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This is for you, Daddy.

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Abstract

Enteric pathogens sense numerous signals specific to the anatomical location in the intestine and integrate them with the complex regulatory networks to temporally and spatially regulate their virulence genes. MarA, SoxS, Rob and RamA are homologous transcription factors that belong to AraC family of proteins in *Salmonella enterica* that primarily were thought to be involved in rendering antibiotic resistance to bacteria by up regulating efflux pumps and down regulating outer membrane porins. The fact that these transcription factors respond to the same intestinal compounds that regulate virulence genes in *Salmonella* motivated us to look for other roles of these transcription factors.

We performed RNA-Seq analysis on *Salmonella* strains overexpressing MarA, SoxS, Rob and RamA and found that the expression of flagellar, fimbrial and SPI1 genes are repressed. Our genetic tests showed *flhDC*, the master regulator of flagellar genes, and *hilA*, the master regulator of the SPI-1 encoded type 3 secretion system (T3SS), are repressed by MarA, SoxS, Rob and RamA. We discovered that MarA and Rob directly repress *flhDC* transcription, while SoxS represses *flhDC* via a post-transcriptional mechanism. Additionally, we delineate direct and indirect contributions of MarA, SoxS, Rob and RamA in repressing *hilA* and currently designing in vitro invasion assays to identify their role in inhibiting infection. Finally, we demonstrate the role of MarA, SoxS, Rob, and RamA in responding to known virulence attenuating compounds.

Flagella, fimbriae, and the SPI1 T3SS are three key components of *Salmonella* virulence. Tight regulation of these genes is necessary for successful

infection by *Salmonella*. Our study identified that multidrug resistance transcription factors MarA, SoxS, Rob and RamA strongly repress these key virulence traits. Chemotherapeutic activation of these transcription factors may reduce the virulence of *Salmonella* before or during infection.

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Chapter 1: Introduction: Multiple roles of multi-drug resistance regulators- MarA, SoxS, Rob and RamA in *Salmonella* Typhimurium

1.1) *Salmonella*: An intestinal human pathogen

a) Taxonomic organization of *Salmonella*

The genus *Salmonella* that belongs to the family *Enterobacteriaceae* is responsible for a food-borne disease in broad range of hosts like – poultry, cattle, and humans. This genus consists of only two species – *S. enterica and S. bongori* but divided into over 2,597 serovars based on the hosts they infected (Jajere, 2019; Kurtz, Goggins, & McLachlan, 2017)

b) Diseases caused by *Salmonella*

Salmonella enterica serovars Typhi and Paratyphi cause typhoid fever. Typhoid fever is life threatening that presents severe symptoms that include high body temperature, diarrhea, stomach pain, weakness and cough. It can only be treated by administering antibiotics to the patients. Typhoid is very rare in developed nations like the United States. Serovar Typhimurium of *Salmonella enterica* causes a self-clearing non-typhoidal salmonellosis (NTS) disease in humans. NTS is characterized by gastroenteritis which presents symptoms like diarrhea, abdominal cramps and fever. NTS rarely enters bloodstream causing bacteremia which present symptoms like fever and chills (Jajere, 2019; Malik-Kale et al., 2011; Rivera-Chávez & Bäumler, 2015).

c) *Salmonella enterica* serovar Typhimurium

Salmonella enterica serovar Typhimurium is the organism of interest in this thesis report – from here onwards referred to as *Salmonella* Typhimurium or just *Salmonella*. According to the CDC, about 1.2 million people fall sick due to *Salmonella* infections annually in the United states (CDC, 2016). Though this disease is self-clearing and symptoms improve within a week, significant numbers of deaths and hospitalizations have been reported in the US and worldwide. *Salmonella* use flagella to reach the epithelial cells of the intestinal lumen; they attach to the epithelial cells with fimbriae and use type III secretion system (consists of needle like structure and effector proteins) to invade epithelial cells. *Salmonella* triggers inflammation in the intestine of the host after invasion of epithelial cells. This causes efflux of water into the intestinal lumen that results in diarrhea (Lou, Zhang, Piao, & Wang, 2019a; Zhou & Galán, 2001). Since the virulent structures – flagella, fibriae and type III secretion apparatus – are key for successful infection by *Salmonella*, acquiring deeper understanding surrounding the regulation of these virulence genes may open new avenues for drug discovery targeting expression of these traits.

