA420ΔiroEΔfes+pWSK29::iroE overnight at 37 degrees with aeration in LB Carb. The resulting cultures were used to infect the mice with approximately 108 bacteria in 100µL. Groups of 5 mice were inoculated per strain by gavage. The infected mice were examined at least twice daily for changes in behavior, body condition and signs of disease. Bacterial cultures used for infection were serially diluted and plated to determine the exact titer of each strain used as the inoculum.

4 days post infection, the mice were humanely sacrificed and the spleen, liver, cecum, mysenteric lymph nodes and the Peyer's patches of each of the 10 mice were removed and placed in a 50 mL conical tube with 3 mL of 1x PBS solution. A piece of each organ collected was placed in a tissue collection cassette and processed for pathology. The remaining organs were homogenized using a tissue grinder, serially diluted using sterile PBS and plated on solid LB containing carbenicillin. The plates were placed in an incubator at 37º C overnight, and the next morning the *S.* Typhimurium colony forming units were counted, recorded, and graphed using the PRISM software. Statistical significance was determined using a Student *t* test.

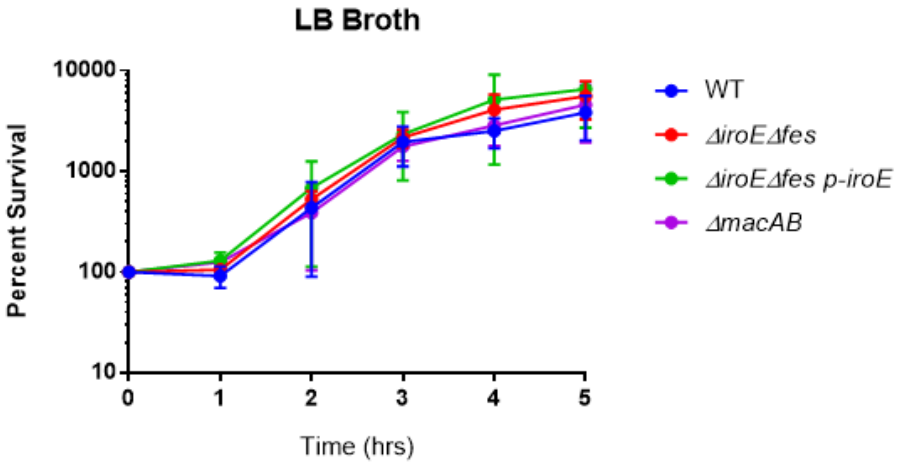
CHAPTER III

RESULTS AND DISCUSSIONS

The role of IroE and Fes esterases in protection of Salmonella from oxidative Stress in vitro

The role of IroE and Fes esterases in protection of Salmonella from oxidative Stress in vitro. All the strains used in this study grew with similar kinetics in the LB broth (Figure 2A). No growth defects were associated with the loss of IroE and Fes. These results show that neither fes nor iroE are required for bacterial growth under normal growth conditions. In contrast, in the presence of peroxide strains showed a different growth pattern (Figure 2B). Wild type Salmonella (HA420 pWSK29) was able to survive and grow the presence of hydrogen peroxide in the second hour, in contrast to the ΔmacAB mutant strain that was killed by peroxide as expected13. In our hands the macAB mutant strain was not able to resume growth at any point in the experiment. The ΔiroEΔfes double mutant strain was also killed in peroxide, similar to the macAB deletion strain (Figure 2B). These results suggest that esterases involved in the processing of enterobactin are required for the protection of Salmonella from oxidative stress. Return of the iroE gene in trans to the ΔiroEΔfes double mutant, reversed the growth defect in the presence of peroxide caused by deletion of the esterases (Figure 2B). This result shows that the iroE can replace the functions of both esterases that are needed for resistance to oxidative stress. We can now directly link the hydrogen peroxide sensitivity phenotype we observe in vitro to the loss of these genes and demonstrate that the presence of the esterase iroE is sufficient to restore growth in the presence of peroxide. In the future we hope to test to see if the esterase fes can be replaced for iroE.

A.



B.

