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Authors
Miller, Halie K
Kwuan, Laura
Schwiesow, Leah
et al.

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IscR Is Essential for *Yersinia pseudotuberculosis* Type III Secretion and Virulence

Halie K. Miller¹, Laura Kwuan¹✉a, Leah Schwiesow¹, David L. Bernick², Erin Mettert³, Hector A. Ramirez¹✉b, James M. Ragle⁴, Patricia P. Chan²✉c, Patricia J. Kiley³, Todd M. Lowe², Victoria Auerbuch¹✉

¹ Department of Microbiology and Environmental Toxicology, University of California Santa Cruz, Santa Cruz, California, United States of America. ² Biomolecular Engineering, University of California Santa Cruz, Santa Cruz, California, United States of America. ³ Department of Biomolecular Chemistry, University of Wisconsin-Madison, Madison, Wisconsin, United States of America. ⁴ Department of Molecular, Cell, and Developmental Biology, University of California Santa Cruz, Santa Cruz, California, United States of America

**Abstract**

Type III secretion systems (T3SS) are essential for virulence in dozens of pathogens, but are not required for growth outside the host. Therefore, the T3SS of many bacterial species are under tight regulatory control. To increase our understanding of the molecular mechanisms behind T3SS regulation, we performed a transposon screen to identify genes important for T3SS function in the food-borne pathogen *Yersinia pseudotuberculosis*. We identified two unique transposon insertions in YPTB2860, a gene that displays 79% identity with the *Escherichia coli* iron-sulfur cluster regulator, IscR. A *Y. pseudotuberculosis* iscr in-frame deletion mutant (ΔiscR) was deficient in secretion of Ysc T3SS effector proteins and in targeting macrophages through the T3SS. To determine the mechanism behind IscR control of the Ysc T3SS, we carried out transcriptome and bioinformatic analysis to identify *Y. pseudotuberculosis* genes regulated by IscR. We discovered a putative IscR binding motif upstream of the *Y. pseudotuberculosis* yscW-lcrF operon. As LcrF controls transcription of a number of critical T3SS genes in *Yersinia*, we hypothesized that *Yersinia* IscR may control the Ysc T3SS through LcrF. Indeed, purified IscR bound to the identified yscW-lcrF promoter motif and mRNA levels of lcrF and 24 other T3SS genes were reduced in *Y. pseudotuberculosis* in the absence of IscR. Importantly, mice orally infected with the *Y. pseudotuberculosis* ΔiscR mutant displayed decreased bacterial burden in Peyer’s patches, mesenteric lymph nodes, spleens, and livers, indicating an essential role for IscR in *Y. pseudotuberculosis* virulence. This study presents the first characterization of *Yersinia* IscR and provides evidence that IscR is critical for virulence and type III secretion through direct regulation of the T3SS master regulator, LcrF.

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**Introduction**

Type III secretion systems (T3SS) are important components in the progression of disease for a number of clinically relevant human pathogens, including those in the genera *Shigella*, *Salmonella*, *Escherichia*, *Chlamydia*, *Vibrio*, *Pseudomonas*, and *Yersinia* [1,2]. The T3SS functions as an injectosome that delivers bacterial effector proteins directly into the host cell cytoplasm [2]. While the T3SS apparatus itself is structurally conserved, the repertoire of T3SS effector proteins used by each group of pathogens is distinct [2]. Thus, the effect of the T3SS on the host is unique to the needs of the pathogen [2]. While the T3SS is generally essential for a T3SS-expressing pathogen to cause disease, several aspects of the T3SS may be detrimental to bacterial growth [2]. For example, T3SS components are recognized by the host immune system [3,4]. In addition, expression of the T3SS is energetically costly and, in some organisms, T3SS induction correlates with growth arrest [5]. Therefore, regulation is essential for proper T3SS function in order to ensure that it occurs only during host cell contact in the appropriate host tissue [2,6].

Members of the genus *Yersinia* that utilize a T3SS are important human pathogens: *Y. pestis*, the causative agent of plague, and the enteropathogens *Y. enterocolitica* and *Y. pseudotuberculosis*. The *Y. pseudotuberculosis* Ysc T3SS is encoded on a 70-kb plasmid termed pYV [7–9] and is made up of approximately 25 known proteins comprising three main structures: the basal body, the needle apparatus, and the translocon [10,11]. The basal body, which displays a high degree of similarity to the flagellar basal body, is made up of rings that span the inner and outer membranes and a rod that traverses the periplasmic space [12]. Basal body associated proteins include YscN, an ATPase that aids in the secretion and translocation of effector proteins [13]. The needle complex, which is thought to act as a molecular channel for effector protein translocation, is a straight hollow appendage approximately 60 nm in length and is made up of helical
Author Summary

Bacterial pathogens use regulators that sense environmental cues to enhance their fitness. Here, we identify a transcriptional regulator in the human gut pathogen, Yersinia pseudotuberculosis, which controls a specialized secretion system essential for bacterial growth in mammalian tissues. This regulator was shown in other bacterial species to alter its activity in response to changes in iron concentration and oxidative stress, but has never been studied in Yersinia. Importantly, Y. pseudotuberculosis experiences large changes in iron bioavailability upon transit from the gut to deeper tissues and iron is a critical component in Yersinia virulence, as individuals with iron overload disorders have enhanced susceptibility to systemic Yersinia infections. Our work places this iron-modulated transcriptional regulator within the regulatory network that controls virulence gene expression in Y. pseudotuberculosis, identifying it as a potential new target for antimicrobial agents.

polimerized subunits of YscF [12]. The translocon is comprised of three proteins: YopD, YopB and LcrV, which are essential for pore formation in the target host membrane and proper translocation of effector proteins YopH/HemoJ/Tk to the host cytoplasm [12,14]. Also encoded on pYV are chaperones important for efficient translocation of a subset of effector proteins [15]. Lastly, several transcriptional and post-transcriptional regulators of the T3SS are found on pYV, including the AraC-like transcriptional regulator LcrF. LcrF is responsible for expression of a number of T3SS structural genes and Yop effectors, specifically the virC and lerGTH-yopBD operons as well as genes encoding effector Yops, the adhesin YadA, and the lipoprotein YlpA [16-22]. LcrF itself is thermoregulated at both the transcriptional and translational levels through the action of the histone-like protein YmoA and a cis-acting RNA thermosensor located on the lerF transcript, respectively [23,24]. This enables Yersinia to express T3SS genes at 37°C within the mammalian host, but not at lower temperatures [23,24]. Importantly, proper LcrF-mediated control of T3SS expression is important for Y. pseudotuberculosis virulence [24].

IscR belongs to the Rrf2 family of winged helix-turn-helix transcription factors [25,26] and has been studied extensively in E. coli, where its DNA-binding activity is dependent on coordination of an iron-sulfur [2Fe-2S] cluster through three conserved cysteines and a histidine [27-30]. E. coli IscR recognizes two distinct DNA motifs, type 1 and type 2, depending on the Fe-S status of the protein [31]. Holo-IscR coordinating an Fe-S cluster binds both type 1 and type 2 motifs, while clusterless apo-IscR recognizes only the type 2 DNA-binding motif [27,32,33]. As iron starvation, oxidative stress, and oxygen limitation affect the holo-IscR/apo-IscR ratio, these environmental cues are thought to have a direct effect on gene expression through IscR in E. coli [28-30]. For example, holo-IscR represses transcription of the housekeeping iscRSUA-hscBA-fdx Fe-S cluster biogenesis operon [32,34], while either holo- or apo-IscR promotes transcription of the inducible sugABCDSE Fe-S cluster biogenesis operon [33,35]. Both pathways function to insert Fe-S clusters onto proteins involved in a range of metabolic processes including electron transfer, substrate binding/activation, iron/sulfur storage, regulation, and enzyme activity [36]. In addition, E. coli IscR is also known to regulate transcription of other Fe-S cluster assembly genes such as ndkR (ndkR) as well as genes integral to oxidative stress resistance, biofilm formation, and anaerobic respiration [28-30,34]. IscR is widely conserved among bacteria [25] and its regulatory activity is integral to the infectious process of the plant pathogen Erwinia chrysanthemi [37]. Furthermore, IscR plays an important role in the virulence of the human pathogens Pseudomonas aeruginosa through modulation of the catalase katA [38], Burkholderia mallei through resistance to reactive nitrogen species [39], and Vibrio vulnificus through induction of several virulence-associated pathways [39,40]. While the iron-dependent transcriptional repressor Fur has been shown to control T3SS expression in Salmonella and Shigella [41,42], IscR has never been linked to regulation of the T3SS in any organism and has not been studied in Yersinia.

In this study, we isolated two independent IscR transposon insertion mutants in a novel screen for Y. pseudotuberculosis genes important for T3SS function. We assessed the impact of iscR deletion on Y. pseudotuberculosis in vitro and in vivo growth, type III secretion, and global gene expression. We found IscR to be essential for full T3SS function and virulence in a mouse model of infection. In addition, we provide evidence that IscR control of the T3SS stems from direct transcriptional regulation of the T3SS master regulator LcrF.