1.2) Challenges faced by *Salmonella* in the host during its transition into an enteric pathogen

Salmonella enters humans through fecal-oral route via contaminated food. This type of entry into the human host lands *Salmonella* in front of two most prevalent systems of human intestine – gut microbiome and the innate immune system (Gart et al., 2016). *Salmonella* has developed strategies to evade these two systems until the adaptive immunity clears the pathogen. During this lag

period of adaptive immune system response, all the symptoms of NTS are presented already (Griffin & McSorley, 2011).

a) Resistance from gut microbiome

The human intestinal system, from the oral cavity to the colon, is a sanctuary for plethora of microbes that constitute the gut microbiota. Different species of bacteria, fungi, archaea and protozoans makeup the gut microbiota. Though some of the species of this microbiota are classified as pathobionts, those which turn pathogenic under unusual circumstances, most of them offer benefits to the host like breaking down complex carbohydrates into simpler sugars that can be absorbed by the human intestine and inhibit the colonization of enteric pathogens (Gart et al., 2016). The 'colonization resistance' of microbiota against *Salmonella* is mediated through competition for nutrients and physical space, by producing toxic compounds, and priming the immune cells that are present under the epithelial lining of the gut (Rivera-Chávez & Bäumler, 2015).

E. coli, the close relative of *Salmonella* in the microbiome, has similar nutritional and physical niche requirements. Owing to its large population in the microbiome compared to the invading *Salmonella, E. coli* will outcompete it for nutrients (fucose and iron) and attachments sites on human gut epithelial cells. Additionally, many *E. coli* produce bacteriocins that are toxic to *Salmonella* as has been demonstrated with the model commensal strain *E.coli* Nissle 1917 (Gart et al., 2016).

Neutrophils and macrophages that constitute the innate immune system are the first line of defense against enteric pathogens. Lipopolysacharides (LPS)

of microbiome activate granulopoiesis of neutrophils in the bone marrow. This helps maintaining the levels of neutrophils that re needed to combat pathogens. Microbiome also triggers secretion of pro-IL-1β a pro-inflammatory interleukin by macrophages that initiates secretion of IL-β, an inflammatory interleukin that is needed to combat enteric pathogens (Franchi et al., 2012; Pickard, Zeng, Caruso, & Núñez, 2017).

b) Host immune system

Peyer's patches and mesentric lymph nodes that lie underneath the intestinal epithelial cells recruit neutrophils and inflammatory monocytes (precursors of inflammatory macrophages) to the intestine that serve as the first line of defense against enteric pathogens like *Salmonella*. Neutrophils prevent *Salmonella* from reaching different organs. They secrete a cytokine called IFN-γ that activates macrophages. Macrophages in response to pathogens produce anti-microbial agents like nitric oxide, TNF-α (cytokine) and IL-1β (Pro-inflammatory cytokine) (Cheminay, Chakravortty, & Hensel, 2004; Mosser & Edwards, 2008). Macrophages also combat *Salmonella* by engulfing it through a process called as phagocytosis. This is followed by directing the engulfed vacuole containing *Salmonella* for degradation via fusion to acidic lysosomes within the macrophage's cytosol (Hu, Yang, Meng, Pan, & Jiao, 2013).

Though *Salmonella* has developed strategies to escape innate immune response, it eventually succumbs to the adaptive immune response. Adaptive immunity begins after activated innate immune cells induce T-cells and B-cells, the key components of adaptive immunity. Dendritic cells and macrophages

present the antigenic peptides of pathogens on their outer membranes by major histocompatibility complexes (MHC) to inactive T-cells. This induces the differentiation of T-cells into cytotoxic T_h1 cells that clear intracellular pathogens and T_h2 cells that activate B-cells, which produce highly variable antibodies that recognize broad range of extracellular antigens and eliminate them (Iwasaki & Medzhitov, 2015).

1.3) *Salmonella* survives colonization resistance and immune response by timely regulation of virulence genes

Salmonella evades gut microbiota's colonization resistance and human innate immune response until the adaptive immunity clears it. Competition for nutrients by gut microbiota and availability of novel nutrients are immediate challenges faced by *Salmonella* after its entry through host's oral-fecal route. It expresses alternative metabolic pathways to assimilate novel nutrients in the gut that are uncommon in its previous habitat and simultaneously turn off expression of structures or mechanisms that consume profound energy which otherwise can be used for cell maintenance under deprived nutritional conditions (Pickard et al., 2017). Flagella and type III secretion system (T3SS) are such structures that consume energy for their production and also simultaneously trigger premature immune response. *Salmonella* will increase its fitness in the human intestine by repressing the genes that code for flagella and T3SS until required (X. Yang et al., 2012),

