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Characterization of the transcription factor IscR in *Yersinia pseudotuberculosis*

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by

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Abstract

Regulation of the Ysc type three secretion system (T3SS) of the human gut pathogen *Yersinia pseudotuberculosis* has been a widely explored field. Located on the *Yersinia* virulence plasmid, the Ysc T3SS has been shown to be regulated by environmental cues such as calcium, temperature, and host cell contact, and by bacterial factor such as AraC-like transcriptional regulators and histone-like proteins. These mechanisms allow for *Yersinia* to respond to environmental changes as well as entry into the host. Through a forward genetic screen to identify novel factors that regulate the T3SS, we discovered IscR, a global transcriptional regulator that coordinates an iron sulfur (Fe-S) cluster. No reports on *Yersinia pseudotuberculosis* IscR have been published, though *Escherichia coli* IscR is well studied. Here we describe the identification and initial characterization of this novel *Yersinia* transcriptional regulator. We constructed an in-frame IscR deletion mutant in *Y. pseudotuberculosis* (Δ*iscR*) as well as a mutant expressing an *iscR* allele unable to coordinate an Fe-S cluster (apo-IscR). Interestingly, both the *Y. pseudotuberculosis* Δ*iscR* and apo-IscR mutants lacked the ability to secrete effector proteins and target macrophages through the T3SS. In contrast, the Δ*iscR* mutant displayed normal flagellar motility while the apo-IscR mutant had a severe motility defect. The

flagellar basal body is a T3SS itself, indicating that the defect in the Ysc T3SS displayed by the Δ*iscR* mutant is not a result of gross abnormalities in secretion systems. Accordingly, the Δ*iscR* mutant showed normal growth in both rich and minimal media, while the apo-IscR mutant displayed a selective growth defect only in rich media. This suggests that the ratio between Fe-S-bound holo-IscR and apo-IscR may be important for balanced growth. Lastly, preliminary data suggest that the Δ*iscR* mutant has increased resistance to hydrogen peroxide in comparison to the parental strain, while apo-IscR was susceptible. Our findings suggest that *Y. pseudotuberculosis* IscR is an important regulator of the Ysc T3SS and plays a role in controlling resistance to ROS, motility, and balanced growth *in vitro*. As the *iscR* gene is almost identical in *Y. pseudotuberculosis*, *Y. enterocolitica*, and *Y. pestis*, we speculate that IscR may be important for the virulence of all three human pathogenic *Yersinia*.

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1 Introduction

1.1 Background

1.1.1 *Yersinia pseudotuberculosis* Type III Secretion System Regulation

Of the 17 *Yersinia* species, only three are pathogenic to humans: *Yersinia pestis*, the causative agent of the bubonic plague and two enterpathogenic *Yersinia*, *Yersinia enterocolitica*, and *Yersinia pseudotuberculosis.* All three have a 70kb plasmid necessary for virulence that encodes the Ysc type three secretion system (T3SS) and its associated effector proteins (Guy R Cornelis, 2002). The T3SS is made up of about 25 proteins and is widely conserved amongst many Gram negative bacteria and is composed of three parts: the basal body, the needle apparatus, and the translocon (Guy R Cornelis, 2006; Yip & Strynadka, 2006).

The basal body is made up of two paired rings that span the inner and outer membrane (IM and OM, respectively) of the bacterial cell envelope and are joined together by a rod (Yip & Strynadka, 2006). At the base of the basal body lies the C-ring, thought to be composed of the protein YscQ. This information however, is only speculative, as there is not enough evidence to fully sustain this assertion. YscN, an ATPase that aids in the secretion and translocation of effector proteins inside the host cytoplasm, is thought to interact with YscQ as well as YscL and YscK. YscL is thought to be the protein tethering YscN to the export channel. YscJ proteins make up the MS ring in the IM (Yip & Strynadka, 2006). Rather than forming a pore in the IM, YscJ is thought to create a specialized patch of membranes for recruiting putative IM-embedded components such as YscR, YscS, YscT, YscU, and YscV. The locations of

these IM-embedded components of the basal structure have yet to be identified (Guy R Cornelis, 2006). On the OM lies another ring made up of a secretin, YscC. Secretins are proteins that are able to form pores on the outer membrane of the bacteria. With the help of YscW, a lipoprotein anchored in the outer membrane, YscC is able to properly insert a pore in the OM as well as function as an anchoring for the needle complex (Guy R Cornelis, 2006; Yip & Strynadka, 2006).

The needle complex is a straight hollow tube approximately 60 nm in length and is made up of helical polymerized subunits of YscF (Guy R Cornelis, 2006). This molecular channel for effector protein translocation is regulated by YscP, a key regulator of needle length. The length of the needle is proportional to the number of residues in the middle region of YscP. It is thought to be the "molecular ruler" that is used to measure the length of the needle to ensure proper extension. Precise length of the needle in response to the distance between the bacterial outer membrane and the host cell is necessary for proper protein translocation (Mota & Cornelis, 2005).

The translocon is comprised of three proteins: YopD, YopB and LcrV. They are found to be encoded under one operon and are collectively essential for pore formation on host target membranes and proper translocation of effector proteins to the host cytoplasm. Both YopB and YopD contain hydrophobic domains and are inserted into the host membrane while LcrV serves as the platform on the YscF needle tip on which YopB and YopD are assembled (C. A. Mueller, Broz, & Cornelis, 2008).

A number of genes encoded on the virulence plasmid are regulated by an AraC-like transcriptional regulator, LcrF (Lambert de Rouvroit, Sluiters, & Cornelis,

1992). Transcription of LcrF in turn is thermoregulated by YmoA, a histone-like protein. YmoA is able to repress transcription of *lcrF* through its interaction sequence located in the 5' UTR of the operon encoding *lcrF*. At 37°C, YmoA is rapidly degraded and alleviates the repression of LcrF (Böhme et al., 2012; Hoe & Goguen, 1993). Translation of LcrF is at temperatures below 37˚C is also inhibited due to a cis-acting RNA thermosensor located in the intergenic region between *yscW-lcrF* (Böhme et al., 2012). At 37°C thermosensor repression is alleviated, allowing the ribosome to bind the ribosomal binding site and allowing translation (Böhme et al., 2012). Translated LcrF is then able to activate transcription of T3SS structural and effector protein genes.

Environment cues are important signals that aid in the regulation of the T3SS upon entry into host organisms. To further investigate alternative regulatory mechanisms of theT3SS, a forward genetic screen was performed (Kwuan, Ramirez, Herrera, and Auerbuch, unpublished data). Using a mariner transposon, a library of approximately 4,600 individual mutations was created. Two independent transposon insertions were identified in one particular gene, IscR, the transcriptional regulator of the iron-sulfur cluster (Isc) biogenesis system (Christopher J Schwartz et al., 2001). In *Yersinia*, not much is known about IscR except its similarity with IscR in *Escherichia coli* (Fig. 1) allows us some insight into the possible functions of IscR in *Y. pseudotuberculosis* (*Y.pstb*).

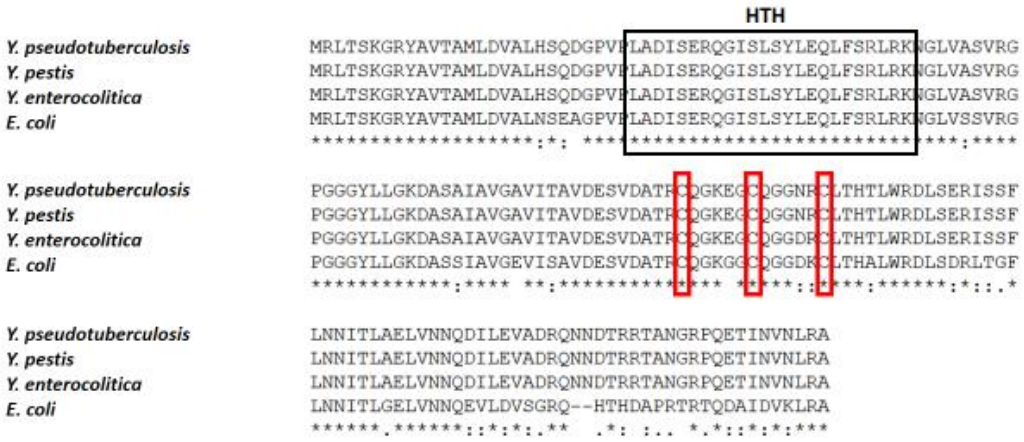


Figure 1. Similarity of E.coli IscR to *Yersinia* IscR. Amino acid sequence alignment of IscR generated with CLUSTALW showed ~79% identical residues (*), 13% similar residues (:), ~4% semi-conserved residues (.), and ~3% gaps. The black box indicates the location of the two helix-turn-helix DNA-binding domains of IscR. The three conserved cysteine residues (C92, C98, C104) of IscR that are thought to coordinate the [2Fe-2S] cluster are outlined in red.

1.1.2 Iron-Sulfur Clusters

As one of the most primordial, ubiquitous and functionally diverse classes of biological prosthetic groups, iron-sulfur (Fe-S) clusters can be found in all three domains of life (Bandyopadhyay, Chandramouli, & Johnson, 2008; Lill & Ulrich, 2006). Fe-S clusters are generated and inserted into proteins by complex Fe-S cluster biosynthesis pathways (Ayala-Castro, Saini, & Outten, 2008; M. Johnson, Cockayne, Williams, & Morrissey, 2005; Py & Barras, 2010). In bacteria, Fe-S clusters are made and incorporated into Fe-S proteins by three identified unique mechanisms: the nitrogen fixation system (Nif), the iron- sulfur cluster system (Isc), and the sulfur utilization factor (Suf) system. Each system is unique in that they carry out Fe-S biogenesis under different environmental conditions for different proteins and yet

they are all unified through their machinery that includes a cysteine desulfurase, a scaffold protein and an iron binding/ scaffold protein.