Results

IscR is required for Y. pseudotuberculosis Ysc T3SS function

To identify regulators of the Y. pseudotuberculosis T3SS, we utilized a novel screen to isolate transposon mutants with defects in T3SS function. We previously showed that Y. pseudotuberculosis expressing a functional T3SS induces NFκB activation in HEK293T cells [43], enabling us to use host cell NFκB activation as a readout for T3SS function in Y. pseudotuberculosis transposon mutants. As some T3SS effector proteins inhibit NFκB signaling [44], we performed the screen using a Y. pseudotuberculosis transposon mutant library in a genetic background that lacked the known T3SS effector proteins YopH/HemoJ (Δyop6; [43]). We identified several transposon mutants with defects in triggering activation of NFκB in HEK293T cells (L. Kwuan, N. Herrera, H. Ramirez, V. Auerbuch, data not shown), suggesting defective T3SS function. Among these were two strains with unique transposon insertions in YPTB2860 (Figure 1A), encoding a protein with 79% identity to the E. coli iron-sulfur cluster regulator IscR, part of the iscRSUA-hscBA-fdx operon involved in Fe-S cluster biogenesis (Figure 1B). Importantly, the helix-turn-helix DNA binding domain as well as the three cysteines and histidine known to coordinate an iron-sulfur (Fe-S) cluster in E. coli IscR are conserved in all three Yersinia species (Figure 1B). These data indicate that Yersinia IscR may coordinate an Fe-S cluster and, as in E. coli, may regulate gene transcription.

To validate that loss of IscR in Y. pseudotuberculosis leads to T3SS defects, we isolated the two iscR transposon mutants (iscR:Tn1 and iscR:Tn2) from our library and again measured their ability to trigger NFκB activation in HEK293T cells compared to the Δyop6 parental strain and a ΔiscN/Y T3SS-null mutant [43]. In addition, we constructed an in-frame iscR deletion mutant in the Δyop6 genetic background (Δyop6/ΔiscR) and tested it in this assay. We found that disruption of iscR led to ~2-fold less NFκB activation relative to the Δyop6 T3SS+ parental strain, although NFκB activation levels were still ~5-fold higher than a strain with complete lack of T3SS function (ΔiscN/Y; Figure 2A), suggesting that loss of iscR leads to partial T3SS loss.

To further verify that deletion of iscR leads to alterations in T3SS function, we assessed the ability of the Δyop6/ΔiscR mutant to insert YopBD pores in target host cell membranes by measuring
entry of ethidium bromide (EtBr) inside Y. pseudotuberculosis-infected bone marrow derived macrophages [45,46]. Pore formation by the \( \text{Dyop6/DiscR} \) mutant was decreased by 7-fold (\( p < 0.05 \)) relative to the \( \text{Dyop6 parental strain} \), which could be restored upon complementation with plasmid-encoded \( \text{iscR} \) (Figure 2B). To determine whether loss of \( \text{iscR} \) affects T3SS function in a wild type genetic background, we constructed an in-frame \( \text{iscR} \) deletion (\( \text{DiscR} \)) in the wild type \( \text{Y. pseudotuberculosis} \) IP2666 strain expressing six of the seven known T3SS effector proteins YopHEMOJK [47]. We then visualized the secretome of the \( \text{DiscR} \) mutant relative to wild type. Deletion of \( \text{iscR} \) led to a dramatic decrease in secretion of T3SS cargo relative to the wild type background, which can be restored upon complementation with plasmid-encoded \( \text{iscR} \) (Figure 2C). Importantly, this lack of type III secretion did not result from a defect in growth of the mutant, as the \( \text{DiscR} \) mutant actually grew better than wild type bacteria under T3SS-inducing conditions (Figure S1A). This is consistent with a T3SS defect in this strain, as wild type \( \text{Yersinia} \) display a characteristic growth arrest upon T3SS expression [5,48,49]. Collectively, these data demonstrate that \( \text{Y. pseudotuberculosis} \) \( \text{IscR} \) is required for proper T3SS function.

\( \text{IscR} \) is required for full virulence of \( \text{Y. pseudotuberculosis} \)

Based on the knowledge that the T3SS plays an important role in the virulence of human pathogenic \( \text{Yersinia} \), we sought to investigate whether the diminished type III secretion observed in the \( \text{Y. pseudotuberculosis} \) \( \text{DiscR} \) strain would lead to a reduction in the infectious capacity of this mutant. Mice were orogastrically infected with \( 2 \times 10^8 \) CFU of either the \( \text{Y. pseudotuberculosis} \) wild type or isogenic \( \text{DiscR} \) mutant strains.

\section*{Results}

\subsection*{Y. pseudotuberculosis DNA sequence}

The \( \text{Y. pseudotuberculosis} \) DNA sequence, which displays the unique insertions sites for the two transposon mutants generated from our genetic screen. A space in the DNA sequence and a solid black line indicate the site of insertion for either \( \text{iscR}::\text{Tn1} \) or \( \text{iscR}::\text{Tn2} \).
infected with Y. pseudotuberculosis displaying significantly decreased colonization of Peyer’s patches and mesenteric lymph nodes (MLN) as well as diminished systemic colonization (Figure 3). Specifically, we noted 10- and 130-fold reductions in CFU recovered from the Peyer’s patches and MLNs, respectively, in mice infected with the ΔiscR mutant strain relative to wild type. Notably, we observed a 1000- to 10,000-fold decrease in bacterial burden in the spleen and liver respectively. The diminished ability of the ΔiscR mutant strain to colonize deep tissue sites is underscored by the fact that bacteria were not detected in seven of the nine livers analyzed. These findings suggest that IscR is essential for Y. pseudotuberculosis virulence in an oral infection model.

IscR deletion leads to global misregulation of gene expression in Y. pseudotuberculosis

To begin to understand the mechanistic contribution of IscR to Y. pseudotuberculosis pathogenesis, we performed high throughput transcriptome sequencing (RNAseq) analysis to determine the T. pseudobrusellosis genes directly and indirectly controlled by IscR under iron replete, T3SS-inducing conditions. Total RNA was collected from wild type Y. pseudotuberculosis as well as the ΔiscR mutant strain grown in M9 at 37°C for 3 h, a point at which the ΔiscR and wild type strains display comparable growth rates (Figure S1A).

For the ΔiscR mutant relative to the wild type, a total of 226 genes demonstrated a statistically significant fold change of ≥2 (Table S1). Of these, 134 genes were up-regulated in the ΔiscR mutant relative to the wild type (Table 1 & Figure 4A), while 92 were down-regulated (Table 2 & Figure 4B). Genes found to be up-regulated in the ΔiscR mutant include key elements of Fe-S cluster biosynthesis, cellular detoxification, metabolism, and protein fate (Figure 4A). The most notable increases in transcription were observed for genes encoding Fe-S cluster biosynthesis proteins including those encoded in the isc operon, iscN (18.7-fold), iscU (21.7-fold) and isiA (13-fold) (Table 1 & Figure S2A). Additional genes encoding proteins involved in Fe-S cluster assembly and their respective fold increases include isiX/yfhJ (10.9), hscB (10.9), hscC (10), hspD (9.3), yadR/erpA (6.8), petB (10.1) and yfjA (7.0). To validate these findings, we performed qRT-PCR analysis on the second gene encoded in the iscRSUA-hscBA-fdx operon, isiS, as well as on the gene encoding the Fe-S biosynthesis protein ErpA. Transcription of isiS was increased by 30-fold, while erpA expression was increased 5-fold (p<0.05; Figure 5A). Bioinformatic analysis identified two IscR type 1 motifs upstream of the isiRSUA operon, as well as on the gene encoding the Fe-S biosynthesis protein ErpA. Transcription of isiS was increased by 30-fold, while erpA expression was increased 5-fold (p<0.05; Figure 5A). Bioinformatic analysis identified two IscR type 1 motifs upstream of the isiRSUA operon, as well as on the gene encoding the Fe-S biosynthesis protein ErpA. Transcription of isiS was increased by 30-fold, while erpA expression was increased 5-fold (p<0.05; Figure 5A). Bioinformatic analysis identified two IscR type 1 motifs upstream of the isiRSUA operon, as well as on the gene encoding the Fe-S biosynthesis protein ErpA. 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IscR is required for transcription of T3SS genes

In total, 92 genes were significantly down-regulated in the ΔiscR mutant relative to wild type Y. pseudotuberculosis (Table 2). These data demonstrate that the majority of pYV-encoded genes are decreased in the ΔiscR mutant relative to the wild type strain, including genes essential for proper T3SS expression and function. The virC and lcrGVH-yopBD operons as well as genes encoding the T3SS cargo YopHEMOJTK were the most affected upon deletion of iscR: the effector proteins YopJ (~2.3-4-fold), YopM (~2.5-fold) and YopT (~3.5-fold), the effector protein and translocation regulator YopK (~2.9-fold), as well as a number of genes encoding T3SS structural proteins. Genes encoding regulators that control T3SS expression and function were decreased in the mutant including lerQ (~2.1-fold), lerF (~3.3-fold), lerG (~2.8-fold) and lerH (~3.9-fold). To verify that T3SS gene expression was indeed decreased in the ΔiscR mutant, we measured the transcript levels of the genes encoding T3SS structural proteins YscN, YscF, and the T3SS transcriptional regulator LcrF via qRT-PCR. As detailed in Figure 5B, we observed fold decreases of 2.8-fold (p<0.05), 6.9-fold (p<0.001), and 5.4-fold (p<0.0001) for yscN, yscF, and lerF, respectively. These data support our RNAseq analysis and confirm that IscR is required for robust transcription of Y. pseudotuberculosis T3SS genes.

In addition to T3SS genes, 25 other pYV-encoded genes were decreased in the mutant, but these are annotated as hypothetical proteins, transposases, and pseudogenes. Analysis of the relative abundance of pYV in the Y. pseudotuberculosis wild type and ΔiscR strains was performed in order to verify that the decreases in pYV-encoded genes were not a result of plasmid loss (Figure S3). The concentration of plasmid isolated from the wild type and ΔiscR mutant was comparable, suggesting that the decreased transcription of pYV-encoded genes, including those encoding the T3SS, are not a result of decreased stability of the pYV plasmid.