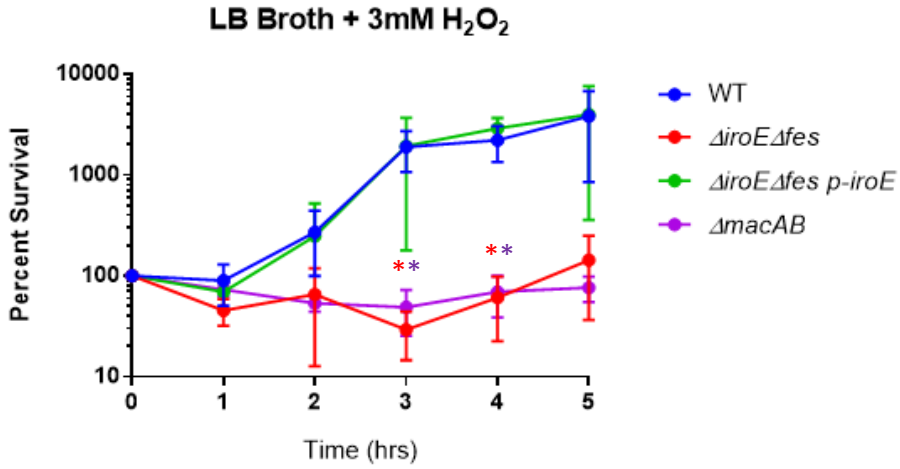


Figure 2. Iron Rescues an ΔiroEΔfes Mutant Strain from Peroxide Mediated Killing in Vitro.

The role of IroE and Fes esterases in protection of Salmonella from oxidative Stress in vivo.

While we found that *iroE* and *fes* are required for the growth of the bacteria in the presence of oxidative stress *in vitro,* we wanted to test the role of these genes during infection. To test these hypotheses, we used the murine colitis model of salmonellosis. We pretreated C57BL6 mice with streptomycin, and subsequently infected them with S. Typhimurium isolates including the wild type, the *ΔiroEΔfes* double deletion, and *ΔiroEΔfes* double deletion complemented with *iroE* (just use actual strain names). In this model, mice infected with S. Typhimurium develop an intestinal infection with this organism characterized by a neutrophilic inflammatory response. Our previous studies show that mutants lacking *iroE* and *fes,* poorly colonize the cecum and other intestinal tissue in streptomycin pre-treated mice (unpublished data). In our experiment, the *ΔiroEΔfes* double mutant bearing an empty vector plasmid (pWSK29), colonized the intestine poorly in the cecum, Peyer's patches, and mesenteric lymph nodes (MLN), concurrent with our previous unpublished findings (Figure 3). The strongest phenotype was observed in the colonization of the cecum where the double mutant colonized ~100 fold less than wild type S. Typhimurium HA420. The cecum is where *Salmonella* creates a niche for itself through induction of inflammation. Our data showed that the *ΔiroEΔfes* double mutant had colonization defects in Peyer's patches, and mysenteric lymph nodes similar to those in the cecum (Figure 3). Finally, the *ΔiroEΔfes* double mutant colonizes the liver and spleen at levels similar to the wild type organism.

Furthermore, the growth of the *ΔiroEΔfes* double mutant during infection was restored when the *iroE* gene was placed *in trans* (pWSK29::*iroE,* Figure 3). In contrast to the *ΔiroEΔfes* double mutant double mutant that colonized the cecum at ~10^6 bacteria/ gram of tissue, the complemented *ΔiroEΔfes* double mutant strain colonized the cecum at 10^8 bacteria/ gram of

tissue. Thus, return of the iroE gene alone reversed the growth defect of the *ΔiroEΔfes* double mutant strain in the inflamed intestine. We also noticed that the complemented *ΔiroEΔfes* double mutant colonized the Peyer's Patches and mysenteric lymph nodes better than the *ΔiroEΔfes* double mutant.