Under low iron conditions, scavenging methods, such as the secretion of sideophores and hemophores, are employed to build up iron stores in the cell (Saha, Saha, Donofrio, & Bestervelt, 2012; Tong & Guo, 2010). Internalization and storage of iron must be carefully regulated because of its ability to become toxic to the cell at high concentration. This can lead to the over-production of reactive oxygen species (ROS) and cause cell damaging reactions like protein carbonylation and DNA damage (Imlay & Linn, 2012; Jang & Imlay, 2007; Touati, 2000). To protect against the dangers of iron mis-regulation, bacteria have put in place regulator proteins that are able to sense and prevent the formation of ROS. Regulators such as the ferric uptake regulator (Fur) and IscR regulate iron and Fe-S cluster biogenesis, respectively, in order to keep the balance of complex iron and free iron in check.

Iron homeostasis is not only important for biological function but also for survival. As seen in organisms such as *Shigella flexneri* and *Pseudomonas aeruginosa*, a lack of iron and Fe-S cluster regulation can lead to an increase in oxidative stress which can be detrimental for survival in the host environment (Kim, Bo-Young, Gee, & You-Hee, 2009; Runyen-Janecky et al., 2008). This review gives a simplified overview of the formation and regulation of prokaryotic Fe-S cluster biogenesis pathways.

Since its first discovery in the early 1960s, over 120 unique types of enzymes and proteins have been identified to have an Fe-S cluster (Bandyopadhyay et al.,

2008; Beinert, 1997). Fe-S clusters have been thought to be one of the first iron cofactors, and that life may have formed on the surface of Fe-S minerals (Wächtershäuser, 1988, 1992). Their utility can be clearly seen through their adaptable structure and unique features, allowing for the Fe-S cluster to be a versatile prosthetic group. Fe-S clusters are made up of iron and sulfur in varying molar ratios. These clusters have been commonly found as the rhombic [2Fe-2S] or the cubane [4Fe-4S] and more rarely [3Fe-4S] or [8Fe-7S]. They generally serve as single electron carriers with the exception of [8Fe-7S], a two electron carrier that is only found in nitrogenases (Peters et al., 1997). Each form of Fe-S cluster possesses a different oxidation state that, depending on the cluster, can be anywhere from -2 to +4 (D. C. Johnson, Dean, Smith, & Johnson, 2005). These Fe-S clusters are particularly good as electron carriers due to their ability to spread the electron density among the Fe and S atoms. The redox potential of the coupled Fe-S cluster can range from -500mV to 150mV and is thought to be a possible oxidant at low-potential redox environments (Beinert & Kiley, 1999; D. C. Johnson et al., 2005).

Fe-S clusters components can be found in the environment. The sulfur comes from free cysteine synthesized by bacteria from serine, while iron, although readily found in the environment, is slightly more difficult to acquire (Kredich & Tomkins, 1966; Lithgow, Hayhurst, Cohen, Foster, & Aharonowitz, 2004; E. G. Mueller, 2006). The average microbe needs about 10^{-7} M of intracellular iron to function properly, but its bioavailability is 10^{-9} – 10^{-18} M (Braun & Braun, 2002; Saha et al., 2012). Environmental iron is generally present in two forms, ferrous and ferric iron.

Ferrous iron (Fe^{2+}) is relatively soluble while ferric iron (Fe^{3+}) is very insoluble. However, microbes have circumvented this obstacle by employing iron scavengers called siderophores and hemophores (Neilands, 1995; Tong & Guo, 2010). Both siderophores and hemophores are secreted or transported out of the cell through means of transport proteins or pumps. Siderophores are transported through three major pathways: the major facilitator superfamily (MFS), the resistance, nodulation and cell division (RND) superfamily, and the ABC super family (Saha et al., 2012). Hemophores on the other hand are secreted though the Type I secretion system, which is characterized by an ABC transporter, a membrane fusion protein and an outer membrane factor (Cescau et al., 2007). Once siderophores or hemophores have scavenged iron from the environment, they are internalized with the help of other membrane receptor proteins such as HasR (for heme) in *Pseudomonas* and FepA (for enterobactin), FhuA (for ferrichrome), and FecA (for ferric citrate) in *Escherichia coli* (Braun & Braun, 2002; Ochsner, Johnson, & Vasil, 2000). The siderophore/ hemophore complex is transported through the inner membrane by either ABC transporters or permeases (Braun & Braun, 2002; Hannauer, Barda, Mislin, Shanzer, & Schalk, 2010; Tong & Guo, 2010)

1.1.3 Iron-Sulfur Biogenesis Pathways in Bacteria

Nif

The nitrogen fixation (Nif) system was the first Fe-S cluster biogenesis system discovered by the lab of Dennis R. Dean during their study of the structural

components of nitrogenase in *Azotobacter vinelandii*, a nitrogen-fixing bacterium (Jacobson, Cash, Weiss, Laird, & Newton, 1989). This system was first discovered to be primarily used for nitrogenase maturation, but has also be found in non-nitrogen fixing bacteria such as *Helicobacter pylori* and *Entamoeba histolytica* (Ali, Shigeta, Tokumoto, Takahashi, & Nozaki, 2004; Olson, Agar, Johnson, & Maier, 2000).

NifS is a pyridoxal phosphate (PLP)-dependent cysteine desulfurase only active under nitrogen fixing conditions. Mechanistically, the PLP of NifS is used to form a Schiff base with the cysteine and form a cysteinyl persulfide through a nucleophilic attack (Fig. 2). This process allows the sulfur to be removed from the cysteine and an alanine to be release (E. G. Mueller, 2006). Cysteine desulfurases involved in Fe-S cluster assembly are divided into two groups defined by consensus sequences surrounding the C-terminal domain Cys residue at the active site. Group I has the sequence SSGSA**C**TS, while Group II has the sequence RXGHH**C**A. NifS belongs to Group I. The significance between groups I and II are have yet to be determined (Ayala-Castro et al., 2008; Mihara, Kurihara, Yoshimura, Soda, & Esaki, 1997).

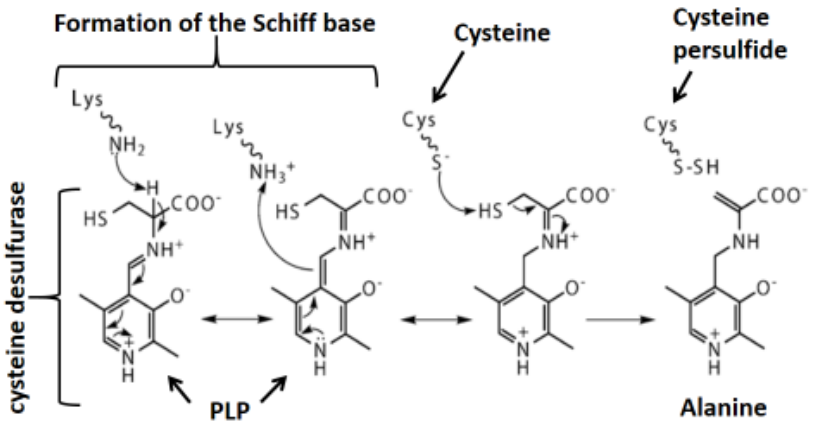


Figure 2. Proposed general mechanism of cysteine desulfurase activity.
Edited from Mihara and Esaki 2002

NifU is a U-type scaffold protein that mediates the formation of the Fe-S cluster (Yuvaniyama, Agar, Cash, Johnson, & Dean, 2000; Zhao, Curatti, & Rubio, 2007). NifU has three highly conserved domains that are capable of coordinating Fe-S clusters. The domains are located at the N-terminal, center and C-terminal regions of the protein. Of the three regions, the N-terminal (Cys35, Cys62, and Cys106) and the C-terminal (Cys137, Cys139, Cys172, and Cys175) regions are capable of coordinating Fe-S clusters and transferring them to respective apo-proteins. The central region of NifU, a ferredoxin-like domain, is reserved for a stable Fe-S cluster that cannot be transferred and is thought to be used in the redox process during assembly (Agar et al., 2000; Fu, Jack, Morgan, Dean, & Johnson, 1994; Yuvaniyama et al., 2000).

IscA^{Nif} is an A-type protein found after the discovery of IscA (discussed below). As an A-type protein it consist of 3 highly conserved Cys residues (**C**X_{42-44}DX_{20}**C**G**C**) in the C-terminal region of the protein. The function of IscA^{Nif} is still unclear though it is thought to have parallel function to IscA but in a nitrogenase specific manner.

Isc

The iron-sulfur cluster (Isc) biogenesis proteins (*iscSUAhscBAfdx*) are highly conserved proteins necessary for the insertion of Fe-S cluster into Fe-S proteins (Chandramouli & Johnson, 2006; D. C. Johnson et al., 2005). The Isc system was first identified in *A. vinelandii* after double deletion of *nifU* and *nifS* continued to allow low levels of active nitrogenase to be detected; this brought about speculation of the presence of housekeeping genes that carry out Fe-S biogenesis.

IscS is the cysteine desulfurase of the Isc pathway. Belonging to Group I of the cysteine desulfurases, IscS shares a great amount of sequence similarity to NifS near the active site cysteine as well as the PLP-binding site (Fig. 2) (C J Schwartz, Djaman, Imlay, & Kiley, 2000; Urbina, Silberg, Hoff, & Vickery, 2001). IscS and IscU form a 1:1 complex that creates a disulfide bond that allows IscS to pass along the sulfur to IscU while enhancing IscS activity (Fig. 3)(Kato et al., 2002).