Figure 3. IscR is required for full virulence of Y. pseudotuberculosis. Mice were infected with 2×10^8 CFU of either WT Y. pseudotuberculosis or ΔiscR mutant via orogastric gavage. At 5 days post-inoculation, the Peyer’s patches (PP), mesenteric lymph nodes (MLN), spleens and livers were collected, homogenized and CFU determined. Each symbol represents one animal. Unfilled symbols indicate that CFU were below the limit of detection. The data presented are from three independent experiments. *p<0.05, ***p<0.001 as determined by an unpaired Wilcoxon-Mann-Whitney rank sum test. Dashes represent the geometric mean.

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Table 1. Genes repressed by IscR, identified by RNAseq analysis.

| Gene Ontology                | ORF ID*   | Description                                      | Gene    | Fold Up Regulationb |
|------------------------------|-----------|--------------------------------------------------|---------|---------------------|
| Fe-S Cluster Biogenesis (11) | YPTB0744  | Fe-S insertion protein                           | yadR/erpA | 6.8                 |
|                              | YPTB2851  | enhanced serine sensitivity protein             | sseB    | 6.6                 |
|                              | YPTB2852  | peptidase B                                     | pepB    | 10.1                |
|                              | YPTB2853  | Fe-S assembly protein                           | iscX/yfhJ | 10.8               |
|                              | YPTB2854  | Isc system ferredoxin                           | fdx     | 10.9                |
|                              | YPTB2855  | Fe-S assembly chaperone                         | hscA    | 9.3                 |
|                              | YPTB2856  | Fe-S assembly chaperone                         | hscB    | 10.0                |
|                              | YPTB2857  | Fe-S assembly protein                           | iscA    | 13.0                |
|                              | YPTB2858  | Fe-S assembly scaffold                          | iscU    | 21.7                |
|                              | YPTB2859  | cysteine desulfurase                            | iscS    | 18.7                |
| Sulfur Metabolism (11)       | YPTB0759  | sulfite reductase, beta (flavoprotein) subunit  | cysJ    | 3.7                 |
|                              | YPTB0760  | sulfite reductase, alpha subunit                | cysI    | 2.2                 |
|                              | YPTB0761  | 3-phosphoadenosine 5-phosphosulfate (PAPS) reductase | cysH | 2.0                 |
|                              | YPTB0764  | Siroheme synthase                               | cysG    | 2.4                 |
|                              | YPTB0765  | ATP-sulfurylase, subunit 2                      | cysD    | 4.8                 |
|                              | YPTB0766  | ATP-sulfurylase, subunit 1                      | cysN    | 4.0                 |
|                              | YPTB0767  | adenosine 5-phosphosulfate kinase                | cysC    | 2.7                 |
|                              | YPTB2714  | cysteine synthase A                             | cysK    | 3.5                 |
|                              | YPTB2732  | ABC sulfate transporter, ATP-binding subunit    | cysA    | 2.1                 |
|                              | YPTB2735  | ABC trans, periplasmic thiosulfate-binding protein | cysP | 3.0                 |
|                              | YPTB2769  | putative sulfatase                              | ydeN    | 2.5                 |
| Cellular Detox (4)           | YPTB0756  | superoxide dismutase precursor (Cu-Zn)          | sodC    | 2.0                 |
|                              | YPTB0811  | catalase hydroperoxidase HPI(I)                  | katY    | 8.6                 |
|                              | YPTB2261  | thiol peroxidase                                | tpx     | 2.0                 |
|                              | YPTB2299  | superoxide dismutase [Fe]                       | sodB    | 6.3                 |
| Protein Fate (33)            | YPTB0017  | secreted thiol/disulfide interchange protein    | dsbA    | 2.0                 |
|                              | YPTB0097  | ATP-binding heat shock protein                  | hslU    | 2.7                 |
|                              | YPTB0102  | 50S ribosomal protein L31                       | rpmE    | 2.4                 |
|                              | YPTB0276  | elongation factor Tu                            | tuf     | 2.7                 |
|                              | YPTB0279  | 50S ribosomal protein L11                       | rplK    | 2.5                 |
|                              | YPTB0280  | 50S ribosomal protein L1                        | rplA    | 2.3                 |
|                              | YPTB0281  | 50S ribosomal protein L10                      | rplU    | 2.7                 |
|                              | YPTB0282  | 50S ribosomal protein L7/L12                    | rplL    | 3.0                 |
|                              | YPTB0404  | 10 kDa chaperonin                               | groES   | 5.2                 |
|                              | YPTB0405  | 60 kDa chaperonin                               | groEL   | 5.7                 |
|                              | YPTB0438  | 30S ribosomal protein S6                       | rpsF    | 2.2                 |
|                              | YPTB0440  | 30S ribosomal protein S18                       | rpsR    | 2.2                 |
|                              | YPTB0441  | 50S ribosomal protein L9                       | rplI    | 2.3                 |
|                              | YPTB0464  | 50S ribosomal protein L21                       | rplU    | 2.3                 |
|                              | YPTB0465  | 50S ribosomal protein L27                       | rpmA    | 2.6                 |
|                              | YPTB0611  | chaperone Hsp70                                 | dnaK    | 3.8                 |
|                              | YPTB0612  | heat shock protein                              | dnaJ    | 3.6                 |
|                              | YPTB0749  | periplasmic serine protease Do, heat shock protein | htrA | 4.4                 |
|                              | YPTB0848  | ATP-dependent, Hsp 100                          | clpB    | 4.1                 |
|                              | YPTB0958  | trigger factor                                  | tig     | 2.5                 |
|                              | YPTB0960  | clpA-clpP ATP-dependent serine protease, chaperone | clpX | 2.7                 |
|                              | YPTB0961  | ATP-dependent protease                          | lon     | 2.9                 |
|                              | YPTB0995  | chaperone Hsp90, heat shock protein C 62.5       | htpG    | 3.3                 |
|                              | YPTB1090  | sec-independent protein translocase protein     | tatE    | 2.9                 |
| Gene Ontology                  | ORF ID* | Description                                    | Gene    | Fold Up Regulation |
|-------------------------------|---------|------------------------------------------------|---------|--------------------|
| Table 1. Cont.                |         |                                                |         |                    |
| YPTB1113                      |         | putative tRNA-thiotransferase                  | miaB    | 2.0                |
| YPTB1141                      |         | heat shock protein GrpE                        | grpE    | 2.7                |
| YPTB1417                      |         | 30S ribosomal protein S1                       | rpsA    | 2.2                |
| YPTB1448                      |         | putative ribosome modulation factor            | rmf     | 3.8                |
| YPTB2820                      |         | putative protease                              |         | 2.1                |
| YPTB3000                      |         | ribosome recycling factor                      | frr     | 2.1                |
| YPTB3026                      |         | protease III precursor                         | ptrA    | 2.4                |
| YPTB3511                      |         | Protease                                       | degQ    | 3.4                |
| YPTB3904                      |         | heat shock protein                             | ibpA    | 3.5                |
| YPTB3905                      |         | heat shock protein                             | ibpB    | 4.5                |
| Misc. Metabolism (33)         |         |                                                |         |                    |
| YPTB0135                      |         | acetolactate synthase isozyme II small subunit | ilvM    | 2.6                |
| YPTB0297                      |         | DNA-binding protein HU-alpha                   | hupA    | 2.1                |
| YPTB0402                      |         | aspartate ammonia-lyase                        | aspA    | 3.1                |
| YPTB0456                      |         | fructose-1, 6-bisphosphatase                    | fbp     | 2.1                |
| YPTB0460                      |         | malate dehydrogenase                           | mdh     | 2.1                |
| YPTB0546                      |         | putative glycoprotein/receptor                  |         | 2.0                |
| YPTB0755                      |         | enolase                                        | eno     | 2.1                |
| YPTB0809                      |         | probable cytochrome b(561)                     | cybB    | 4.0                |
| YPTB0810                      |         | putative cytochrome b(562)                     | cybC    | 6.7                |
| YPTB1117                      |         | putative N-acetylglucosamine regulatory protein | nagC    | 2.5                |
| YPTB1118                      |         | N-acetylglucosamine-6-phosphate deacetylase    | nagA    | 4.1                |
| YPTB1119                      |         | putative glucosamine-6-phosphate isomerase     | nagB    | 5.9                |
| YPTB1120                      |         | N-acetylglucosamine-specific IIA/B component   | nagE    | 5.2                |
| YPTB1148                      |         | dihydrolipoamide succinyltransferase           | sucB    | 2.1                |
| YPTB1149                      |         | succinyl-CoA synthetase beta chain             | succD   | 3.0                |
| YPTB1150                      |         | succinyl-CoA synthetase alpha chain            |         | 3.1                |
| YPTB1358                      |         | glutaredoxin 1                                 | grxA    | 2.3                |
| YPTB1418                      |         | integration host factor beta-subunit           | ihbB    | 2.7                |
| YPTB2047                      |         | pyruvate kinase II                             | pykA    | 2.3                |
| YPTB2143                      |         | aconitate hydratase 1                          | acnA    | 2.2                |
| YPTB2216                      |         | putative acetylactate synthase large subunit   | ilvB    | 2.2                |
| YPTB2217                      |         | putative acetylactate synthase small subunit   | ilvN    | 2.3                |
| YPTB2306                      |         | pyruvate kinase I                              | pykF    | 2.0                |
| YPTB2845                      |         | nucleoside diphosphate kinase                  | ndk     | 2.3                |
| YPTB2870                      |         | flavohemoprotein                               | hmp     | 2.1                |
| YPTB2943                      |         | urease beta subunit                            | ureB    | 2.0                |
| YPTB2944                      |         | urease gamma subunit                           | ureA    | 2.1                |
| YPTB3202                      |         | Biosynthetic arginine decarboxylase            | speA    | 2.1                |
| YPTB3572                      |         | biotin carboxylase                              | accC    | 2.1                |
| YPTB3966                      |         | ATP synthase epsilon subunit protein            | atpE    | 2.5                |
| YPTB3967                      |         | ATP synthase beta subunit protein               | atpD    | 2.1                |
| YPTB3968                      |         | ATP synthase gamma subunit protein              | atpG    | 2.2                |
| YPTB3969                      |         | ATP synthase alpha subunit protein              | atpA    | 2.1                |
| Regulatory Functions (6)      |         |                                                |         |                    |
| YPTB0784                      |         | putative transcriptional regulatory protein     |         | 2.0                |
| YPTB1955                      |         | putative phosphate starvation-inducible protein | phoH    | 2.2                |
| YPTB3068                      |         | putative carbonic anhydrase                    |         | 2.2                |
| YPTB3418                      |         | RNA polymerase sigma factor RpoD               | rpoD    | 2.3                |
| YPTB3527                      |         | putative sigma N modulation factor              | yhbH    | 2.1                |
| Transport and Binding Proteins (9) | YPTB0306 | putative sodium:phenylacetate symporter       | actP    | 2.4                |
**Table 1.** Cont.