In the ileum, *Salmonella* crosses the epithelial cell barrier through epithelial cells and M-cells and enters lamina propia where it comes in contact with neutrophils, macrophages and dendritic cells (**Figure 1**) (Urdaneta & Casadesús, 2017). *Salmonella* use T3SS-1 coded by *Salmonella* pathogenicity island – 1 (SPI-1) genes for invasion into non-phagocytic epithelial cells and intracellular replication in epithelial cells and macrophages (Lou et al., 2019a). The T3SS-1 system constitutes a needle complex and effector proteins that catalyze the rearrangement of the actin filaments in the epithelial cells. The rearrangement of actin filaments leads to the ruffling of outer membrane that will surround the *Salmonella* to form vacuoles. These salmonella containing vacuoles (SCV) detach from the outer membrane into the host cytoplasm (Zhou & Galán, 2001). *Salmonella* divides in the SCV by secreting T3SS-2 effector proteins coded by SPI-2 genes. Macrophages in the lamina propia engulf *Salmonella* by phagocytosis. *Salmonella* uses T3SS-2 effector proteins to survive by inactivating NADPH oxidase that produces superoxides inside macrophages (Gallois, Klein, Allen, Jones, & Nauseef, 2001). Intracellular *Salmonella* activates macrophages, neutrophils and dendritic cells in lamina propria. These activated immune cells migrate to the lumen and produce inflammatory cytokines and nitric oxide. Nitric oxide simultaneously kills various species of microbiota that out compete *Salmonella* for nutrients and oxidizes thiosulfate, the oxidized product of H2S released by gut microbiota, to tetrathionate. *Salmonella* efficiently uses tetrathionate as the final electron acceptor to respire in an anaerobic intestinal environment and proliferate faster than other gut microbiota, which have to rely

on lesser efficient form of respiration, fermentation to meet their energy requirements (Gart et al., 2016; Pickard et al., 2017; Rivera-Chávez & Bäumler, 2015).

Early expression of virulence traits like flagella and T3SS-1 will be a burden for *Salmonella* to maintain these structures and also illicit host immune response prematurely. *Salmonella* expresses T3SS-1 needle complex and effectors specifically in the ileum to cross the epithelial barrier and invade immune cells; it activates T3SS-2 inside the immune cells for its survival and activation of immune cells. This spatial and temporal expression of virulence genes render *Salmonella* the ability to successfully combat colonization resistance and innate immune response.

1.4) *Salmonella* senses its anatomical location in the intestine to regulate virulence genes

For spatial and temporal regulation of flagellar and T3SS genes, *Salmonella* must be aware of its anatomical location in the intestine. It uses several cues specific to a location in the intestine to sense the environment. *Salmonella* senses bile salts, fatty acids, iron, fucose and anaerobic environment, and to regulate flagella and SPI-1 genes (Gart et al., 2016).

Bile, secreted into upper and lower intestinal regions is known to represses SPI-1 and flagellar genes. 3,4-dimethylbenzoic acid, *o*-coumaric acid and hydrocinnamic acid of bile specifically destabilize HilD, the upstream regulator of SPI-1 genes (Hung et al., 2014; Peixoto et al., 2017). The mechanism by which *Salmonella* senses and responds to bile, specifically

destabilization of HilD is not well understood. Short chain fatty acids (SCFAs) were also observed to regulate SPI-1 genes. The length of carbon chain of SCFAs determines how SPI-1 genes are regulated. Short chain acetate and formate activate SPI-1 genes but longer carbon chained SCFAs like propionate and butyrate repress them. The mixture of different SCFAs present in colon and cecal region repress SPI-1 genes but the mixture present in ileal region activates SPI-1 genes. SCFAs are sensed by SirA/BarA two-component system in which BarA is the sensor kinase and SirA is the response regulator of downstream genes (Gart et al., 2016; Hung, Bullard, Gonzalez-escobedo, & Gunn, 2016).

Low oxygen environment in the ileum is another factor that activates SPI-1 genes. ArcB/ArcA two-component system senses low oxygen environment and activates low oxygen inducing factor A gene(*loiA*). LoiA activates transcription of *hilD,* directly. Intestine of humans is an organ with diverse chemical composition specific to an anatomical location (Jiang et al., 2017). *Salmonella* efficiently senses these compounds to perceive its location in the gut and regulate the virulence genes.