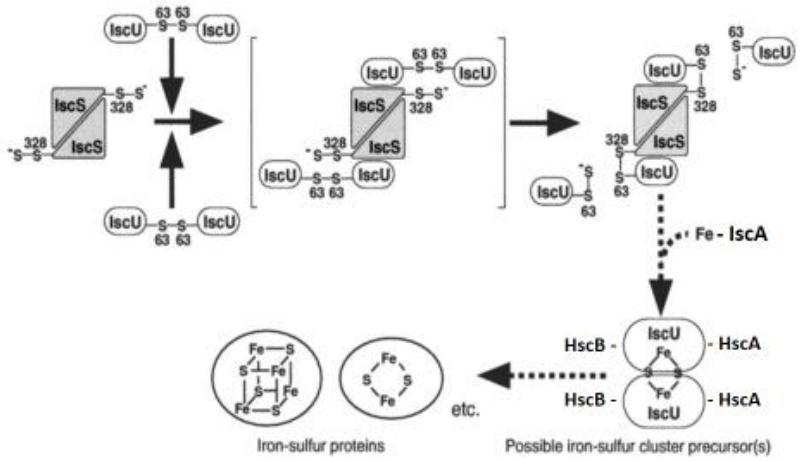


Figure 3. Proposed mechanism of the IscS/IscU complex. Steps noted in the dotted arrows are unknown. Edited from Kato et al 2002

Similar to NifU, IscU is a U-type scaffold protein that aids in the formation and transfer of Fe-S clusters to apo-protiens (Chandramouli & Johnson, 2006; Hoff, Silberg, & Vickery, 2000; Yuvaniyama et al., 2000). Yet unlike NifU, IscU needs molecular chaperones, HscB and HscA, to assist it the placement of Fe-S clusters onto apo-proteins. HscB and HscA (heat shock cognate) proteins are homologues to molecular chaperones DnaJ and DnaK (Bandyopadhyay et al., 2008). IscU is thought to interact with HscA at a conserved set of amino acids (LPPVK) to stimulate its ATPase activity. In addition, HscB can bind to the IscU/HscA complex to augment the ATPase activity of HscA. This ATPase activity then allows for an ATP-dependent transfer of the Fe-S cluster to an apo-protein. Uniquely, other U-type proteins do not require HscBA-like molecular chaperones in order to transfer the Fe-S cluster (Chandramouli & Johnson, 2006).

IscA, an A-type protein, has been shown to both act as a scaffold protein and an iron-binding protein (Krebs et al., 2001; Lu, Yang, Tan, & Ding, 2008). IscA has an iron association constant of $2.0\text{-}3.0 \times 10^{-19}$ M when in the presence of thioredoxin reductase system and dithiothreitol (Yang, Bitoun, & Ding, 2006). Though the mechanism of action is still unclear, IscA seems more likely to be an iron-binding protein first due to its high association constant with iron and a scaffold protein second, as IscU has already been shown to be the primary scaffold protein (Ding, Clark, & Ding, 2004; Ding & Clark, 2004). Upon binding to iron, IscA subsequently passes along its iron for the iron-sulfur cluster to the main scaffold, IscU (Ding et al., 2004; Yang et al., 2006).

While the complete function of Isc Fdx has yet to be elucidated, its redox potential is thought to initiate reductive coupling on the IscU complex. With the help of Isc Fdx, IscU would be more capable of forming mature [2Fe-2S] and [4Fe-4S] clusters for the respective apo-proteins (Bandyopadhyay et al., 2008). Isc Fdx also shares similar functionality with the ferredoxin-like center of NifU, though the two share no primary sequence similarities (Jung, Gao-sheridan, Christiansen, Dean, & Burgess, 1999).

Suf

The sulfur utilization factor (Suf) system is critical during oxidative stress or low iron conditions as an alternative for the Isc operon. Under conditions such as oxidative stress or low iron, the Isc operon is incapable of making Fe-S clusters (Jang

& Imlay, 2010). The transcription factor IscR triggers activation of the *sufABCDSE* operon to synthesize Fe-S clusters (see below) (Jang & Imlay, 2010; Outten, Djaman, & Storz, 2004; Xu & Møller, 2008). As seen in *Escherichia coli*, either the Isc or Suf pathway is essential; if both are absent the cell becomes nonviable (Desnoyers, Morissette, Masse, & Pre, 2009; Outten et al., 2004). The Suf operon is needed for the repair of Fe-S clusters that have undergone oxidative damage and is regulated by four transcriptional regulators: OxyR, IHF, Fur and IscR (J.-H. Lee, Yeo, & Roe, 2004; K.-C. Lee, Yeo, & Roe, 2008).

SufS, similar to NifS and IscS, is a cysteine desulfurase (Ayala-Castro et al., 2008; D. C. Johnson et al., 2005; Loiseau, Ollagnier-de-Choudens, Nachin, Fontecave, & Barras, 2003). It ensures the mobilization of the sulfur from free cysteines, however, it is slightly more complex. SufS also associates with SufE to become a novel cysteine desulfurase complex. This partnership allows for SufS to pass along the sulfur to SufE, which in turn passes it to SufB in the scaffold complex. The interaction between SufS and SufE also allows for higher activity of the cysteine desulfurase portion up to 8-fold (Outten, Wood, Munoz, & Storz, 2003).

SufBCD complex make up the scaffold protein of the Suf system. SufB and SufD form a complex with SufC in a 1:1:2 ratio, respectively, to form a scaffold also bound to a redox cofactor, $FADH_2$ (Wollers et al., 2010). SufB and SufD share sequence homology throughout the C-terminal region but not in the N-terminal domain. SufB subsequently contains a Fe-S motif, **C**X_2**C**X_3**C**, in the N-terminus that is thought to coordinate with SufE for the transfer of the sulfur (Wollers et al., 2010).

SufC is an ATPase that is activated by complexing with SufB. The SufBCD complex aids in enhancing the activity of SufE and, therefore, the cysteine desulfurase activity of SufS from 8- to 32-fold (Outten et al., 2003).

The activity of the A-type protein, SufA has yet to be fully determined but it may be an alternative iron-sulfur scaffold protein, an iron-binding protein or both (Layer et al., 2007; Lu et al., 2008).

1.1.4 Regulation of Iron-Sulfur Clusters in Response to Environmental Cues

Ferric Uptake Regulator (Fur)

As an essential element needed for the fundamental processes of life in most organisms, it is no surprise that the biological consumption of iron is regulated by the availability of iron. The ferric uptake regulator (Fur) is a homodimer made up of a helix turn helix motif in the N-terminal domain and two metal binding sites at the C-terminal domain important for dimerization (Escolar et al., 1999; Ochsner et al., 2000). Fur also contains a zinc binding site essential for activity, though its function is still a mystery (Pecqueur et al., 2006). As seen in *E.coli*, Fur is a global regulator that regulates over 90 genes that fall into three major categories: iron metabolism, energy production and miscellaneous/unknown (McHugh et al., 2003). Fur requires an iron cofactor in order to bind to specific DNA sequences called “fur boxes” which are typically found at the -35 and -10 regions of the promoter relative to the transcriptional start site (Chen et al., 2007; Lorenzo, Wee, Herrero, & Neilands, 1987). Under iron rich conditions, holo-Fur (Fur with iron) binds to fur boxes and

represses transcription of a number of genes, including the *fur* gene itself (Lorenzo et al., 1987). While under iron-poor conditions, apo-Fur (Fur without iron) is not able to bind to *fur* boxes and, thus, repression is relieved.

An additional component of iron regulation by Fur occurs through the small, 90 nucleotide long RNA RyhB and the RNA chaperone Hfq (Masse, Vanderpool, & Gottesman, 2005; Murphy & Payne, 2007). RyhB and Hfq, found in bacteria such as *E. coli*, *Vibrio cholera*, and *Shigella spp*, aid in the post-trascriptional regulation of iron homeostasis (Masse et al., 2005; Payne et al., 2006; Wyckoff, Mey, & Payne, 2007). Under iron starved conditions, RyhB mediates the degradation of mRNA encoding non-essential iron-utilizing proteins. It is thought that RyhB pairs with the ribosome binding site and targets the mRNA for degradation through recruitment of the degradosome (Massé, Escorcia, & Gottesman, 2003). Whereas in iron replete conditions, expression of RyhB is repressed by Fur (Jacques et al., 2006; Massé & Gottesman, 2002; Salvail & Massé, 2012).

IscR

IscR, the iron sulfur cluster regulator, is a member of the Rrf2 family of transcriptional regulators, a typical winged helix turn helix superfamily of transcriptional regulators in prokaryotes (Aravind, Anantharaman, Balaji, Babu, & Iyer, 2005). It is the first gene in the Isc operon and in *E. coli* is known to regulate the expression of its own operon and many other genes depending on its association with an Fe-S cluster. There are two types of IscR binding sites known for transcriptional

regulation, Type I and Type II (a D. Nesbit, Giel, Rose, & Kiley, 2009). In order to bind to Type I sequences, IscR requires a [2Fe-2S]. When iron is abundant and/or under low oxidative stress, IscR is predominantly in the holo-IscR form and acts as a repressor for genes such as *iscRSUA* (Christopher J Schwartz et al., 2001). On the other hand, Type II binding sites can be bound by either apo- or holo-IscR. In iron-limited conditions and/or high oxidative stress, IscR loses its [2Fe-2S] cluster (apo-IscR) and now can act as an activator for genes such as *sufABCDSE* (Yeo, Lee, Lee, & Roe, 2006). Coordination of the Fe-S cluster in IscR is mediated through its three conserved cysteines (Cys92, Cys98, and Cys104). As seen previously in *E.coli*, an amino acid change from a cysteine to an alanine in any one of the three cysteines will cause a loss of [2Fe-2S] cluster binding, causing IscR to remain in an apo-locked form (a D. Nesbit et al., 2009).

Although it has been shown that the *E. coli* Isc operon is not directly regulated by Fur, the Fur-regulated sRNA RyhB modulates the post-transcriptional activities of IscR under iron depleted conditions (Desnoyers et al., 2009). By binding to the mRNA intergenic region between *iscR* and *iscS*, RhyB coordinates recruitment of RNA degradosomes to degrade the *iscSUA* cistrons.

IscR has been found to regulate genes under both aerobic and anaerobic environments. Under aerobic conditions in *E.coli*, 31 genes have been identified as being repressed by IscR while a smaller subset of genes were activated (Giel, Rodionov, Liu, Blattner, & Kiley, 2006). Among the repressed genes were some Fe-S containing respiratory enzymes found only under anaerobic growth like the *hya* and

hyb operons that allows cells to couple the oxidation of H_2 with reduction of electron acceptors other than O_2 (Giel et al., 2006). Another gene identified as being repressed by IscR in *E. coli* was the general regulator of oxidative stress, SoxS. SoxS along with SoxR regulate genes during exposure to redox-cycling (described below). IscR is thought to regulate SoxS in an indirect manner through the Fe-S cluster on SoxR (Giel et al., 2006).