| Gene Ontology        | ORF ID*         | Description                                                      | Gene   | Fold Up Regulationb |
|----------------------|-----------------|------------------------------------------------------------------|--------|---------------------|
| YPTB1718             | putative cystine-binding periplasmic protein                  | fltY    | 2.4                |
| YPTB2463             | PTS system, glucose-specific IIBC component                   | ptsG    | 2.0                |
| YPTB2682             | ABC transporter, periplasmic iron(III)-binding protein         | sfuA    | 2.6                |
| YPTB2771             | PTS system glucose-specific IIA component, permease            | crr     | 2.3                |
| YPTB2770             | probable ABC transporter, ATP-binding subunit                   |         | 2.8                |
| YPTB2771             | putative ABC iron transporter                                   |         | 2.7                |
| YPTB2772             | ABC transporter, periplasmic iron binding protein               |         | 4.1                |
| YPTB3957             | ABC transporter, periplasmic amino acid binding protein         |         | 4.6                |
| YPTB1334             | pH 6 antigen precursor (antigen 4) (adhesin)                   | psaA    | 2.5                |
| YPTB2123             | putative exported protein                                      | ompW    | 3.3                |
| YPTB2287             | putative lipoprotein                                           | slyB    | 2.0                |
| YPTB2867             | attachment invasion locus protein                               | all     | 4.3                |
| YPTB3584             | outermembrane protein                                          | pcp     | 2.5                |
| YPTB0439             | primosomal replication protein n                               | prtB    | 2.2                |
| YPTB0693             | tubulin-like GTP-binding protein and GTPase                    | ftZ     | 2.1                |
| YPTB0782             | putative dihydroxyacetone kinase                               |         | 2.7                |
| YPTB0830             | quorum sensing protein                                          | luxS    | 2.4                |
| YPTB1162             | quinolinate synthetase A                                       | nadA    | 2.1                |
| YPTB1182             | biotin synthase                                                | bioB    | 2.2                |
| YPTB1468             | cytotoxic necrotizing factor (partial)                         |         | 6.1                |
| YPTB1517             | formaldehyde dehydrogenase                                     |         | 2.2                |
| YPTB2248             | D-lactate dehydrogenase                                        | ldhA    | 2.1                |
| YPTB2395             | probable N-acetylmuramoyl-L-alanine amidase                    |         | 2.4                |
| YPTB2791             | putative arsenate reductase                                     | ygfD    | 2.0                |
| YPTB2887             | pyridoxal phosphate biosynthetic protein                       | pdxJ    | 2.2                |
| YPTB0391             | putative exported protein                                      |         | 2.1                |
| YPTB0449             | hypothetical protein                                           |         | 3.3                |
| YPTB0458             | putative exported protein                                      |         | 2.2                |
| YPTB1093             | hypothetical protein                                           |         | 3.6                |
| YPTB1571             | hypothetical protein                                           |         | 2.1                |
| YPTB2255             | putative exported protein                                      |         | 2.3                |
| YPTB2277             | hypothetical protein                                           |         | 2.6                |
| YPTB2496             | hypothetical protein                                           |         | 2.8                |
| YPTB3109             | hypothetical protein                                           |         | 4.1                |

*ORF IDs are derived from the *Y. pseudotuberculosis* IP 32593 genome unless otherwise stated.

bFold change is of the ΔiscR mutant relative to the wild type strain.

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**Y. pseudotuberculosis** expressing only apo-locked IscR has a proton motive force defect and cannot secrete Yops

To assess the contribution of Fe-S cluster ligation to IscR control of the T3SS, we constructed an IscR mutant strain in which the three conserved cysteines were substituted with alanines (C92A, C98A, C104A; apo-locked IscR). Identical mutations in *E. coli* IscR render the protein unable to coordinate an iron-sulfur cluster, yet able to bind type 2 DNA binding motifs and to regulate target gene transcription [28–30]. We analyzed the secretome of the *Y. pseudotuberculosis* apo-locked IscR strain under T3SS-inducing conditions and found that the mutant was just as defective as the ΔiscR strain in Yop secretion (Figure 2C). This defect could be complemented with plasmid-encoded wild type IscR. As apo-locked IscR is insufficient to promote type III secretion, holo-IscR-mediated regulation of gene expression through one or more type 1 motifs may be specifically involved in regulating T3SS gene expression. Alternatively, forcing all IscR expression within the cell to the clusterless form, which leads to IscR overexpression, may lead to alterations of bacterial pathways that indirectly affect type III secretion.

Consistent with this latter explanation, the apo-locked IscR mutant exhibited decreased colony size on LB agar, slower growth in rich media (Figure S1), and decreased motility (Figure 6A). The flagellar basal body is a T3SS itself, indicating that the defect in the Ysc T3SS for this strain may be a result of gross abnormalities in secretion systems. Based on these findings, we set out to examine

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Dissertation of the (potential of the addition of wild type relative to the wild type strain, which can be complemented upon decrease in membrane potential in the apo-locked IscR mutant conditions. As demonstrated in Figure 6B, there is a notable apo-IscR in order maintain normal membrane potential. For mutant has a proton motive force defect, leading to decreased type type. Collectively, these data suggest that the apo-locked IscR operon were observed for the apo-locked IscR strain when compared to both the wild type and ΔiscR strains (Figure S4). As IscR is overexpressed by 30-fold (p<0.05) in the apo-locked iscR mutant compared to wild type (Figure S2A), we speculate that the suf operon is positively regulated by IscR in Yersinia as in E. coli. In contrast, the extensively studied E. coli IscR target, hycABCDEF, is not encoded in the Y. pseudotuberculosis genome.

Importantly, our RNAseq analysis demonstrated that transcription of genes within the visA, visB, visC, yscW-lcrF, and lcrGVH-yopBD operons was restored in the apo-locked IscR mutant compared to the ΔiscR mutant (Figure 7 and Table S2). However, we observed a decrease in transcription of genes encoding the T3SS effector proteins YopH (~4.4-fold), YopM (~3.0-fold), YopK (~7.1-fold), and YopE (~2.1-fold) in the apo-locked IscR mutant compared to wild type. Transcription of yopE has been shown to be regulated by Yop secretion through a positive feedback loop [51,52], suggesting that the defect in YopHEMK transcription observed in the apo-locked IscR mutant may be caused by the lack of Yop secretion we observed in this strain. Together, these data suggest that both hol- and apo-IscR can promote T3SS gene transcription, possibly through binding to one or more type 2 DNA motifs.