1.5) The Global Regulators - MarA, SoxS, Rob and RamA

Transcription factors (TFs) MarA and its homologs SoxS, Rob and RamA belong to AraC family of proteins (Robert G. Martin & Rosner, 2001). These are well conserved across many bacterial species of the family – *Enterobacteriaceae*. Most of the species belonging to this family have at least one of these homologs. These TFs were first identified to give bacteria multidrug resistance (MDR) (Cohen, Hachler, & Levy, 1993). Eventually their role in

regulating numerous physiological and metabolic pathways necessary for bacterial survival in ever changing environment were also observed. These TFs have several common and unique gene targets, which together make-up the *mar-sox-rob* regulon. Though MarA, SoxS and Rob directly regulate the transcription of many genes but the affinity with which they bind their promoters and the extent to which they activate them differs. Transcriptomics in *Escherichia coli* revealed that *mar-sox-rob* regulon contains around 80 genes that are involved in rendering antibiotic resistance, acid tolerance, oxidative stress tolerance to the bacteria along with a few metabolic genes to prepare bacteria to environmental challenges. The different mechanisms regulated by these transcription factors discussed in this chapter are based mostly on research conducted in *E. coli*, unless mentioned (Duval, 2013).

a) Role in antibiotic resistance

Transposon insertions that lead to increased expression of *marA* and *soxS* had first thrown light on the role of transcription factors - MarA and SoxS in rendering *E. coli* with resistance to antibiotics and superoxide. Ectopic expression of MarA and SoxS further confirmed it. Rob (*R*ight *o*rigin *b*inding, 33-kDa) that binds the right border of *oriC* in *E.coli* is a much larger protein compared to MarA (15.1 kDa) and SoxS (12.9 kDa). The N-terminal region of Rob is homologous to MarA and SoxS. This motivated (authors) to observe the role of Rob in antibiotic resistance in *E.coli.* They observed overexpression of Rob lead to decrease in susceptibility of *E.coli* to antibiotics (Cohen, Hachler, et al., 1993; Li & Demple, 1994; Skarstad, Thony, Deog Su Hwang, & Kornberg,

1993). The mechanisms that bacteria use to obtain antibiotic resistance and genes involved are discussed below.

1) Increased expression of AcrAB - TolC Efflux Pump

MarA, SoxS and Rob activate the *acrAB* operon and *tolC* that code components of the AcrAB-TolC efflux pump. AcrB, the key component of this efflux pump belongs to the protein super family resistance-nodulation-division (RND). AcrB forms a channel that connects cytoplasm to periplasm. TolC extends this channel from periplasm into the external environment of bacteria. Two AcrA proteins serve as adapters that connect AcrB to TolC on either side. These pumps render resistance to bacteria from lipophilic antibiotics like penicillin G, oxacillin, nafcillin, cloxacillin, novobiocin etc., by excreting them outside. Apart from antibiotics, AcrAB-TolC pump also excrete organic solvents. Microarray and transcriptional fusion data suggest that the TFs MarA, SoxS and Rob, increase the transcription of *acrAB* and *tolC* (H. Nikaido & Takatsuka, 2009)

2) Decreased Outer-membrane Permeability

Gram-negative bacteria first take in compounds from their environment through porins in the outer membrane of the cell wall. These compounds include both nutrients and toxins. OmpF and OmpC are most common porins in the outer membrane of *E.coli*. There is evidence that shows down regulation of the OmpF/C ratio causes decreased assimilation of antibiotics like penicillin, cephalosporin and tetracycline into the cell. MarA, SoxS and Rob, whose levels increase when he bacteria sense antibiotics in the environment activate the expression of a sRNA called MicF that represses the translation of OmpF. MicF

sRNA represses OmpF translation by pairing with the sequence spanning Shine-Delgarno region of the *ompF* mRNA. YedS is another porin seen in *E.coli* that is also repressed by MicF. Repression of YedS makes bacteria less susceptible to carbapenem (Cohen, McMurry, & Levy, 1988; Masi & Pagès, 2013).

b) Acid Tolerance

The biological role of MarA in acid tolerance is still unclear. HdeA and HdeB are periplasmic chaperones in *E.coli* that protect proteins from denaturing under low pH conditions. MarA regulates the expression of the *hdeAB* operon in stationary phase grown in rich medium (LB). Specifically, transcription of *hdeAB* is repressed by MarA at pH 5.5 in the stationary phase. MarA does this by occupying the binding site of GadE, an activator of *hdeAB*. Additionally, MarA represses *hdeAB* in a H-NS dependen mechanism that is unresolved. The extent to which *hdeAB* is repressed by MarA is too low to have any effect on bacterial survival in stationary phase at acidic pH. Though the biological significance of this repression is unclear, this mechanism can be exploited to make pathogenic bacteria more susceptible to stomach acids by overexpressing *marA* (Ruiz, McMurry, & Levy, 2008).