Besides its repressor activities, IscR can also be an activator such as in the case of the Suf operon. Under iron limiting condition/oxidative stress, IscR activates the transcription of the Suf operon, known to be capable of synthesizing Fe-S clusters under these conditions (Giel et al., 2006; Outten et al., 2004; Yeo et al., 2006). SodA, a Mn-containing superoxide dismutase is another gene activated by *E. coli* IscR, though its expression is also governed by six other global regulators (Compan & Touati, 1993; Giel et al., 2006).

1.1.5 Oxygen /Oxidative stress

Reactive oxygen species (ROS) are natural byproducts from living organisms that rely on aerobic respiration (Fridovich, 1995). In combination with free ferric iron, ROS can be perpetuated in the cell through the Fenton/Harber-Weiss reactions (Fig. 4). Oxidative stress is sensed by two major regulatory proteins OxyR and SoxRS, both of which are able to trigger the transcriptional activation of *fur* under

these conditions.

$$Fe^{3+} + {}^{\bullet}O_2^{-} \rightarrow Fe^{2+} + O_2$$

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^{-} + {}^{\bullet}OH$$

$$\text{Net reaction: } {}^{\bullet}O_2^{-} + H_2O_2 \rightarrow {}^{\bullet}OH + OH^{-} + O_2$$

Figure 4. Fenton/Harber-Weiss reaction. Fenton/Harber-Weiss reaction triggered by "free ferric iron." Borrowed from Cornelis et al 2011

OxyR, part of the LysR type transcriptional regulator family, is a global regulator that senses the cellular response to hydrogen peroxide stress (Ming Zheng et al., 2001). Using a helix turn helix DNA binding motif at the N-terminal domain, OxyR binds DNA as dimers or tetramers. A conformational change is triggered by hydrogen peroxide or change in the redox status of thiol-disulfide in the cytoplasm. (P. Cornelis, Wei, Andrews, & Vinckx, 2011). This induction causes OxyR to change into its tetrameric form, allowing for the transcriptional activation of regulated genes. This enables OxyR to bind the upstream region of the *fur* gene to activate *fur* transcription.

A member of the MerR family of transcriptional regulators, SoxR senses redox-cycling agents through the oxidation of its Fe-S cluster (Kiley & Beinert, 2003). Redox-cycling agents induce the process of single-electron transfers leading to

a buildup of superoxides inside the cell. As SoxR is oxidized, it triggers the transcription of *soxS*. This in turn allows SoxS to bind to the promoter region of flavodoxin, *fldA*, which is able to activate transcription of both flavodoxin and Fur allowing them to activate downstream genes needed for resistance (Eiamphungporn, Nakjarung, Prapagdee, Vattanaviboon, & Mongkolsuk, 2003; Fang, Vazquez-Torres, & Xu, 1997; M Zheng, Doan, Schneider, & Storz, 1999).

1.1.6 Iron—Sulfur Clusters and Virulence

Pathogenic bacteria must be able to survive under the stressful conditions of the host environment. During an infection, the release of ROS by macrophages can be deleterious to the invading microbe (Paauw, Leverstein-van Hall, Van Kessel, Verhoef, & Fluit, 2009; Runyen-Janecky et al., 2008). In *Pseudomonas aeruginosa*, modulation of catalase A (KatA) activity by IscR is critical for its resistance to peroxides and complete virulence (Kim et al., 2009). KatA is a heme dependent protein (requiring ferric iron for activity) that catalyzes the decomposition of hydrogen peroxide to water and oxygen gas (Kim et al., 2009). Without the regulation of IscR, KatA is not able to regulate the formation of peroxides and therefore the cell loses its ability to detoxify ROS. In *Burkholderia mallei*, the presence of IscR allows *B. mallei* to become less susceptible to the reactive nitrogen species created by inducible nitro oxide synthase (iNOS) in IFNγ-primed macrophages, by repairing the enzymatic activity of the Fe-S protein aconitase (Jones-Carson et al., 2008).

Similarly, in *Shigella flexneri* the presence of oxidative stress induces transcription of the Suf operon through OxyR and apo-IscR (Runyen-Janecky et al., 2008) and this mediates prolonged survival under oxidative stress conditions (J.-H. Lee et al., 2004; Outten et al., 2004; Runyen-Janecky et al., 2008). Also in the case of the plant pathogen, *Erwinia chrysanthemi*, the Isc operon is an important factor for virulence and resistance to oxidative stress, through IscR-dependent expression of the Suf operon (Rincon-Enriquez, Crété, Barras, & Py, 2008).

Another survival mechanism regulated by Fe-S clusters is the formation of biofilms. Biofilms are matrix-encased, surface-associated microbial communities formed by many bacteria (Fong, Karplus, Schoolnik, & Yildiz, 2006). *E. coli* apo-IscR regulates type I fimbriae through *fimE* by binding to the *fimB-fimE* intergenic region and activating transcription of FimE which leads to the decrease in biofilm formation under iron limited conditions (Wu & Outten, 2009).

1.2 Justification for this Research

As the T3SS can be found in numerous Gram negative pathogens, what we learn through our research can hopefully be applied to other bacteria. Understanding the regulatory pathways of the T3SS would allow for further development of compounds that could inhibit the T3SS. This manner of inhibition through the T3SS would prevent the evolution of resistance, which is a growing problem. My research on the transcriptional regulator of iron-sulfur cluster biogenesis, IscR, will provide

new and important insights into the regulation of the T3SS in response to environmental cues such as bioavailable iron and oxidative stress.

2 Methods and Materials

2.1 Bacterial growth conditions.

Y. pseudotuberculosis IP2666 was grown in 2xYT or M9 media at 26˚C shaking overnight. When necessary the cultures were back-diluted into low calcium media (media plus 20mM sodium oxalate and 20mM $MgCl_2$) to an OD_{600} of 0.2 and grown for 1.5h at 26°C shaking followed by 1.5h at 37˚C shaking to induce Yop synthesis as previously described (Merriam et al., 1997).

2.2 Bacterial mutants.

The *Y. pseudotuberculosis* strains generated for this study were constructed by splicing by overlap extension PCR. Primers were designed using MacVector and Primer 3 software (http://fokker.wi.mit.edu/primer3/input.htm). Amplified PCR fragments, encoding ~500 bp of homology on either side of the intended mutation, were subcloned into the TOPO TA cloning vector (Invitrogen) and further cloned into a BamHI- and NotI-digested pSR47s suicide plasmid (λpir-dependent replicon, Kan^R, *sacB* gene conferring sucrose sensitivity)(Andrews, Vogel, & Isberg, 1998; Merriam et al., 1997). Recombinant plasmids were transformed into *E. coli* S17-1 λpir competent cells and later introduced into *Y. pseudotuberculosis* IP2666 via

conjugation. The resulting kanamycin^{R}, irgansan^{R} integrants were grown in the absence of antibiotic selection and plated on sucrose-containing media to identify clones that had lost *sacB* (and by inference, the linked plasmid DNA). Kanamycin^{S}, sucrose^{R}, congo red-positive colonies were screened by PCR and subsequently sequenced to confirm the presence of the intended mutation.

2.3 Growth Curve

Yersinia strains were cultured overnight in 2xYT and M9 at 26°C and then sub-cultures to an OD_{600} of 0.2 in 25ml of their respective media (note: M9 O/N was used to sub-culture into M9-Fe, as M9-Fe does not support overnight growth). Cultures were incubated shaking at 250rpm at 26°C and 37°C for 9h. Optical density was measured at 600nm every hour.

2.4 Motility

Overnight cultures were normalized to OD_{600} 2.5 and pipetted onto motility plates (1% tryptone and 0.25% agar) in 1ul spots. Plates were incubate overnight at room temperature. Diameter for growth was measured in comparison to the other strains.

2.5 Colony morphology

Overnight cultures were serial diluted in 1x phosphate buffer saline (PBS) and plated onto LB plates. Plates were incubated for 2 days at 26°C prior to observations and pictures.

2.6 Intracellular Iron Content

Intracellular iron content was measure similarly to Ma et al 1999 (Ma et al., 1999). Briefly, 400ml *Yersinia* was grown at 37°C in both iron replete and iron depleted media for 3h. Cultures were spun down at 10,000 x g for 10min at 4°C than washed twice with 200ml 1x PBS with 1mM EDTA (pH 7.4). Cultures were washed again with 200ml 1xPBS without EDTA and then spun down. Pellet was resuspended in 2ml trace metal grade nitric acid and incubated at 80°C for 1h. Final volume was brought to 20ml with Milli-Q water. Intracellular iron content was analyzed by inductively coupled plasma-optical emission spectroscopy (ICP-OES) by the PerkinElmer Optima 4300DV.

2.7 Ethidium Bromide Entry Assay

Done as previously described in Kwuan, Adams and Auerbuch 2013 (Kwuan, Adams, & Auerbuch, 2013). Briefly, $2x10^4$ immortalized C57Bl/6 BMDMs were plated in a 96-well clear bottom black plate in 100ul DMEM + 10% FBS Infection proceeded in triplicates at an MOI of 25 and spun down at 750 x g at 4°C for 5min to initiate contact. Cell are then incubated at 37°C with 5% CO_2 for 2h. After incubation media was aspirated and replaced with 30ul of PBS with 25ug/ml ethidium bromide (EtBr) and 12.3ug/ml Hoechst dye. The cell monolayer was visualized using an ImageXpressMICRO automated microscope and MetaXpress analysis software. The percent of EtBr-positive cells was calculated by dividing the number of EtBr-stained

cells by the number of Hoechst-stained cells. Data from three separate wells was averaged for each independent experiment.