To determine whether IscR might directly regulate T3SS gene expression, we carried out bioinformatic analysis to search pYV for sequences resembling the E. coli IscR type 2 motif (xxWWWWCCxYAxxxxxxxTRxGGWWWWxx) [30,31,33], as the DNA-binding domain of Yersinia IscR is 100% identical to that of E. coli IscR (Figure 1A). We searched within the 150 nucleotides upstream of the 99 genes encoded on the pYV plasmid and obtained a ranked list of putative type 2 motifs (data not shown). Among these was a site located within the yscW-lcrF promoter region (Figure 8A) [24]. To test whether IscR bound specifically to this site, we performed equilibrium DNA competition assays utilizing purified E. coli IscR-C92A (apo-IscR) [33], with a fluorescein-labeled E. coli hyc type 2 site previously identified by Nesbit et al. [33]. Purified E. coli IscR was utilized in this assay, as complementation of the Y. pseudotuberculosis ΔiscR mutant strain with IscR of E. coli encoded on a plasmid fully restored secretion of T3SS cargo (Figure 8B). Competitor DNA included unlabeled E. coli hyc as a positive control, the identified site within the Yersinia yscW-lcrF promoter region, a mutated version of this sequence (mutF), where nucleotides previously demonstrated in E. coli to be important for type 2 motif binding were altered [33], as well as one of the Y. pseudotuberculosis isc type 1 motif sites we identified as a negative control (Figure S2B & Figure 8C). We found that unlabeled lcrF DNA competed as well as unlabeled hyc DNA (IC50 27 nm and 61 nm, respectively), suggesting that IscR can indeed bind to the identified type 2 motif upstream of lcrF (Figure 8D). Furthermore, mutation of key nucleotides in the lcrF promoter sequence led to alleviation of competition and increased the IC50 to greater than 1000 nM, a level comparable to that of the isc negative control type 1 motif site.
| Gene Ontology | ORF ID | Description | Gene | Fold Up Regulation |
|---------------|--------|-------------|------|-------------------|
| pYV-encoded (50) | pYV0002 | YpkA chaperone | sycO | 4.6 |
|               | pYV0003 | putative transposase remnant |      | 2.2 |
|               | pYV0008 | possible transposase remnant |      | 2.6 |
|               | pYV0009 | hypothetical protein |      | 3.3 |
|               | pYV0010 | hypothetical protein |      | 3.3 |
|               | pYV0012 | hypothetical protein |      | 4.2 |
|               | pYV0014 | possible transposase remnant |      | 3.2 |
|               | pYV0015 | possible transposase remnant |      | 3.9 |
|               | pYV0016 | trpA putative transposase protein |      | 2.6 |
|               | pYV0021 | putative transposase |      | 2.4 |
|               | pYV0022 | putative transposase |      | 2.3 |
|               | pYV0023 | possible transposase remnant |      | 2.8 |
|               | pYV0034 | putative transposase remnant |      | 3.3 |
|               | pYV0035 | hypothetical protein |      | 3.0 |
|               | pYV0036 | hypothetical protein |      | 3.6 |
|               | pYV0037 | C-term conjugative transfer: surface exclusion |      | 4.2 |
|               | pYV0038 | N-term fragment conjugative transfer: surface exclusion |      | 8.3 |
|               | pYV0039 | putative transposase |      | 7.5 |
|               | pYV0040 | yop targeting protein | yopK | 9.3 |
|               | pYV0041 | yop targeted effector | yopT | 5.5 |
|               | pYV0044 | hypothetical protein |      | 4.1 |
|               | pYV0046 | putative transposase remnant |      | 2.9 |
|               | pYV0047 | targeted effector protein | yopM | 5.3 |
|               | pYV0049 | hypothetical protein |      | 2.4 |
|               | pYV0056 | low calcium response protein H | lcrH | 3.9 |
|               | pYV0057 | V antigen, antihost protein/regulator | lcrV | 3.5 |
|               | pYV0058 | Yop regulator | lcrG | 2.8 |
|               | pYV0061 | type III secretion protein | yscY | 2.2 |
|               | pYV0062 | type III secretion protein | yscX | 2.5 |
|               | pYV0063 | type III secretion protein | yscN | 2.5 |
|               | pYV0064 | Yop secretion and targeting protein | yvaA | 2.1 |
|               | pYV0068 | type III secretion protein | yscO | 2.0 |
|               | pYV0069 | type III secretion protein | yscP | 2.1 |
|               | pYV0075 | Yop targeting lipoprotein | virG | 2.5 |
|               | pYV0076 | putative thermoregulatory protein | lcrF | 3.3 |
|               | pYV0078 | hypothetical protein | yscB | 3.5 |
|               | pYV0079 | type III secretion protein | yscC | 2.0 |
|               | pYV0080 | type III secretion protein | yscD | 2.0 |
|               | pYV0082 | type III secretion protein | yscF | 2.8 |
|               | pYV0083 | type III secretion protein | yscG | 2.9 |
|               | pYV0084 | type III secretion protein | yscH | 2.1 |
|               | pYV0087 | type III secretion protein | yscK | 3.2 |
|               | pYV0088 | type III secretion protein | yscL | 2.2 |
|               | pYV0089 | type III secretion regulatory | lcrQ | 2.1 |
|               | pYV0090 | putative transposase |      | 2.7 |
|               | pYV0091 | putative transposase |      | 3.1 |
|               | pYV0092 | putative transposase |      | 3.2 |
|               | pYV0093 | putative transposase |      | 2.2 |
|               | pYV0098 | targeted effector protein | yopJ | 3.4 |
|               | pYV0099 | hypothetical protein |      | 4.8 |
Table 2. Cont.

| Gene Ontology                        | ORF ID*       | Description                                      | Gene      | Fold Up Regulationb |
|--------------------------------------|---------------|--------------------------------------------------|-----------|---------------------|
| Hemin Transport (4)                  | YPTB0336      | ABC hemin transporter, ATP-binding subunit        | hmuV      | 2.4                 |
|                                      | YPTB0337      | ABC hemin transporter, permease subunit           | hmuU      | 2.4                 |
|                                      | YPTB0338      | ABC transporter, periplasmic hemin-binding protein| hmuT      | 2.4                 |
|                                      | YPTB0339      | hemin degradation/transport protein               | hmuS      | 2.2                 |
| Anaerobiosis Associated (5)          | YPTB0209      | anaerobic glycerol-3-phosphate dehydrogenase subunit A | glpA      | 2.3                 |
|                                      | YPTB0518      | anaerobic ribonucleotide reductase activating protein | nrdG      | 2.6                 |
|                                      | YPTB0805      | anaerobic dimethyl sulfoxide reductase, subunit A | dmsA      | 2.3                 |
|                                      | YPTB0806      | anaerobic dimethyl sulfoxide reductase, subunit B | dmsB      | 2.1                 |
|                                      | YPTB2688      | putative dimethyl sulfoxide reductase chain A protein | dmsA      | 2.1                 |
| Regulatory Functions (3)             | YPTB0247      | lysR-family transcriptional regulatory protein    | metR      | 2.0                 |
|                                      | YPTB0386      | L-rhamnose operon regulatory protein               | rhaS      | 2.4                 |
|                                      | YPTB3808      | putative hybrid two-component system regulatory protein | 2.0        |                     |
| Protein Fate (3)                     | YPTB0877      | translation initiation factor EF-2B, GDP-GTP exchange factor (alpha subunit) | eif       | 2.2                 |
|                                      | YPTB1266      | putative outer membrane-associated protease       | pla2      | 2.2                 |
| Transport and Binding Proteins (8)   | YPTB0502      | ABC type sugar transport system, permease        | 2.0       |                     |
|                                      | YPTB0868      | putative amino acid ABC transporter, permease     | 2.5       |                     |
|                                      | YPTB1724      | SSS family proline sympporter                    | putP      | 2.4                 |
|                                      | YPTB1956      | calcium/proton antiporter                        | chaA      | 2.7                 |
|                                      | YPTB2011      | SulP family sulfate permease                      | ychM      | 2.1                 |
|                                      | YPTB2022      | MFS multidrug efflux antiporter                  | yctE      | 2.1                 |
|                                      | YPTB2491      | proton dependent di-tripeptide transporter       | ycdE      | 2.0                 |
|                                      | YPTB2815      | AcrB/AcrD/AcrF (HAE1) family drug efflux pump    | yegO      | 2.2                 |

*ORF IDs are derived from the Y. pseudotuberculosis IP 32593 genome unless otherwise stated.

bFold change is of the ∆iscR mutant relative to the wild type strain.

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(Figure 8D). These findings suggest that IscR may regulate transcription of the T3SS through a type 2 motif within the yscW-lcrF promoter region.

Discussion

In this study, we present the first characterization of the iron-sulfur cluster regulator, IscR, of Yersinia. Initially identified through a genetic screen for modulators of Ysc T3SS function, iscR-deficient Y. pseudotuberculosis had a dramatic defect in secretion of T3SS effector proteins and in targeting macrophages through their T3SS, yet displayed normal growth in broth culture and wild type T3SS effector proteins and in targeting macrophages through their T3SS, yet displayed normal growth in broth culture and wild type flagellar motility. Bioinformatic and DNA binding analysis revealed an IscR binding site upstream of the operon encoding the T3SS master regulator LcrF, indicating that IscR controls expression of the Ysc T3SS. Collectively, these findings indicated that IscR is a central component of the Y. pseudotuberculosis T3SS regulatory cascade.

Both E. coli holO- and apo-IscR are active transcription factors with distinct DNA binding targets. Holo-IscR can bind both type 1 and 2 motifs whereas apo-IscR can only bind type 2 motifs. IscR of E. coli autoregulates the isc operon, iscRSUA-hscaA-fdx, through binding to type 1 motifs within the isc promoter region [34]. In addition, Giel et al. described increased transcription of the genes located immediately downstream of the isc operon, yfhJ-pepB-sseB, in an iscR mutant, suggesting a negative regulatory effect on these genes as well [30]. We observed derepression of the iscRSUA-hscaA-fdx operon and the yfhJ-pepB-sseB locus in the Y. pseudotuberculosis ∆iscR mutant as well as the mutant expressing apo-locked IscR. Furthermore, we identified two sites within the Y. pseudotuberculosis isc operon that closely match the E. coli IscR motif consensus sequence. These data indicate that the iscRSUA-hscaA-fdx operon, and possibly the yfhJ-pepB-sseB locus, are negatively regulated by holo-IscR in Yersinia as they are in E. coli (Figure 9A). IscR in E. coli is known to activate transcription of the sufABCDSE operon through binding to a type 2 motif [29]. Our analysis revealed that the Y. pseudotuberculosis apo-locked IscR mutant overexpresses the sufABCDSE operon compared to the wild type and ∆iscR strains, which we predict results from the overexpression of IscR observed in the apo-locked mutant as found in E. coli [32,33]. We identified a site within the Y. pseudotuberculosis suf promoter region that closely resembles an E. coli IscR type 2 motif (data not shown). Together, these data indicate that the suf operon is positively regulated by IscR in Yersinia as in E. coli. Thus, we propose that IscR of Y. pseudotuberculosis modulates transcription of both the isc and suf Fe-S cluster biosynthesis pathways via mechanisms established for its E. coli ortholog.

In addition to control of Fe-S cluster biogenesis pathway expression, we present evidence that IscR controls expression and function of the Y. pseudotuberculosis T3SS. Bioinformatic analysis revealed a type 2 motif within the promoter of the T3SS master regulator LcrF that contained all nine bases previously found to be important for IscR binding (Figure 8A) [33]. Indeed, DNA
binding assays demonstrated that IscR is able to specifically recognize this type 2 motif, suggesting that IscR may be acting directly to promote transcription of lcrF [Figure 9B]. In support of this, we observed a marked decrease in transcription of numerous T3SS genes in the ΔiscR mutant strain. These include the gene that encodes LcrF, as well as a number of LcrF-regulated genes including the wuc operon, yopK, yopT, yopM, yopH, and lcrGVH-yopBD [17,20,22,33,54]. The lcrF type 2 motif is further upstream of the -10/-35 region previously identified by Bohme et al. [24] as other IscR binding sites that promote transcription [33], as we propose this site does. However, there may be an alternative -10/-35 region closer to the identified motif 2 site that might be used under specific growth conditions. Together, these data suggest that IscR is required for full expression of lcrF and LcrF-regulated genes through binding to a type 2 motif in the yscW-lcrF promoter [Figure 9B].