c) Oxidative Stress

SoxS is the primary candidate of MarA homologs that responds to oxidative stress. Bacteria experience oxidative stress as a result of cellular metabolism that releases reactive oxygen species (ROS), super oxides and hydroxyl radicals; exposure to redox cycling drugs like paraquat, plumbagin and menadione; or release of Nitric oxide (NO) by host's immune cells. Under

oxidative stress, structural proteins and enzymes of bacteria are damaged due to oxidation. SoxS activates respiratory enzyme genes like *zwf*, *pgi* and *deoB* genes of pentose phosphate pathway that code for glucose-6-phosphate dehydrogenase (G6PDH), glucose-6-phosphate isomerase, deoxyribouratase enzymes, respectively. Increased expression of these enzymes results in elevated levels of NADPH that reduces the oxidized proteins and enzymes and protect them from oxidative damage. MarA and SoxS also activate superoxide dismutase genes (*sodA*) and catalase genes (*katG*, *katE*, *ahpC*). Superoxide dismutase in concert with catalases convert toxic superoxide ions and intermediary product hydrogen peroxide into water (Baez & Shiloach, 2013; Ding & Demple, 2000; Gu & Imlay, 2011).

d) Virulence

Experiments in animal models and microarray data suggest that MarA, SoxS, Rob and RamA have a role in virulence in the genera *Escherichia*, *Salmonella* and *Klebsiella* of *Enterobacteriaceae*. A triple knockout of *marA*, s*oxS* and *rob* in clinically isolated *E.coli* was less effective in causing ascending pyelonephritis in mice. Biofilm formation is a key trait of many human pathogenic bacteria that cause nosocomial diseases. Mutations in uropathogenic *E.coli* (UPEC) that lead to increased expression of *marA* inhibited biofilm formation. This occurs through downregulation of fimbrial genes that are key for biofilm attachment to the surfaces (Casaz et al., 2006).

Cationic antimicrobial peptides (CAMPs) that are secreted by human epithelial cells mucosal cells and neutrophils protect the host against pathogens.

CAMPs bind the negatively charged cell wall of bacteria and form hydrophilic channels that cause disruption of osmolarity balance between the cytoplasm of bacteria and the environment (Le, Fang, & Sekaran, 2017). Over-expression of *marA* decreases the susceptibility of *E.coli* to CAMPs. MarA activate the expression of AcrAB-TolC efflux pumps that excrete CAMPs out of cell. In this way, MarA protects *E. coli* from CAMPs secreted by host cells and play a key role in the survival of pathogen during virulence. Over-expression of RamA was also shown to decrease susceptibility of *Klebsiella pneumoniae* to CAMPs, also (Warner & Levy, 2010).

RamA was shown to have a role in adhesion and invasion in to host cells by *Salmonella* and *Klebsiella*. Over-expression of *ramA* inhibited the adhesion and survival of *Salmonella* in RAW 264.7 macrophages and their killing of *C.elegans*. This is attributed to repression of SPI1 and activation of SPI2 genes from the transcriptomic data. SPI1 genes are responsible for attachment and invasion of host cells by *Salmonella* and SPI2 is responsible for its survival inside the host cell. SPI1 and SPI2 genes and their mechanism will be discussed in detail in chapter 4 of this thesis. The attachment defect of *ramA* over-expression *Klebsiella pneumoniae* strain to murine RAW macrophages is attributed to the increased expression of *lpxO*, *lpxC* and *lpxL-2* genes involved in lipid A synthesis. RamA was shown to directly interact with these lipid biosynthesis genes and activate them. It is hypothesized that altered lipid A moiety under *ramA* over-expression conditions changes the outermembrane LPS

of *Klebsiella* leading to inhibition of phagocytosis by the host cells(A. M. Bailey et al., 2010; de Majumdar et al., 2015) .

Sensing of anatomical location in the intestine and regulating virulence genes require regulatory networks that are responsive to intestinal cues. Homologous proteins MarA, SoxS, Rob and RamA are TFs that are regulated by several compounds found in the intestine. Bile that represses virulence of *Salmonella* activates Rob post-translationally and Indole, a metabolic byproduct of the gut microbiota that represses *Salmonella* virulence also activates the transcription of *ramA* (A. M. Bailey et al., 2010; Rosenberg, Bertenthal, Nilles, Bertrand, & Nikaido, 2003). It was also observed that the expression of *marA*, *soxS*, *rob* and *ramA* are increased inside macrophages, concomitant with the repression of flagellar and SPI-1 genes (Avital et al., 2017). Overlap of the intestinal cues (bile, indole) that regulate MarA, SoxS, Rob and RamA, and virulence genes suggests a possible role of these homologous TFs in virulence of *Salmonella*.