2.8 Quantitative Real Time PCR

Overnight cultures were back diluted to OD_{600} 0.2 and grown for 3h are 37°C. After incubation RNA was isolated using the RNeasy Mini Kit (Qiagen). DNA-free kit (Ambion) was used to remove any contaminating genomic DNA from the total RNA samples harvested. 2mg RNA was used to make cDNA as previously described Auerbuch et al 2004 (Auerbuch, Brockstedt, Meyer-Morse, O'Riordan, & Portnoy, 2004). SYBR Green PCR master mix (Applied Biosystems) was used for qRT-PCR reactions according to the manufacturer's instructions and a 60°C annealing temperature. The results were analyzed using the Bio-Rad CFX software.

2.9 β-Galactosidase Assay

β-Galactosidase assay was done as previously described in Nagel et al 2001 (Nagel, Lahrz, & Dersch, 2001). Briefly, cell were grown overnight in M9 media. Overnight cultures were back diluted to OD_{600} 0.2 the next day and grown for 3h at 26°C. 2ml of culture was spun down and resuspended in an equal amount of 1x Z-Buffer. Cells were lysed with the addition of 40ul 0.1% SDS and 80ul chloroform and incubation at room temperature for 10mins. The reaction was started with 400ul ONPG (4mg/ml) and subsequently stopped with 1ml of 1M $NaCO_2$ after the appearance of a yellow color. Cell free extract was aliquoted in triplicates in a 96-well clear plate and read by

the Victor X3 plate reader. Calculations was done as followed: OD_{420} x 6.75 x OD_{600}^{-1} x $\Delta t\ (min)^{-1}$ x volume $(mL)^{-1}$.

2.10 ROS sensitivity

ROS sensitivity assay was done similarly to Schiano et al 2010 (Schiano, Bellows, & Lathem, 2010). Briefly, overnight cultures are back diluted to OD_{600} 0.1 and grown until OD_{600} 0.4-0.6 (mid-log phase) and then diluted 1:10 in the respective media. Hydrogen peroxide at a final concentration of 100mM or water alone was added to the bacterial cultures and incubated on a roller drum. At 10 and 30 minutes aliquots were taken and serial diluted to plate. CFU/mL is calculated of treated versus untreated after a two day incubation at 26°C.

3 Results

3.1 Isc operon is Fur regulated in *Y.ptsb*.

In *E. coli*, the *isc* promoter is not directly targeted by Fur (Desnoyers et al., 2009; Giel et al., 2006; Christopher J Schwartz et al., 2001). Upstream of the *iscR* in *Y. pstb* we have identified a putative Fur box (Fig. 5). Through an in silico search using the *Y. pestis* Fur box as the consensus sequence (5'-AATGATAATNATTATCATT-3'), for which we saw a 63.16% consensus. To test whether this putative Fur box regulates Isc expression, we constructed a *lacZ* fusion plasmid containing the promoter region of *iscR* with and without the putative Fur box. *Y. pstb* containing the *lacZ* constructs were incubated in both M9 media and M9-

Fe+DP (DP: 2,2'-dipyridyl, an iron chelator) at 26°C for 3h prior to measuring β-galatosidase activity. No significance was found between the two stains when grown under low iron conditions (M9-Fe+DP). However, β-galatosidase activity was significantly higher from the strain containing the *lacZ* construct lacking the putative Fur box (p-value = 0.035). Contrary to what has been shown for *E. coli*, our results seem to suggest that the *isc* operon in *Y. pstb* may be regulated by a putative Fur box in the *isc* operon promoter region (Fig. 6).

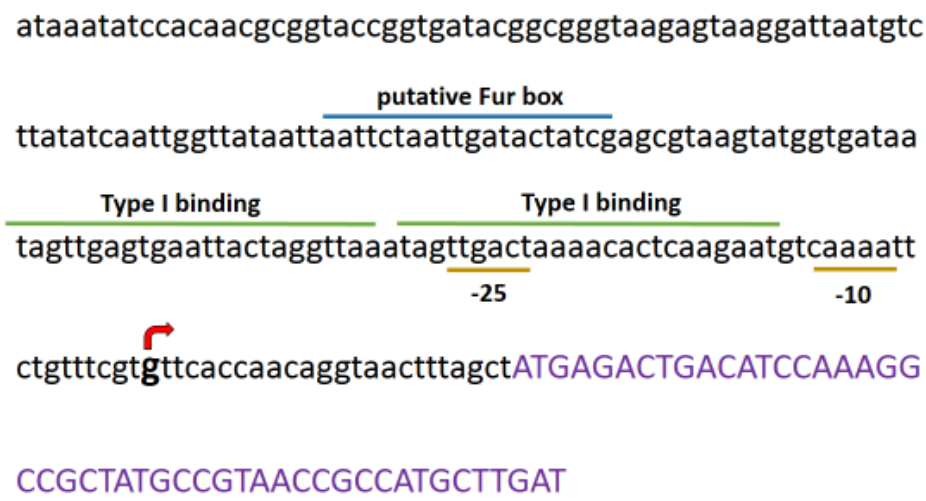


Figure 5. *Y. pstb* IscR contains a putative Fur box located upstream of the IscR Type I binding sites. *Y. pstb* sequence was obtained from UCSC Microbial Genome Browser. Bolded **g** denotes transcriptional start site (http://microbes.ucsc.edu/).

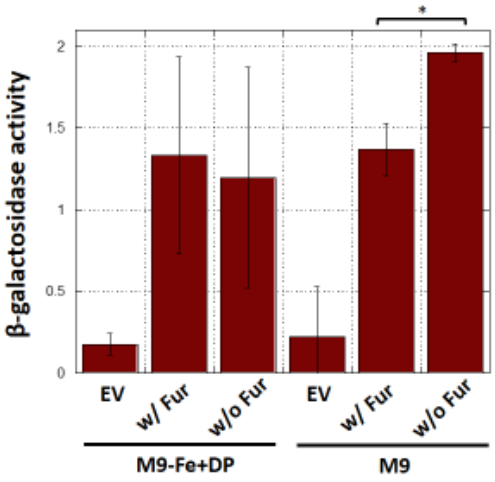


Figure 6. Gene expression in the presence and absence of Fur box. *Y. pstb* strain harboring the empty vector (EV) or different *iscR* w/Fur-*luxCDABE* or *iscR* w/out Fur-*luxCDABE* reporter fusions encoded on a low copy pSC101* were diluted 1:50 in fresh M9 from overnight cultures and grown for 3h. The averages ± sem from three experiments are shown. * p=0.035, as determined by one-way ANOVA followed by Bonferroni post hoc test.

3.2 WT IscR is necessary for full T3SS expression.

From our forward genetic screen, we identified IscR as a candidate for T3SS regulation. To further analyze the impact of IscR on the Ysc T3SS, we performed quantitative real time PCR (qRT-PCR) measuring T3SS gene transcript levels in relation to our IscR mutants. The Δ*iscR* mutant was constructed to include the start codon and 9 terminal codons while, the apo-IscR mutant contains alanine substitutions of the three conserved cysteines.

Overnight cultures were back diluted to OD_{600} 0.2 in M9 media and grown for 3h at 37°C. RNA was isolated followed by a DNase treatment to remove DNA contamination and then proceeded with cDNA synthesis and qRT-PCR. Results show increased IscS transcription in the Δ*iscR* (p-value = <0.004) and apo-IscR (p-value = <0.0004) mutants compared to wildtype *Y. pstb*, indicating that, as in *E. coli*, *Y. pstb* holo-IscR represses transcription of its own operon (Fig. 7A). In contrast,

transcription of LcrF was decreased in the Δ*iscR* (p-value = <0.0001) mutant but not in the apo-IscR mutant (Fig. 7B), indicating that holo-IscR may induce transcription of this T3SS master regulator. YscF and YscN transcipts were decreased in both Δ*iscR* (YscN, p-value = <0.02 YscF, p-value = <0.0004) and apo-IscR (YscF, p-value = <0.02) (Fig. 7 C and D), indicating that T3SS expression is decreased in both mutants despite the normal LcrF transcript levels in the apo-IscR mutant.