Based on these findings, an IscR mutant unable to coordinate an Fe-S cluster (apo-locked IscR) should lead to restoration of T3SS expression. Indeed, transcription of the yscW-lcrF and virC operons, as well as the majority of genes in the lcrGVH-yopBD operon, were not significantly decreased in the apo-locked IscR mutant compared to the ΔiscR strain. However, decreased transcription of yopE, yopK, yopM, and yopH as well as a severe defect in secretion of Yops was still observed. This could be explained by a deficiency in the apo-locked mutant’s membrane potential, but not in the ΔiscR strain [Figure 9B]. Wilharm et al., demonstrated that Y. enterocolitica motility and type III secretion requires the proton motive force [50]. Indeed, the apo-locked Y. pseudotuberculosis strain displayed a significant motility defect while the ΔiscR mutant was fully motile. Therefore, the type III secretion defect of the Y. pseudotuberculosis apo-locked IscR mutant can be explained by a deficiency in the proton motive force. Furthermore, the defect in YopHEMK transcription in the apo-locked IscR mutant may be explained by the fact that Yop secretion has a positive regulatory effect on Yop transcription [51,52]. Together, these data suggest that apo-IscR can promote LcrF transcription, but that locking iscR is the apo form causes a proton motive force defect that prevents effector Yop transcription and secretion [Figure 9B].

It is unclear why locking IscR in the apo-locked form leads to a proton motive force defect. We observed ~9-fold more suf transcript in the apo-locked IscR mutant compared to the ΔiscR strain that does not have a proton motive force defect, whereas the isc operon was expressed to the same degree in both mutants. Ezraty et al. recently showed that expression of the suf, but not the isc, operon in E. coli leads to a proton motive force defect, possibly as a result of impaired loading of Fe-S clusters into aerobic respiratory complexes [55]. Although the isc operon is expressed in the apo-locked Y. pseudotuberculosis mutant, perhaps overexpression of the suf pathway leads to misassembly of the Fe-S complexes of the electron transport chain that drive the proton motive force.

Both holo- and apo-IscR are predicted to bind to the type 2 motif within the yscW-lcrF promoter [33]. Based on previous data on E. coli IscR [28–30,34,56], low iron, aerobic growth, or high oxidative stress conditions are predicted to result in high expression of IscR through derepression of the isc operon, which in turn should increase T3SS gene expression. Likewise, high iron, anaerobic, or low oxidative stress conditions should lead to decreased IscR levels and therefore lower T3SS expression. Under normal aerobic culture conditions, we do not observe a change in wild type Y. pseudotuberculosis type III secretion when iron levels are altered (data not shown). However, in vivo, bacteria may be present in microaerophilic or anaerobic niches, where changes in iron

Figure 5. Deletion of IscR leads to increased transcription of Fe-S cluster biogenesis genes and robust transcription of T3SS genes. Quantitative real-time PCR analysis of WT and ΔiscR Y. pseudotuberculosis was performed (A) for the Fe-S cluster biogenesis genes, iscS and erpA and (B) for the T3SS genes, yscF, yscN and lcrF. Experiments were carried out from cultures grown in M9 at 37°C for 3 h. Shown are the averages ± SEM from three independent experiments. *p<0.05, **p<0.001, ***p<0.0001 as determined by a Student t test.

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bioavailability and reactive oxygen species production may impact  
isR  and T3SS gene expression. Upon ingestion by a host animal,  
Y. pseudotuberculosis  enters the lumen of the intestine, which receives  
approximately 15 mg of iron per day [57,58]. In the small  
intestine,  Y. pseudotuberculosis  can cross the gut barrier and enter the  
bloodstream and deeper tissues, which have very low iron  
bioavailability ([10^2] M free serum iron) [59–61]. Sequestration  
of iron by iron carriers in mammalian tissues is an important host  
defense mechanism to prevent growth of bacterial pathogens, the  
majority of which require iron for growth [62]. The Ysc T3SS has  
been shown to be required for  Y. pseudotuberculosis  pathogenesis in  
these deep tissue sites that are low in iron bioavailability [44].  
Perhaps  Y. pseudotuberculosis  uses IscR to sense iron, O_2, and/or  
ROS concentration in order to optimally control T3SS expression  
in vivo.

Consistent with the severe T3SS expression defect displayed by  
the  Y. pseudotuberculosis  ΔiscR strain, this mutant was deficient in  
colonization of the Peyer’s patches, spleen, and liver. Interestingly,  
the ΔiscR mutant was also defective in colonization of the mesenteric  
lymph nodes (MLN), yet T3SS mutants were previously shown to  
persist in the MLN and chromosomally-encoded factors were found  
to be important for  Y. pseudotuberculosis  survival in this tissue  
[24,63,64]. These results indicate that the virulence defect of the  
Y. pseudotuberculosis  ΔiscR strain may not be due solely to misregulation  
of the T3SS, suggesting the existence of other IscR gene targets  
important for virulence. IscR of  Pseudomonas aeruginosa  has been  
shown to be important for full virulence through its ability to  
upregulate KatA, encoding a catalase that protects against oxidative  
stress [38,65–67]. In  Vibrio vulnificus , IscR upregulates two genes  
encoding the antioxidants peroxiredoxin and glutaredoxin 2, and is

Figure 6. The apo-IscR mutant strain displays decreased motility and disruption of electrical potential. (A) Motility was analyzed by spotting 1 µl aliquots of either a nonmotile strain (Δyop6/flhD_Y.pestis), WT, ΔiscR, or apo-locked IscR  Y. pseudotuberculosis  onto motility agar plates. The diameters of the colonies were determined one day later and used to calculate percent motility relative to WT, which was set at 100%. Shown is the average percent motility ± SEM and is representative of three independent experiments. ***p≤0.0001 as determined by one-way ANOVA followed by Bonferroni post hoc test where each indicated group was compared to the appropriate negative (Δyop6/flhD_Y.pestis) and positive (WT) controls. (B) Proton motive force (PMF) was measured using JC-1 dye for  Y. pseudotuberculosis  IP2666 wild type (WT), iscR deletion mutant (ΔiscR), iscR complemented (ΔiscR pIscR), apo-iscR, and apo-iscR complemented (apo-iscR pIscR) strains grown in M9 at 37°C for 3 hours. The protonophore CCCP was added to a WT sample as a negative control (CCCP). Decreases in PMF were measured as a decrease in red (590 nm) fluorescent cells relative to green (530 nm). The data is presented as total fluorescence intensities at 590 (red) relative to 530 (green) ± SEM and is representative of three independent experiments. *p≤0.05, as determined by one-way ANOVA followed by Bonferroni post hoc test where each indicated group was compared to the appropriate negative (CCCP) and positive (WT) controls.

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essential for survival during exposure to reactive oxygen species [40]. Interestingly, our analysis suggests that *Y. pseudotuberculosis* IscR plays an opposite regulatory role, as IscR negatively affects expression of the genes encoding cellular detoxification proteins KatY, Tpx, SodC and SodB. Furthermore, hydrogen peroxide sensitivity assays showed comparable levels of survival between the *Y. pseudotuberculosis* wild type and *ΔiscR* strains (Figure S5). This suggests that the virulence defect observed for the *ΔiscR* *Y. pseudotuberculosis* mutant is not due to increased susceptibility to oxidative stresses encountered during infection. Pathways other than the T3SS, such as the *hmu* hemin uptake system, were found to be misregulated in the *Y. pseudotuberculosis* *ΔiscR* strain (Table 2 & Figure 4B). While the *hmu* operon was shown to not affect *Y. pestis* virulence, it is possible that IscR control of the *Y. pseudotuberculosis hmu* pathway is important for virulence.

In summary, we present the first characterization for the iron-sulfur cluster regulator, IscR, of *Yersinia*. We reveal that IscR regulates genes involved in Fe-S cluster assembly in a manner akin to that of *E. coli*. Most notably, we demonstrate that mutation of IscR leads to decreased function of the *Y. pseudotuberculosis* T3SS and that this is due to a decrease in transcription of genes encoding structural, regulatory, and effector proteins. Furthermore, we present evidence showing that IscR is essential for the virulence of *Y. pseudotuberculosis* and that this attenuation is likely due, in part, to direct regulation of the T3SS by IscR. Collectively, this study argues for the important and novel role of IscR in the virulence of *Y. pseudotuberculosis*.
Y. pseudotuberculosis as well as regulation of the Ysc T3SS, and identifies IscR as a potential target for novel antimicrobial agents.

Materials and Methods

All animal use procedures were in strict accordance with the NIH Guide for the Care and Use of Laboratory Animals and were approved by the UCSC Institutional Animal Care and Use Committee.

| A | IscR type 2 site |
|---|-----------------|
| -123 | GCGATTTTTTACCATATATACGAGTGGTATGTTGCTATTTCCTGCTGATTTTAAATTACATATAAA -57 |
| -56 | CTAGACCAATTTAGGATATACACGTTGTGGTTGCTATTCCGATTATAACATCTTTCTTTTTAATGAAAGAAAGGA |
| 12 | ACTATTACATGTGTTTTGCTGATTCCGATTACAACATCTTTTTTTTTAATGAAAGAAAGGA |
| 79 | TGTHTGCTTATTATTCGAAAGTTGTTGATATGCTAGTCTGTAATTAAATGATGTTGTTGCC |
| 146 | GTGGAGATAATTGGAATGCTGGTGATATTTTTCAATAAATAAATTTACCACATATTGGCGGAACCTCG |
| 213 | GATTTCTACTTACCTACTGCTACTTTTTGGTTGGAACCTCAAGGGAGGGAGCTG |

Y. pseudotuberculosis as well as regulation of the Ysc T3SS, and identifies IscR as a potential target for novel antimicrobial agents.