We demonstrated that the presence of *iroE* is sufficient to rescuing the double mutant lacking *iroE* and *fes* during infection. We can therefore assume that it is sufficient to have just have one of the esterases, *iroE*, to provide linearized enterobactin trimer- and restore bacterial growth in hydrogen peroxide. In the future we hope to test to see if *fes alone is also sufficient to restore growth to the ΔiroEΔfes* double mutant both in vitro and during infection. We suggest that this linear trimer of enterobactin, $DHBS_3$, is produced from the cleavage of enterobactin by esterases IroE and Fes.

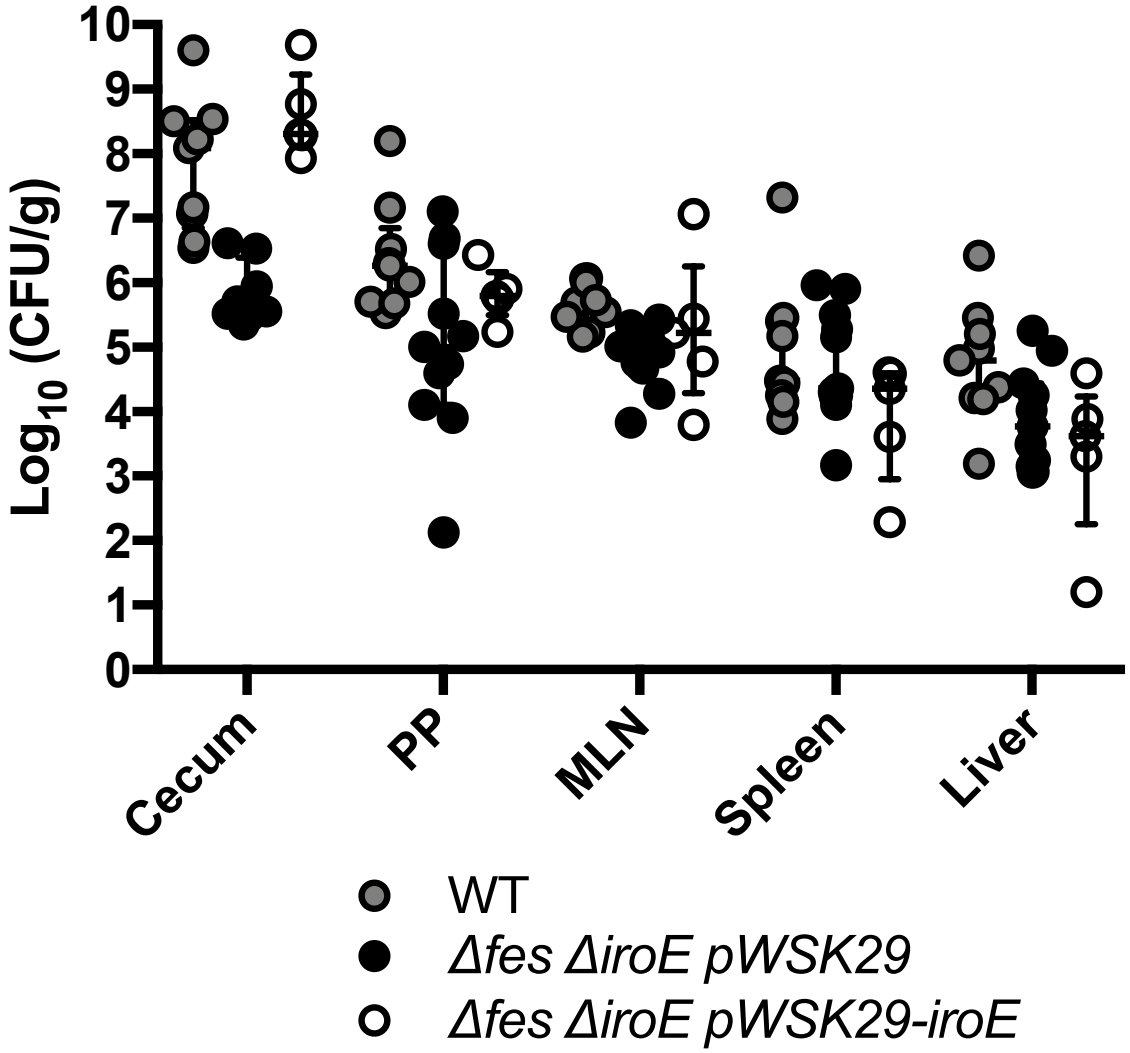


Figure 3: Iron Rescues an ΔiroEΔfes Mutant Strain from Peroxide Mediated Killing in Vivo