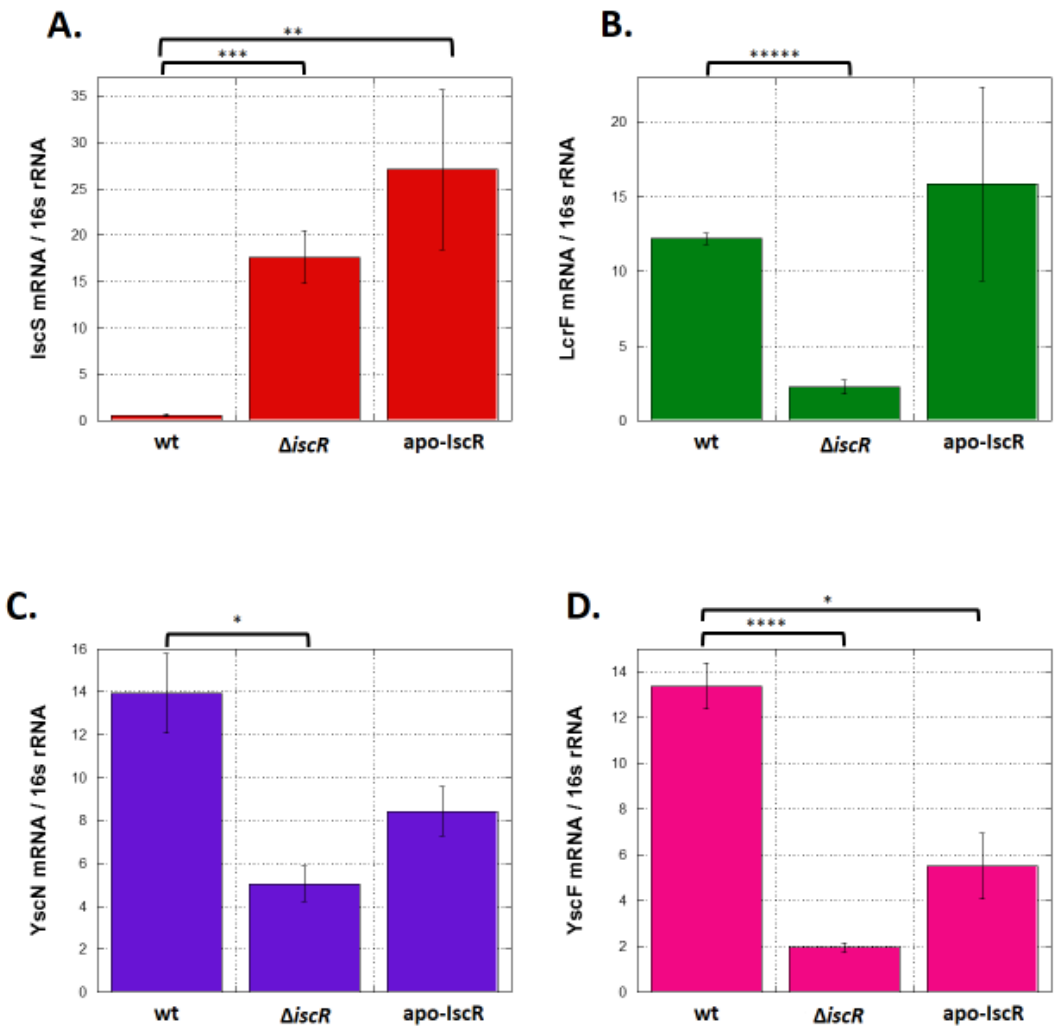


Figure 7. Transcript expression of IscR by qRT-PCR. IscS (A), LcrF (B), YscN (C), and YscF (D) gene expression in response to WT, Δ*iscR* and apo-IscR. RNA was isolated at 3h and qRT-PCR analysis performed. mRNA levels normalized to 16s rRNA. The averages ± sem from three experiments are shown. p-values: * = p<0.02, ** = p<0.04, *** = p<0.004, **** = p<0.0004, ***** = p<0.0001 were determined by Studetnt T-test.

3.3 IscR is required for pore formation in *Y. pstb*

To determine the involvement of IscR in T3SS function we sought to identify the ability of the Δ*iscR* and apo-IscR mutants to form T3SS-dependent pores in macrophages. Macrophages are one of many targets of *Yersinia* in the host and was a

reasonable candidate for this assay (Durand, Maldonado-Arocho, Castillo, Walsh, & Mecsas, 2010; Köberle et al., 2009; Marketon, DePaolo, DeBord, Jabri, & Schneewind, 2005). Visualization of pore formation was achieved through the entry of a small molecule, ethidum bromide (EtBr), into bone marrow derived macrophages (BMDM) infected with *Y. pstb* by fluorescence microscopy (Kirby, Vogel, Andrews, & Isberg, 1998; Kwuan et al., 2013). Two hours post-inoculation, EtBr fluorescence was measured. Results indicated a significant defect in the ability to form pore for both IscR mutants (p-value = <0.0001), suggesting that IscR is required for normal T3SS function (Fig. 8).

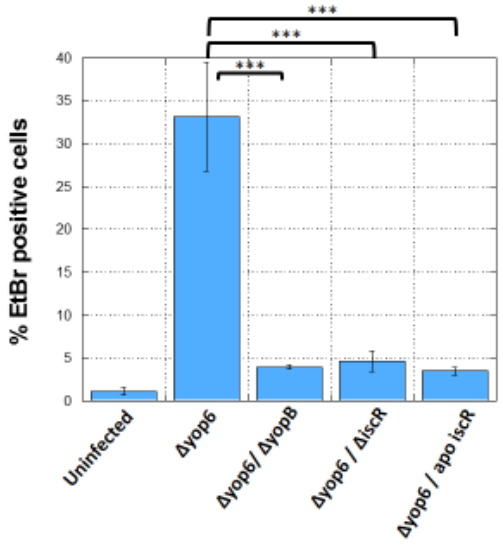


Figure 8. Analysis of pore formation in Δ*iscR* and apo-IscR by EtBr entry. Pore formation of Δ*iscR* and apo-IscR was analyzed through the entry of ethdium bromide (EtBr) into macrophages. Entry of EtBr into C57Bl/6 BMDMs was examined after 2h post-infection. Analysis was achieved by calculating the number of EtBr-positive cells over the total Hoechst-positive cells. The averages ± sem from three experiments are shown. *** p≤0.0001, as determined by one-way ANOVA followed by Bonferroni post hoc test.

3.4 Locking IscR in the apo form leads to decreased flagellar motility

In order to further investigate the effect of IscR on the T3SS we examined motility, as the flagellar basal body is considered a T3SS and the flagellar T3SS is

evolutionarily conserved with virulence-associated T3SSs (Macnab, 1999). *Y.pstb* cultures were spotted into motility agar plates and the diameter of bacterial colonies measured after 24 and 48h incubation at room temperature. As controls, we used the WT strain, which has normal motility, and $flhD^{Y.pestis}$, a mutant containing the flagellar master regulator *flhD* allele from *Y. pestis* which is defective due to a frame shift mutation (Atkinson et al., 2011). We observed no change in motility for Δ*iscR* as compared to the parental strain (Fig. 9). However, apo-IscR displayed a significant decrease in motility in comparison to $flhD^{Y.pestis}$ (day 1, p-value = 0.0025; day 2 p-value = <0.0001), WT (both days, p-value = <0.0001) and Δ*iscR* (both days, p-value = <0.0001) (Fig. 9 A and B). The motility defect of apo-IscR was restored to WT levels with complementation *in trans* (data not shown). This data suggests that apo-IscR has a motility defect.

A light-refractive substance, possibly a biosurfactant, was also observed surrounding apo-IscR colonies on motility agar plates. Biosurfactants are known to be produced by organisms to assist in the reduction of surface tension (Desai & Banat, 1997). To test the unknown refractive substance for properties of biosurfactant, a drop of water was placed inside and outside of the refractive substance. Inside, the water droplet immediately dispersed displaying decreased surface tension similar to biosurfactant, while the droplet outside remained intact (data not shown).

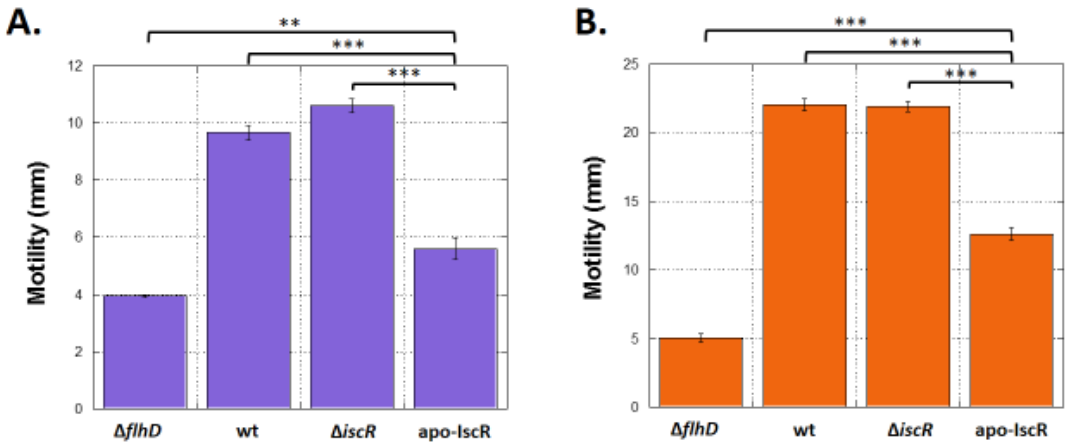


Figure 9. Analysis of motility in Δ*iscR* and apo-IscR. Motility was analyzed by spotting onto low percentage agar plates. Observed after incubation at room temperature for 1-2 day. Graphs are a representative of 3-5 individual experiments. The averages ± sem from 5 technical replicates are shown. *** = p <0.0001, ** = p <0.0025, as determined by one-way ANOVA followed by Bonferroni post hoc test.

3.5 The apo-IscR *Y.pstb* mutant has a selective growth defect in rich media

Although IscR has been shown to regulate genes involved in metabolic processes, the absence of IscR has not been shown to lead to any observable growth defects in *E. coli* (Giel et al., 2006; Kim et al., 2009; Christopher J Schwartz et al., 2001). In order to determine if IscR impacts growth of *Y. pstb*, we performed a growth curve study at both 26°C and 37°C, in rich media versus minimal media, and in iron replete and depleted conditions. Iron concentration of our media was first analyzed using inductively coupled plasma atomic emission spectroscopy (ICP-OES). 2xYT contained the most iron, followed by M9 media, and M9-Fe media not containing added iron (Fig. 10A). There was no change in intracellular iron concentration among strains in each media type (Fig. 10B), indicating that the Δ*iscR* and apo-IscR mutants do not have a defect in maintenance of intracellular iron pools.

An initial OD_{600} of 0.2 was used to start the cultures in 2xYT, M9, and M9-Fe media. At both 26°C and 37°C in 2xYT, we observed a significant growth defect in apo-IscR (p-value = <0.03) but not Δ*iscR* in 2xYT (Fig. 11 A and B). This growth defect seen in apo-IscR was complemented *in trans* with WT IscR (Fig. 11A). In M9 and M9-Fe media at 26°C, neither IscR mutant showed any defect in growth in comparison to the parental strain (Fig. 11 C and E). However, in M9 and M9-Fe media (data not shown) at 37°C, we observed an intermediate growth (Fig. 11D). Induction of the *Yersinia* T3SS coincides with a cessation of vegetative growth (Wiley, Rosqvist, & Schesser, 2007), a phenomenon called growth restriction. M9 media enables induction of the T3SS at 37˚C (Cheng, Anderson, & Schneewind, 1997) (data not shown). Thus, the Δ*iscR* and apo-IscR mutants display an intermediate growth restriction phenotype in M9 media at 37˚C, correlating with their T3SS defect as described above.