Bacterial strains, plasmids and growth conditions

All strains used in this study are listed in Table 3. Y. pseudotuberculosis strains were grown in 2xYT low calcium media at 37°C to induce type III secretion in the absence of host cells. Proteins in the bacterial culture supernatant were precipitated and visualized alongside a protein molecular weight marker (Ladder) on a polyacrylamide gel using commassie blue. Sample loading was normalized for OD600 of each culture. These results are representative of three independent experiments.
Construction of \textit{Y. pseudotuberculosis} mutant strains

The \textit{iscR} deletion mutant (\textit{ΔiscR}) was generated via splicing by overlap extension PCR [70]. Primer pairs F5'iscR/R5'iscR and F3'iscR/R3'iscR (Table S3), designed using MacVector and Primer 3 software (http://fokker.wi.mit.edu/primer3/input.htm), were used to amplify \(500\) bp \(5'\) and \(3'\) of the \textit{iscR} coding region, respectively. Amplified PCR fragments served as templates in an overlap extension PCR using the outside primers F5'iscR and R3'iscR. Nucleotide changes within the internal primers R5'apo-IscR and F3'apo-IscR allowed for amplification of \textit{iscR} target containing sequences coding for alanine substitutions of the three conserved cysteines that coordinate an Fe-S cluster.

The resulting \(1\) kb fragments were cloned into the TOPO TA cloning vector (Invitrogen) and further subcloned into a BamHI- and NotI-digested pSR47s suicide plasmid (\textit{lpir}-dependent replicon, kanamycin R (KanR), \textit{ sacB} gene conferring sucrose sensitivity) [71,72]. Recombinant plasmids were transformed into \textit{E. coli} S17-1 \textit{lpir} competent cells and later introduced into \textit{Y. pseudotuberculosis}.

Figure 9. Regulation of the \textit{isc} and \textit{lcrF} operons by IscR. (A) Model of \textit{isc} operon transcriptional control in the \textit{Y. pseudotuberculosis} wild type and apo-locked \textit{iscR} strains based on previous work on \textit{E. coli} IscR [32,34] and on data shown here. In wild type bacteria, the Isc Fe-S cluster biogenesis pathway loads a \([2Fe-2S]\) cluster onto IscR (holo-IscR) [32], which recognizes a type 1 DNA-binding motif in the \textit{isc} promoter to repress transcription in a negative feedback loop. Expression of the apo-locked IscR allele (***, \textit{IscR-C92A/C98A/C104A}) results in loss of holo-IscR-mediated repression, thereby increasing transcription of the \textit{isc} operon relative to wild type, resulting in a 30-fold increase in \textit{iscR}. (B) Model depicting the mechanism by which IscR controls the \textit{Y. pseudotuberculosis} Ysc T3SS. Holo- and apo-IscR are predicted to bind a newly identified type 2 DNA-binding site within the \textit{yscW-lcrF} operon encoding the LcrF T3SS master regulator. Subsequently, LcrF expression leads to transcription of the LcrF regulon, which includes the \textit{crGW-yopBD} and \textit{virC} operons and \textit{yop} genes [17,20,22,53,54]. These genes encode the majority of T3SS structural, regulatory, and effector proteins. However, through an as yet undefined mechanism, overexpression of apo-locked IscR leads to a decrease in the proton motive force, which is required for type III secretion [50]. As Yop secretion positively regulates \textit{yop} gene transcription [51,52], the secretion defect of the apo-locked \textit{iscR} mutant is predicted to lead to a decrease in effector \textit{yop} transcription.

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Table 3. \textit{Y. pseudotuberculosis} strains used in this study.

| Strain          | Background | Mutation(s)                          | Reference |
|-----------------|------------|--------------------------------------|-----------|
| WT              | IP2666     |                                      |           |
| Δyop6           | IP2666     | ΔyopHMOJ                             | [43]      |
| Δy sucNU        | IP2666     | Δy sucNU                             | [63]      |
| pYVR            | IP2666     | ΔyopBL pYVR cured                    | [43]      |
| Δyop6/Δyop8     | IP2666     | ΔyopHMOJ Δyop8                       | [43]      |
| Δyop6/Δyop8/FhD\_\textit{ppestis} | IP2666 | ΔyopHMOJ inactive, \textit{Y. pestis} allele of \textit{fhD} | [43] |
| Δyop6/Tn1       | IP2666     | ΔyopHMOJ iscR\_\textit{R}_{\textit{Tn}}=Himar1 | This work|
| Δyop6/Tn2       | IP2666     | ΔyopHMOJ ISC\_\textit{R}_{\textit{Tn}}=Himar1 | This work|
| Δyop6/ΔiscR     | IP2666     | ΔyopHMOJ ΔiscR                       | This work|
| ΔiscR           | IP2666     | ΔiscR                                | This work|
| ΔiscR plscR     | IP2666     | ΔiscR pACYC184::iscR*                 | This work|
| apo-iscR        | IP2666     | IscR-C92A/C98A/C104A                 | This work|
| apo-iscR plscR  | IP2666     | IscR-C92A/C98A/C104A pACYC184::iscR* | This work|

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**Yersinia IscR**

*Yersinia pseudotuberculosis* IP2666 via conjugation. The resulting Kan\(^R\), irgansan\(^R\) (*Yersinia* selective antibiotic) integrants were grown in the absence of antibiotics and plated on sucrose-containing media to select for clones that had lost sacB (and by inference, the linked plasmid DNA). Kan\(^R\), sucrose\(^R\), congo red-positive colonies were screened by PCR and subsequently sequenced to verify loss of the intended *iscR* coding region.

The *iscR* complement construct was generated by insertion of a fragment containing the *iscR* coding region as well as 530 bp of 5\' upstream sequence. This was PCR amplified using primer pair FiscRC and RiscRC, and cloned into the vector pACYC184 via BamHI/Sall restriction sites [73,74]. Recombinant plasmids were transformed into *E. coli* S17-1 λpir competent cells and later introduced into *Y. pseudotuberculosis* IP2666 ΔiscR via a modified transformation method [75]. Briefly, recipient *Yersinia* strains were grown overnight in LB containing 2% glucose at 26\(^\circ\)C. Cultures were centrifuged at 3,500 rpm for 3 min then washed with 750 μl of ice-cold sterile diH\(_2\)O and repeated for a total of three washes. Washed pellets were resuspended in 100 μl of sterile diH\(_2\)O, combined with 3 μl of plasmid and electroporated at 42. Cells were allowed to recover in 1 ml SOC media for 1 h at 26\(^\circ\)C followed by plating on LB containing carbenicillin to select for *Yersinia* bearing the plasmid of interest. Clones were confirmed by PCR analysis, using a combination of gene- and vector-specific primers, to construct both the ΔiscR complemented strain (ΔiscR pLsR) and the apo-iscR complemented strain (apo-iscR pLsR).

The nonmotile *Yop6p/flipDC::Tn1* mutant was generated by crossing in the *Y. pestis* flipDC gene into *Y. pseudotuberculosis*. *Y. pestis* flipD has a frameshift mutation, resulting in suppression of flagellin *flhD*.*Y. pestis* Y. *pestis* (*Y. pestis*) was mated with *Y. pestis* ΔiscR::Tn1 and *Y. pestis* ΔiscR complemented strain (*iscR*::Tn1) was grown on LB containing 2% glucose at 26\(^\circ\)C. Cultures were then used to infect the HEK293T cells containing the luciferase reporter plasmid at an MOI of 10. After 4 h incubation at 37\(^\circ\)C, 100 μl of 1:1 NeoLite:PBS solution was added to each well of the 96-well clear-bottom white plate (Corning), and luminescence was measured using a Victor\(^3\) plate reader (PerkinElmer). Data from three separate wells were averaged for each independent experiment.

**Type III secretion assay**

Visualization of T3SS cargo secreted in broth culture was performed as previously described [46]. Briefly, *Y. pseudotuberculosis* in M9 low calcium media (M9 plus 20 mM sodium oxalate and 20 mM MgCl\(_2\)) was grown for 1.5 h at 26\(^\circ\)C followed by growth at 37\(^\circ\)C for 2 h. Cultures were normalized by OD\(_{600}\) and pelleted at 13,200 rpm for 10 min at room temperature. Supernatants were removed and proteins precipitated by addition of trichloroacetic acid (TCA) at a final concentration of 10%. Samples were incubated on ice for 20 min and pelleted at 15,200 rpm for 15 min at 4\(^\circ\)C. Resulting pellets were washed twice with ice-cold 100% EtOH and subsequently resuspended in final sample buffer (FSB) containing 20% dithiothreitol (DTT). Samples were boiled for 5 min prior to running on a 12.5% SDS PAGE gel.

**Ethidium bromide entry assay**

Evaluation of pore formation was performed via the ethidium bromide (EtBr) entry assay as previously described [46]. Briefly, 2×10\(^4\) immortalized C57Bl/6 BMDMs were plated in a 96 well clear bottom black plate (Corning) in 100 μl DMEM +10% FBS. Infection was performed in triplicate at an MOI of 25. Plates were centrifuged at 750 xg at 4 °C for 3 min to facilitate contact. Infections were carried out at 37 °C with 5% CO\(_2\) for 2 h, at which point media was aspirated and replaced with 30 μl of PBS containing 25 μg ml\(^{-1}\) ethidium bromide (EtBr) and 12.3 μg ml\(^{-1}\) Hoechst dye. The cell monolayer was visualized using an ImageXpressMICRO automated microscope and MetaXpress analysis software (Molecular Devices). 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.