1.6) Distribution of MarA, SoxS, Rob and RamA in *Enterobacteriaecea*

These TFs are well conserved across *Enterobacteriaceae* though all the candidates of the family do not have all four homologs. RamA is currently identified to be present only in *Salmonella*, *Klebsiella*, *Citrobacter* and *Enterobacter*. The presence of other 3 homologs MarA, SoxS and Rob have been experimentally verified to be present in *E.coli*, *Salmonella*, *Shigella*, *Klebsiella*, *Citrobacter* and *Enterobacter* genera. Based on the sequence

similarity to *marA* of *E.coli*, *Yersinia pestis* was identified to have 2 homologs. These are termed as MarA47 and MarA48 (Duval, 2013).

1.7) *marRAB* system

The *mar* locus has two divergently transcribing units – the *marC* and *marRAB* operons that together code for MarC, MarR, MarA and MarB proteins. The operon *marRAB* is autoregulated by MarR and MarA. $MarR_2$ dimer binds at 2 sites in the intergenic region of *marRAB* and *marC*, termed as *marO* and represses transcription of *marRAB* (**Figure 2A**). Each of the two sites that $MarR_2$ binds has palindromic sequences: TTGCC and GGCAA. This repression of *marRAB* is alleviated in the presence of aromatic compounds like salicylate, sodium salicylate, benzoate, 2,4-dinitrophenol (**Figure 2B**). Salicylate binds to $MarR_2$ and inhibits $MarR_2$-*marO* interaction. MarA binds to a 20 base pair element in the *marO* region known as the *marbox* and activates the *marRAB* operon. Apart from activating its own transcription, MarA regulates other genes that have a marbox in their promoters. There is a second marbox in the *marR* coding region from which basal levels of *marA* transcription occurs. In the presence of aromatic compounds mentioned above, MarR-*marO* interaction is hindered and the basal levels of MarA protein produced bind to the marbox in the *marO* region and activates the *marRAB* operon (Robert G. Martin, Gillette, Rhee, & Rosner, 1999; Prajapat, Jain, & Saini, 2015; Vila & Soto, 2012).

Though the role of MarC is still unknown, it is clear that it has no role in antibiotic resistance like MarA. MarB is a periplasmic protein that represses the transcription of the *marRAB* operon by an unknown mechanism. MarA is also

post-translationally regulated by proteolysis of Lon protease (Bhaskarla et al., 2016; McDermott et al., 2008). MarA belongs to AraC/XylS family of transcriptional regulators. Proteins of this family have an effector region at their N-terminal end that interacts with signal molecules and two helix-turn-helix (HTH) domains that comprise the recognition domain with which these TFs bind the DNA. MarA is different than other AraC/XylS family members as it lacks the effector region. It is hypothesized that the effector domain coding region diverged into *marR* during evolution.

MarA recognizes two types of marbox containing promoters. Class I has a marbox in reverse orientation (B) upstream of -35 promoter element. Reversing the marbox to forward orientation (F) in class I promoters resulted in repression of the downstream genes. Class II promoters have marbox overlapped with the -35bp element. In this case, the marbox is in a forward orientation. SoxS and Rob also recognize marbox but bind relatively with low affinity compared to MarA (Robert G. Martin et al., 1999).

1.8) *soxRS* system

This system consists of two divergently transcribing genes *soxR* and *soxS* that code for 17-kDa and 13-kDa proteins, respectively. The promoter for *soxS* is located in the intergenic region and that of *soxR* is located in the *soxS*. SoxR belongs to the MerR family of transcription factors, which bind DNA as dimers (Chander & Demple, 2004; J. Wu & Weiss, 1991). Each monomer of this family of proteins have iron-sulfur clusters. Under anaerobic conditions, the iron-sulfur clusters of $SoxR_2$ dimer are in a reduced state. Reduced $SoxR_2$ dimer, though it

binds the *soxS* promoter region, does not activate it (**Figure 3A**). When the iron-sulfur clusters of the $SoxR_2$ dimer oxidize, conformational change in the protein alters the spacing between -35 and -10 regions of the promoter leading to the activation the transcription of *soxS* (**Figure 3B**) (J. Wu, Dunham, & Weiss, 1995).