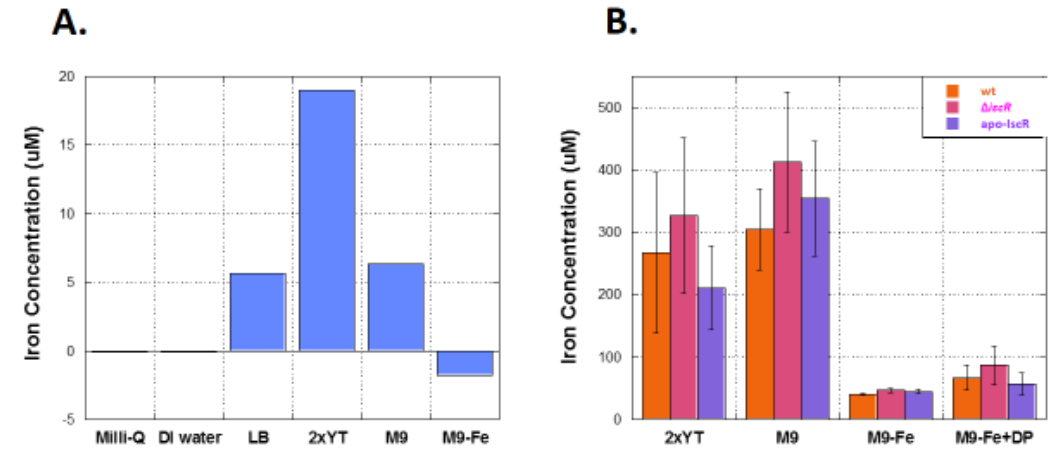
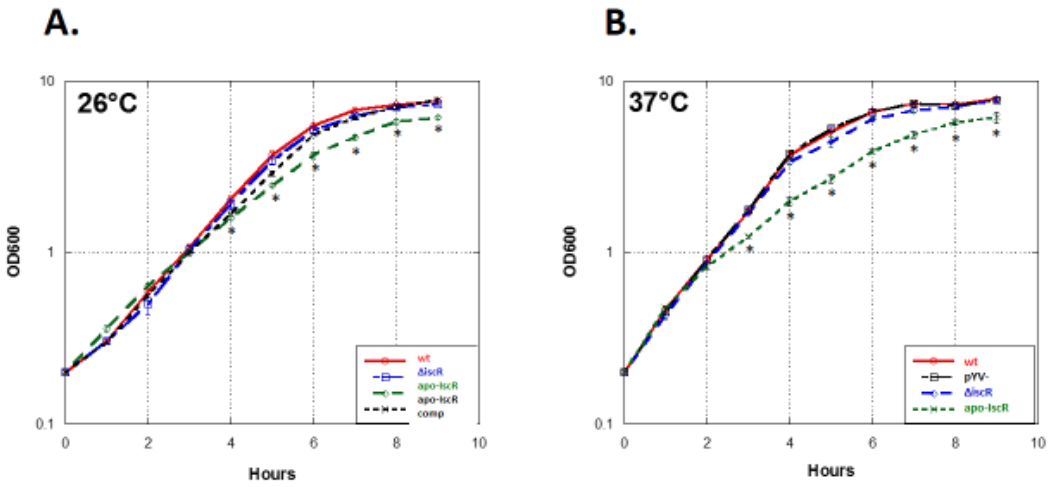


Figure 10. Analysis of iron concentration. The concentration of iron in media (A) and intracellular iron concentration of wt, Δ*iscR* and apo-IscR when grown in various media with different iron concentrations (B) were measured using an ICP-OES.



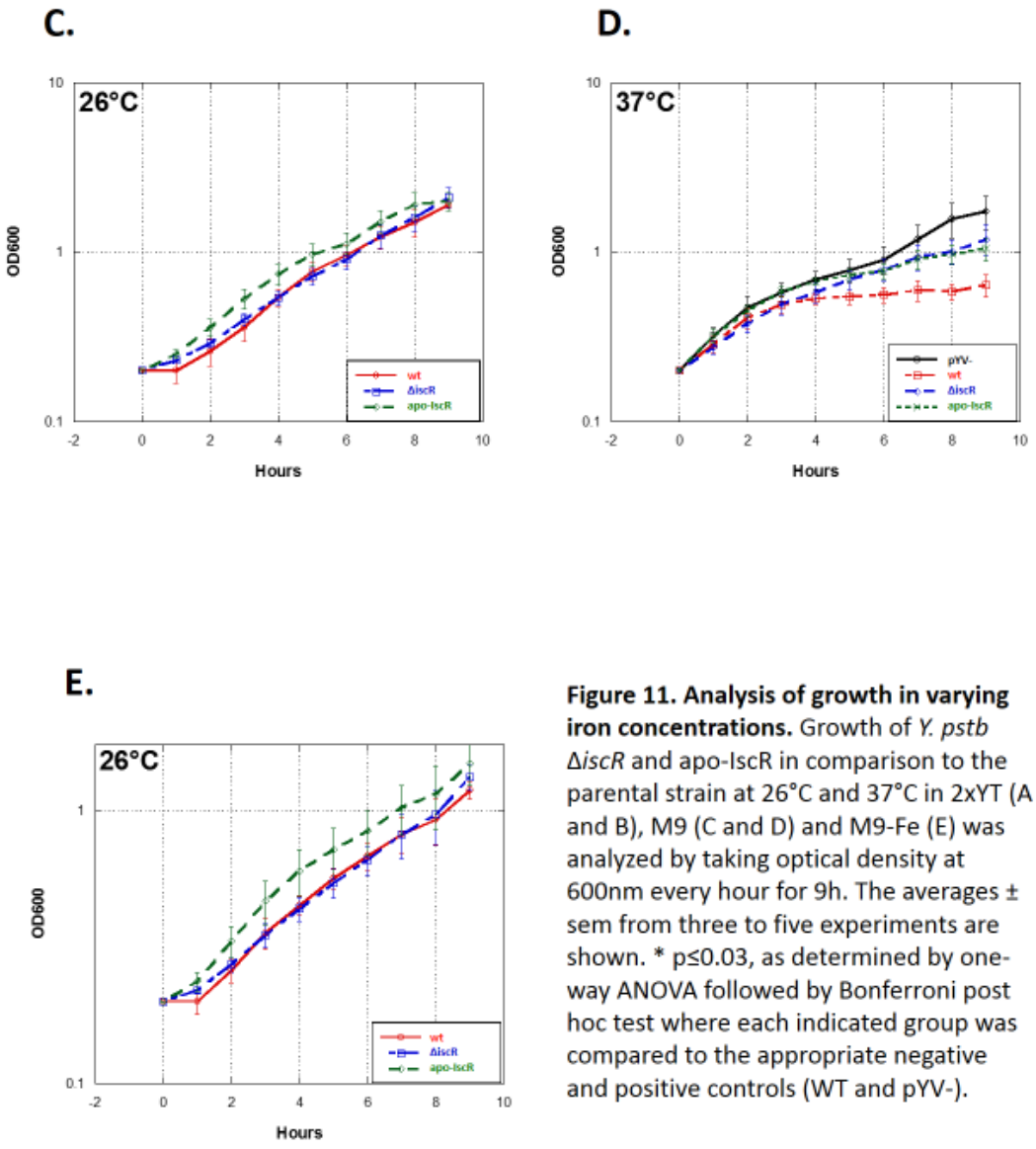


Figure 11. Analysis of growth in varying iron concentrations. Growth of *Y. pstb* Δ*iscR* and apo-IscR in comparison to the parental strain at 26°C and 37°C in 2xYT (A and B), M9 (C and D) and M9-Fe (E) was analyzed by taking optical density at 600nm every hour for 9h. The averages ± sem from three to five experiments are shown. * p≤0.03, as determined by one-way ANOVA followed by Bonferroni post hoc test where each indicated group was compared to the appropriate negative and positive controls (WT and pYV-).

3.6 The apo-IscR *Y. pstb* mutant has a smaller colony morphology

Given the growth defects we observed in liquid culture, we analyzed growth of IscR on solid media. Overnight cultures were serial diluted and plated on LB plates. Observation were made after incubation at 26°C for 2 days. The Δ*iscR* mutant

colonies were similar in size as compared to the parental strain, while apo-IscR appeared to have small colonies (Fig. 12). The morphology defect seen in apo-IscR was complemented *in trans* with WT IscR

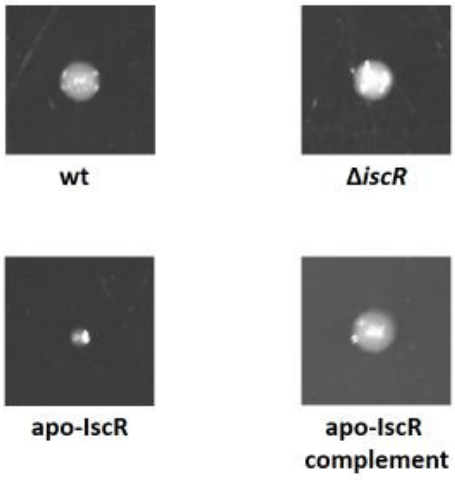


Figure 12. Analysis of colony morphology of Δ*iscR* and apo-IscR. Colony morphology of Δ*iscR* and apo-IscR was analyzed by plating serial dilution of each strain and observed morphology after incubation at 26°C for 2 days.

3.7 Susceptibility to hydrogen peroxide

As seen in *E. coli* as well as *Pseudomonas aeruginosa*, the loss of IscR causes each bacteria to become more sensitive to reactive oxygen species (ROS) (Choi, Chung, Kim, Heo, & Cho, 2007; Giel et al., 2006; Kim et al., 2009). Thus, we set out to examine the effect of IscR on ROS. Overnight cultures of the WT, Δ*iscR*, and apo-IscR strains were back diluted to OD_{600} 0.1 in M9 media and grown until mid-log (OD_{600} 0.4-0.6). Cultures were then diluted 1:10 and then treated with a final concentration of 100mM H_2O_2 or water and incubated on a roller drum for 10 and 30min. Aliquots were taken, serial diluted and plated at each time point. After a two day incubation at 26°C, CFU were counted to determine H_2O_2 resistance or

sensitivity. Preliminary data (data not shown) suggest that the absence of IscR in *Y. pstb* can lead to enhanced resistance to ROS, while conversely the apo-locked form of IscR seemed to be associated with extreme susceptible to ROS. The experimental designed used during this experiment was adapted from Schiano et al 2010 and will need to be modified in future experiments to fit our low iron conditions (Schiano et al., 2010).