**Growth curves**

*Y. pseudotuberculosis* strains were cultured overnight in 2xYT or M9 at 26 °C and sub-cultured to an OD\(_{600}\) of 0.2 in 25 μl of either 2xYT or M9. Cultures were incubated at either 26°C or 37°C with shaking at 250 rpm and optical density measured at 600 nm every hour for 9 h.

**Mouse infections**

All animal use procedures were in strict accordance with the NIH Guide for the Care and Use of Laboratory Animals and were approved by the UC Santa Cruz Institutional Animal Care and Use Committee. Eleven to twelve-week-old 129S6/SvEvTac...
mice from our breeding facilities were used for oral infections as previously described [79]. Briefly, mice were orogastrically inoculated with 2 × 10^6 CFU in a 200 μl volume using a feeding needle. Mice were given food and water ad libitum and were euthanized at 5 days post-inoculation. Peyer’s patches, mesenteric lymph nodes, spleens, and livers were isolated and homogenized for 30 s in PBS followed by serial dilution and plating on LB supplemented with 1 μg mL⁻¹ iganas for CFU determination.

RNAseq analysis

RNA was isolated from the IP2666 wild type and isogenic ΔiscR and apo-IscR strains grown for 3 h at 37°C in M9, using the RNaseasy Mini Kit (Qiagen) as per the manufacturer’s protocol. We chose M9 media for our RNAseq analysis because this condition enables expression of T3SS genes and secretion of T3SS cargo at 37°C [68]. Contaminating DNA was removed from the RNA samples using a DNA-free kit (Life Sciences). Samples were subjected to removal of contaminating rRNA via the Ribo-Zero Magnetic Kit for Gram-negative bacteria (Epicentre). The cDNA library was created using the NEBNext Ultra Directional RNA Library Prep Kit for Illumina (NEB). These studies were performed with three biological replicates per condition. Six indexed samples as described previously [82] were sequenced per single lane using the HiSeq2500 Illumina sequencing platform for 30 bp single reads (UC Davis Genome Center) and subsequently analyzed and visualized via the CLC Genomics Workbench version 5.5.1 (CLC bio). Samples were normalized for both sequence depth and gene size by determining RPKM (Reads Per Kilo base per Million reads) normalized for both sequence depth and gene size by
described previously [82]. Results were analyzed using the Bio-
systems) was used for qPCR reactions according to the manufactur-

ting a fold change with an absolute value of 2 or greater. Statistical significance was determined by baySeq test with a
g was used to make cDNA as previously described [81]. SYBR Green PCR master mix (Applied Biosys-
ted for qPCR reactions according to the manufacturer’s instructions and a 60°C annealing temperature. Primers used are listed in Table S4. Control primers were for the 16S rRNA as
correct FDR post hoc test where p

Real-time PCR

Total RNA generated from our RNAseq analysis at a concentration of 2 μg was used to make cDNA as previously described [81]. SYBR Green PCR master mix (Applied Biosys-
ted for qPCR reactions according to the manufacturer’s instructions and a 60°C annealing temperature. Primers used are listed in Table S4. Control primers were for the 16S rRNA as
correct FDR post hoc test where p

Virulence plasmid map generation

Average RPKM values generated from RNAseq analysis for the wild type, ΔiscR and apo-IscR mutants were converted to log-ratios \( \log_{2}(\text{RPKM}_{\text{mutant}}/\text{RPKM}_{\text{wt}}) \) for each gene encoded on the virulence plasmid, pYV. These values were converted to a Circos heatmap [83] and plotted against the respective pYV base coordinate positions from Y. pseudotuberculosis IP32953.

Motif identification and search

Position specific scoring matrix (PSSM) was generated by the alignment of the known E. coli IscR type 2 motifs (Table S4) (Maverix Biomics, Inc) [31]. PSSM of type 2 was used to scan against the 150-nt upstream of 99 genes encoded on the Y. pseudotuberculosis pYV plasmid and obtained a ranked list of putative type 2 motifs.

DNA binding fluorescence anisotropy assays

Fluorescence anisotropy was measured similar to Nesbit et al., [33]. E. coli apo-IscR lacking the [2Fe-2S] cluster (IscR

correct FDR post hoc test where p

Measurement of the membrane potential

The electrical potential was measured similar to the JC-1 red/
green dye assay previously described for E. coli [84]. JC-1 is a membrane-permeable dye that emits green fluorescence (~530 nm) upon excitation when the dye is in the monomeric form. Due to the membrane potential of the bacterial cell, JC-1 dye will form J aggregates which emit red fluorescence (~590 nm). If the membrane potential decreases, there will be a decrease in J aggregate formation and subsequently a decrease in red fluorescence. As such, membrane potential can be displayed as a ratio of red/green fluorescence. Briefly, Y. pseudotuberculosis isogenic ΔiscR, ΔiscR complemented (ΔiscR pIscR), WT, ΔisR, or apo-IscR strains onto motility agar plates (1% tryptone, 0.25% agar) from overnight cultures standardized to an OD_600 of 2.5. Plates were incubated at room temperature for 1 day, at which point the diameters of the colonies were determined and used to calculate percent motility relative to WT, which was set at 100%.
microscope (Zeiss) fitted with a Plan-Apochromat 63x/1.4 Oil DIC objective and analyzed using the LSM 510 software (Zeiss). Quantification of image intensities was performed using ImageJ [85].

Supporting Information

Figure S1 IscR does not affect \( Y. \) pseudotuberculosis growth under non-T3SS-inducing conditions, but partially alleviates T3SS-associated growth restriction. The \( \mu \) pseudotuberculosis WT, \( \Delta \)iscR, apo-IscR and, where applicable, \( \Delta \)iscR and apo-IscR complemented strains \( \Delta \)iscR plScR and apo-IscR plScR, respectively) and \( Y. \) pseudotuberculosis lacking the virulence plasmid pPYV (pPYV\(^{+-}\)), were grown (A) in M9 at 37°C, (B) in 2xYT at 37°C, (C) in M9 at 26°C or (D) in 2xYT at 37°C. Optical density of the cultures were monitored at 600 nm every hour for 9 h. The averages ± SEM from three independent experiments are shown. * \( p < 0.05 \), ** \( p < 0.01 \), *** \( p < 0.001 \) as determined by a Student \( t \) test relative to the type wild.

Figure S2 Deletion of IscR leads to increased transcription of Fe-S cluster biogenesis genes. A) RPKM expression levels generated from RNAseq analysis of \( Y. \) pseudotuberculosis \( \Delta \)iscR and apo-IscR mutants relative to WT for 12 genes involved in Fe-S cluster biogenesis are displayed. \( \Delta \)iscR vs WT, B) Displayed is the nucleotide sequence of a region 130 bp upstream of the putative IscR start codon in \( Y. \) pseudotuberculosis IP 92953 including the putative transcriptional start site (arrow; UCSC Microbial Genome Browser) and putative sigma70 promoter elements (−10) and (−35), as well as the two putative IscR type 1 binding sites (brackets).

Figure S3 Mutation of iscR does not affect pPYV virulence plasmid yield. Relative amounts of the virulence plasmid, pPYV, were analyzed from standardized cultures of the wild type (WT), \( \Delta \)iscR mutant \( \Delta \)iscR and pPYV\(^{-}\) strains grown in M9 at 37°C for 3 hours through midiprep analysis (Promega) according to the manufacturer’s protocol. Plasmid yield was quantified via spectrophotometric analysis (Nanodrop). The data is displayed as \( \mu \)g of plasmid isolated per mL of culture ± SEM and is an average of 3 independent experiments. * \( p \leq 0.05 \), ** \( p < 0.01 \), as determined by Student \( t \) test.

Figure S4 Expression of the suf operon is increased in the apo-locked IscR mutant strain. RNAseq analysis was performed on WT, \( \Delta \)iscR and apo-IscR \( Y. \) pseudotuberculosis strains after growth in M9 at 37°C for 3 h (T3SS-inducing conditions). The data is presented as mean RPKM ± SEM and is an average of 3 independent experiments. *** \( p \leq 0.001 \), as determined by Bayseq followed by FDR post hoc test.

Figure S5 IscR is not required for survival post-exposure to hydrogen peroxide stress. Hydrogen peroxide assays were performed similar to Schiano et al. [87]. \( Y. \) pseudotuberculosis wild type (WT), \( \Delta \)iscR, and \( \Delta \)iscR complemented \( \Delta \)iscR plScR strains were grown overnight in 2xYT at 26°C. Cultures were standardized to an OD\(_{600}\) of 0.1 and grown at 26°C with shaking to mid-log phase, at which point they were diluted 1:10 into fresh 2xYT. Samples were supplemented with 50 \( \mu \)L of either sterile water (negative control) or hydrogen peroxide to a final concentration of 50 mM. Samples were incubated with shaking at 26°C and CFU determined via serial dilution and plating 10 min after the start of treatment. The data is displayed as percent survival (CFU \( H_2O_2 \)/CFU \( H_2O \))*100 ± SEM and is an average of 3 independent experiments.

Table S1 RNAseq RPKM values for wild type \( Y. \) pseudotuberculosis and the \( \Delta \)iscR mutant.

Table S2 Total pPYV-encoded genes differentially regulated by IscR, identified by RNAseq analysis.

Table S3 \( Y. \) pseudotuberculosis primers used in this study.

Table S4 Known type 2 DNA-binding sequences used for in silico search.

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Author Contributions

Conceived and designed the experiments: HKM VA. Performed the experiments: HKM LS HAR JMR. Analyzed the data: HKM VA HK LS DDB PPC. Contributed reagents/materials/analysis tools: HKM LS JMR PPC TML EM PJK. Wrote the paper: HKM VA.

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