The iron-sulfur clusters of SoxR is oxidized during oxidative dress due to superoxides. SoxR, under these conditions activate *soxS*. SoxS is a transcription factor that belongs to AraC/XylS family of transcription factors that activates genes like superoxide dismutase (*sodA*) and catalases that convert DNA damaging super-oxides into H_2O and O_2. (Li & Demple, 1994) . Though MarA, SoxS and Rob activate or repress many of the same genes, SoxS is the primary regulator that responds to the oxidative stress. Paraquat that belongs to the chemical class viologen is known to activate *soxS* by oxidizing SoxR. SoxR quickly goes back to reduced state when the inducer is removed. This recycling of reduced SoxR occurs by transfer of electrons form NADPH to SoxR via the products of *rseC* and *rsxABCDGE* (Koo et al., 2003).

Several constitutively active SoxR mutants have been isolated in *E.coli* through chemical mutagenesis. There is no evidence that these *soxR* mutants are active because of a locked oxidation state. These mutants, for example soxR105 is a truncated wild-type SoxR that lacks a portion of its C-terminal end. The constitutive active state of these mutants is attributed to change in conformation of the proteins that keep the *soxS* promoter in active state (Chubiz, Glekas, & Rao, 2012).

1.9) *rob* system

Rob (*R*ight *o*rigin *b*inding protein) is the largest of all the four multidrug resistance genes regulators (33-kDa). Initial research on this protein indicated that it binds the replication origin (*oriC*) of *E.coli*. The N-terminal 100 amino acid sequence of Rob that has a helix-turn-helix domain is similar to MarA and SoxS. Rob has an additional 175 amino acid C-terminal region that is absent in MarA and SoxS. Unlike MarA and Soxs, Rob is post-translationally regulated. *rob* is constitutively transcribed and around 5000-10,000 copies of Rob are present in the cell under normal conditions (**Figure 4A**) (Chubiz et al., 2012; Rosner, Dangi, Gronenborn, & Martin, 2002). In uninduced state, Rob proteins are sequestered in clustered aggregates and inactive. In the presence of inducers like dipyridyl or decanoate, a bile-salt, Rob proteins are dispersed into monomers and become active (**Figure 4B**). The inducers are hypothesized to bind to the C-terminal tails of Rob and lead to its dispersal. Rob belongs to AraC/XylS family, which actives numerous genes of *mar-sox-rob* regulon including *marRAB*.

1.9.1) *ram* system

The *ram* locus has been identified only in *Salmonella*, *Klebsiella*, *Citrobacter* and *Enterobacter* species. In *Salmonella*, this locus consists of genes *ramR* and *ramA* transcribed divergently (**Figure 5A**). The intergenic regions between *ramR* and *ramA* contains two promoter elements – P I and P II to which both RamR and RamA bind. Both of these promoter elements contain inverted repeat elements – *ATGAGTGcgtactCACTCAT*. RamR has been shown to

repress the *ramA* expression and RamA is speculated to be an auto-activator of *ramA*. The *ram* locus in *Klebsiella* has an extra gene called *romA* with unknown function. *romA* is in same orientation as *ramA* but has a different promoter. The P II promoter element in *Klebsiella* from which *ramA* is transcribed is located in the *romA*. *romA* is shown to be co-transcribed with an sRNA sRamA5 in that de-represses *ramA* by sequestering RamR (**Figure 5B**).

RamA in *Salmonella* confers MDR by increasing expression of the AcrAB-TolC efflux pump. It has been shown that the MDR conferred by RamA in *Klebsiella* has no involvement of AcrAB efflux pumps. It is speculated that the MDR by RamA in *Klebsiella* can be via efflux pumps other than AcrAB (A. M. Bailey et al., 2010; de Majumdar et al., 2015).

1.9.2) Cross-talk between *marRAB*, *soxRS*, *rob* and *ramRA* loci

All four homologous MDR regulators – MarA, SoxS, Rob and RamA regulate numerous metabolic and physiological genes. It is already mentioned above that these transcription factors auto-regulate their own expression directly. Apart from regulating other genes and their own expression, MarA, SoxS and Rob regulate transcription of each other when ectopically expressed. SoxS and Rob activate *marRAB* transcription. MarA and Rob repress *soxS*, and SoxS and MarA repress r*ob* (**Figure 6**). At physiological levels, which is mimicked by the presence of inducers, only cross-talk between *marRAB* and *rob* was observed. Salicylic acid and decanoic acid activate *marRAB* transcription via Rob. This integration of signal increases the amplitude of regulation of common

downstream gene targets of MarA and Rob (Chubiz et al., 2012). The degree of cross-talk between RamA and the *marRAB*, *soxRS*, and *rob* systems has not been fully resolved.