CHAPTER IV

CONCLUSION

The multidrug efflux system MacAB secretes linearized enterobactin trimer ($DHBS_3$) from the cell. This linear trimer of enterobactin, $DHBS_3$, is produced from the cleavage of enterobactin by the esterases IroE and Fes. Figure 4 shows a schematic diagram of the method the enterobactin uses to move the iron the *Salmonella* needs for survival into the close proximity of the bacteria cell, and of the locations and putative functions of both esterases.

We show that the esterases IroE and Fes are necessary for the protection of *Salmonella* against oxidative stress both *in vitro* and during infection. Previously, the reason why the IroE esterase was needed in the bacterial periplasm was unclear, but we now know that *Salmonella* requires the proteins encoding for *iroE* to process enterobactin in the periplasm, where it is likely passed to the MacAB efflux system for secretion to help S. Typhimurium survive oxidative stress (Figure 4).

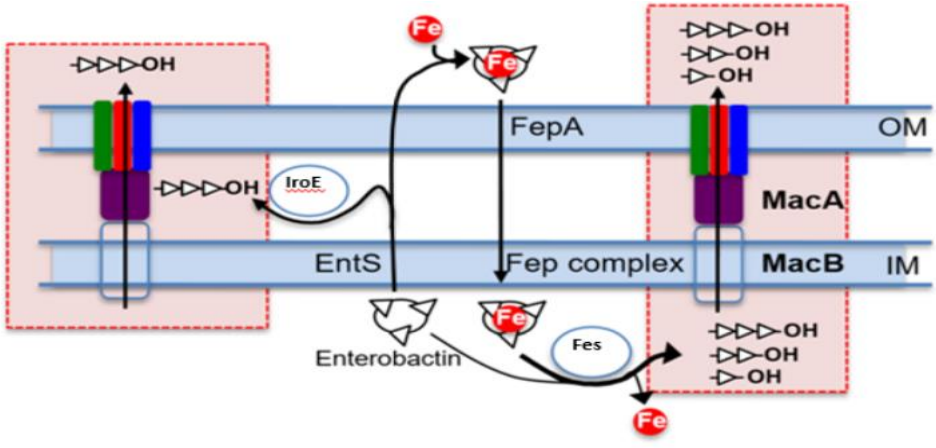
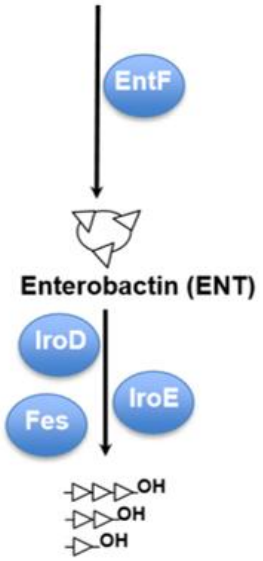


Figure 4. Model for Export of Linearized Enterobactin from *Salmonella*

Figure 5 shows several of the genes that are required for the protection of *Salmonella* against ROS. This data suggests that MacAB, IroE, and Fes are all required for survival in peroxide containing media. IroE and Fes are known to cleave enterobactin to $DHBS_3$, and we confirm that these enzymes are necessary both in vitro to survive peroxide mediated death and during infection in inflammatory conditions.



Strain	H_2O_2 survival, %	Residual $[H_2O_2]$, %
WT	167.7±37.4	5.5±1.7
ΔmacAB	29.8±6.3	81.6±10.0
ΔentF	27.3±9.4	83.6±5.8
ΔiroD	128.2±39.7	6.9±2.9
ΔiroE	53.5±13.6	28.9±14.5
Δfes	58.6±3.5	55.5±14.0
ΔiroE Δfes	23.5±16.7	83.8±2.0

Figure 5. Esterases IroE and Fes are Required for the Resistance of H_20_2

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