4 Discussion

The T3SS is regulated by many environmental cues, as it is only required by pathogens during infection of a host organism and may be deleterious to express when it is not needed. It remains unclear whether environmental cues other than temperature influence expression of the *Y. pstb* T3SS. In this work we sought to discover alternative T3SS regulators in *Y. pstb* through a forward genetic screen. In this screen, we identified IscR, a gene with a high degree of similarity to *E. coli* IscR which acts as a transcriptional regulator of iron-sulfur cluster biogenesis. In *Y. pstb*, IscR has yet to be characterized; therefore in this study we sought to characterize IscR of *Y. pstb* to elucidate its role in resistance to ROS and in regulation of the T3SS.

4.1 Regulation

It has been previously demonstrated in *E. coli* that Fur is a global regulator controlling over 90 genes that fall into two major categories: iron metabolism and energy production (McHugh et al., 2003). Interestingly, the *isc* operon in *E. coli* has

not been shown to be regulated by Fur but instead only by IscR. However, in the case of the *isc* operon in *Y. pstb*, our results suggest that it may be regulated by Fur. This regulatory function has not been previously seen in the literature and thus is a novel regulatory component to the *isc* operon in *Y. pstb*. This unique regulation can be seen as a division of work, similar to what was propped in *E. coli* which suggested that in the Suf operon, OxyR and IscR contribute to the expression of Suf during oxidative stress, while iron limitation is regulated by Fur (K.-C. Lee et al., 2008; Outten et al., 2004; Yeo et al., 2006). This thought process can be used with the Isc operon: perhaps IscR contributes to regulation under oxidative stress while Fur contributes during iron limited states.

4.2 T3SS regulation

With the help of our collaborators in the Lowe lab at UCSC, we were able to determine possible IscR binding sites in the *Y. pstb* genome. One possible type II binding site was located upstream of the T3SS transcriptional regulator LcrF (Chan, Lowe, Miller, Kwuan, and Auerbuch, data not shown). In agreement with this data, qRT-PCR analysis showed that LcrF transcript levels are low in the Δ*iscR* mutant but are WT in the apo-IscR mutant under T3SS-inducing conditions. However, we believe that another layer of T3SS regulation may be active in the apo-IscR mutant, as this mutant showed lower transcripts of other T3SS genes and could not induce T3SS-dependent pore formation in macrophages.

Both apo-IscR and Δ*iscR* mutants display secretion of minimal amounts of T3SS cargo under *in vitro* inducing conditions (Miller, Kwuan, and Auerbuch, data not shown). To further analyze T3SS function, we attempted to conduct a translocation assay to measure the ability of the mutant strains to deliver T3SS effector proteins inside target host cells. Transformation of a plasmid encoded YopM-β-lactamase fusion reporter protein into our WT, Δ*iscR* and apo-IscR strains would allow us to measure translocation during an infection using a fluorescent β-lactamase substrate CCF2 (Dewoody, Merritt, Houppert, & Marketon, 2011; Kwuan et al., 2013). We transformed the WT, Δ*iscR*, and apo-IscR strains with a plasmid encoding the YopM-β-lactamase fusion reporter protein. After performing transformations in *Y. pstb*, we routinely screen for the ability of the resulting transformants to take up the dye congo red on Congo Red-sodium oxalate plates. Congo red is an indicator dye taken up only by *Yersinia* actively undergoing T3S (Bhaduri & Smith, 2011) and T3SS$^-$ mutants are white on congo red plates. Surprisingly, Δ*iscR* pYopM-Bla transformants were all white on congo red-containing plates despite Δ*iscR* lacking the pYopM-Bla plasmid being red on congo red plates and and Δ*iscR* pYopM-Bla transformants harboring the pYV virulence plasmid that encodes the Ysc T3SS (Kwuan and Auerbuch, data not shown). However, apo-IscR pYopM-Bla transformants were mostly red on congo red, indicating the presence of at least a partially active T3SS. The reason for these findings are unknown, but future work will address the mechanisms by which IscR controls T3SS expression and function in *Y. pstb*.

4.3 Motility

As function of the T3SS is disrupted in our IscR mutants, speculation of whether other T3SSs, such as the flagellar T3SS, was disrupted came to be questioned. In *E. coli*, IscR was shown to regulate motility genes (Giel et al., 2006; Wu & Outten, 2009). In *Y. pstb*, Δ*iscR* showed no defect in motility while apo-IscR showed significantly decreased motility. In addition, apo-IscR appeared to produce a biosurfactin-like compound on motility agar. Similar biosurfactin release has been previously seen a Δ*hfq* mutant.(Schiano et al., 2010). Hfq is an RNA-binding protein that helps mediate the interaction between small, noncoding RNA (sRNA) and its target mRNA in posttranslational regulation of gene expression (Massé et al., 2003; Masse et al., 2005; Schiano et al., 2010). In relation to IscR, Hfq assists RyhB, a small noncoding RNA, in the degradation of the downstream region of the *isc* operon during low iron conditions while the upstream region stays intact. Recent RNASeq data show an up-regulation of *hfq* in apo-IscR (data not shown). The connection between Hfq, IscR, and the biosurfactin-like substance produced by the apo-IscR mutant will be the work of future experiments.

4.4 Growth

IscR in *E. coli* has been shown to regulate genes involved in metabolism (Giel et al., 2006). To determine the impact of IscR on metabolic processes in *Y. pstb*, we performed a growth curve. *Y. pstb* containing a functional T3SS display normal

growth at 26°C but will display a growth restriction upon a temperature shift to 37°C and low calcium conditions (Wiley et al., 2007). As previously mentioned, at 28°C, thermoregulation of LcrF by YmoA prevents transcription of T3SS genes (Böhme et al., 2012). Upon a shift to 37°C, LcrF is translated and directs the transcription of T3SS genes. Paradoxically, under T3SS-inducing conditions (37˚C in the absence of calcium), *Yersinia* undergo growth restriction, the cause of which remains unclear (G R Cornelis et al., 1989; Hueck, 1998). Hence, active type III secretion and growth seem to be inversely regulated in *Yersinia*. In *E. coli*, an IscR deletion has been shown not to have detectable growth defects when grown in rich media (A. D. Nesbit, 2009; Christopher J Schwartz et al., 2001; Wu & Outten, 2009). Similar to our results, in all three media we did not observe a growth defect in Δ*iscR* when grown at 26°C. However, a significant growth defect was seen in the apo-form of IscR only in rich media when grown at 26°C. This slow growth can also be seen on solid media, where apo-IscR produces smaller colonies compared to WT and Δ*iscR*. Growth defects of apo-IscR in both liquid and solid media were complemented *in trans* with WT IscR. This growth defect shown in apo-IscR has not been shown in the literature. The apo-form of IscR is more prevalent in the cell during low iron conditions (Giel et al., 2006; Christopher J Schwartz et al., 2001; Yeo et al., 2006). We speculate that when IscR is locked in the apo form, it acts an activator for genes that are not necessary in iron rich conditions, thus wasting energy and causing a significant defect in growth. Furthermore we see only partial growth restriction for both Δ*iscR* and apo-IscR mutants when grown at 37°C in M9 and M9-Fe, conditions which allow

expression and function of the T3SS (data not shown). These data support our results demonstrating a defect for both Δ*iscR* and apo-IscR mutants in secretion of T3SS cargo and T3SS-dependent pore formation in macrophages. However, the mechanism of how IscR disrupts the T3SS is not fully understood.

4.5 Reactive Oxygen

IscR has been shown to be a key player in the resistance to oxidative stress in bacteria such as *P. aeruginoas* and *B. mallei* (Jones-Carson et al., 2008; Kim et al., 2009), although this is contrary to our preliminary results (data not shown) which suggest that in the absence of IscR, *Y. pstb* is actually more resistant to ROS, although the opposite may be true for apo-IscR. These results could be explained though RNASeq results (Miller, Kwuan, and Auerbuch, data not shown) showing differential regulation of dismutases, catalases and peroxidases that encourage the forward reaction of ROS such as hydrogen peroxide to water (Bakshi et al., 2006; Gabrielsen et al., 2012; Han et al., 2008). One catalase in particular shown to be regulated by IscR, KatA, has been shown to be crucial for peroxide sensitivity and virulence in *P. aeruginosa* (Choi et al., 2007; Kim et al., 2009). Data from our RNASeq analysis showed no significant change in expression of KatA in Δ*iscR*, but found a decrease in expression in the apo-IscR mutant. Apo-IscR sensitivity to hydrogen peroxide due to lack of KatA expression, inversely correlates with results seen in *P. aeruginosa*, as sensitivity of an Δ*iscR* mutant to hydrogen peroxide is restored to WT levels with an

extra copy of *katA* (Kim et al., 2009). More work is needed to determine the cause of the alteration in ROS resistance in the IscR *Y. pstb* mutant strains.

5 Conclusions

In summary, our data suggest that IscR in *Y. pstb* is a regulator of the T3SS. From our observations, IscR seems to be an important factor in T3SS gene transcription, pore formation, secretion and translocation, and may play some role in regulation of the flagellar T3SS. In addition, an appropriate holo-IscR/apo-IscR ratio is needed for WT growth in rich media, normal colony morphology as well as provide resistance to ROS. And surprisingly, Fur regulation was observed to regulate the *Y. pstb* Isc operon, in contrast to what is known for *E. coli* IscR. Although more research is needed to fully determine its role in the regulation of the T3SS, work done in this study has allowed us gain insight on a possible new regulator. Future work will address how iron and oxidative stress might control the T3SS as well as the mechanism behind this control.

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