1.9.3) Conclusion

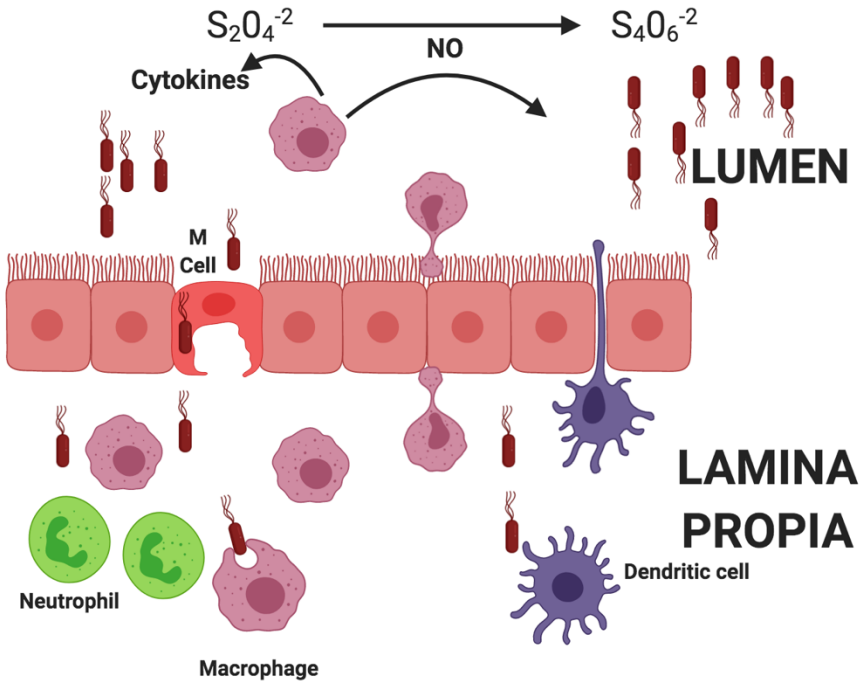
Salmonella and other enteric pathogens face numerous challenges during transition from a non-pathogen to virulent form. These challenges include colonization resistance from gut microbiome and host immune response. *Salmonella* represses flagellar and T3SS genes whose products are metabolically expensive which otherwise can be appropriated for cell maintenance. Repression of flagella and T3SS complex also benefits *Salmonella* since the activation of immune response is delayed. SPI-1 genes that code for T3SS-1 are activated after *Salmonella* reaches the ileum. This location specific activation of the SPI-1 T3SS is required for successful crossing of epithelial barrier by *Salmonella* (Hung et al., 2016, 2014; Pickard et al., 2017).

The spatial and temporal regulation of SPI-1 genes requires numerous regulatory networks that respond to the anatomical location in the intestine. Multidrug resistance gene regulators (MDR), MarA, SoxS, Rob and RamA are known to be induced by the same intestinal factors that regulate virulence genes expression. Some of these factors are short chain fatty acids (SCFAs), low pH, bile salts, anaerobic environment and nitric oxide. Transcriptomic analysis showed an inverse relation between expression of MarA homologs and SPI-1 genes in intracellular *Salmonella*. All the above evidences suggest the potential role of MarA homologs as direct repressors of SPI-1 genes. Acquiring knowledge

about the regulatory aspect of SPI-1 virulence genes will pave ways to identify new drug targets (Avital et al., 2017; Gart et al., 2016; Hung et al., 2016; Pickard et al., 2017).

In the following chapters, I present the research I conducted in Dr. Chubiz lab during the period January 2015 – December 2019. In chapter 3, I show differential gene expression in strains that over-express MarA, SoxS, Rob and RamA compared to a control strain. This transcriptomic data provided the foundation on which I based the rest of my research. Although this transcriptomic data suggested changes in levels of expression of numerous genes, my attention was drawn particularly towards three groups of genes that were downregulated. These included flagellar, fimbrial and SPI-1 needle genes that code for virulence traits in *Salmonella*. In chapter 4, I elucidated how MarA, SoxS, Rob and RamA regulated flagellar genes. I found that these transcription factors regulate the master regulator of flagellar genes, *flhDC*, at both transcriptional and post-transcriptional levels. In chapter 5, I have shown that *hilA* and *hilC*, the regulators of SPI-1 genes are reduced in strains that over-expressed MarA, SoxS, Rob and RamA. It must be further elucidated if the reduction in *hilA* and *hilC* is directly or in-directly dependent on MarA, SoxS, Rob and RamA. In chapter 6, I present my initial experiments that were designed to identify direct targets of Rob transcription factor. Finally, in chapter 7, I discuss the significance of my work, future questions that remain unanswered and experiments I designed to answer them.

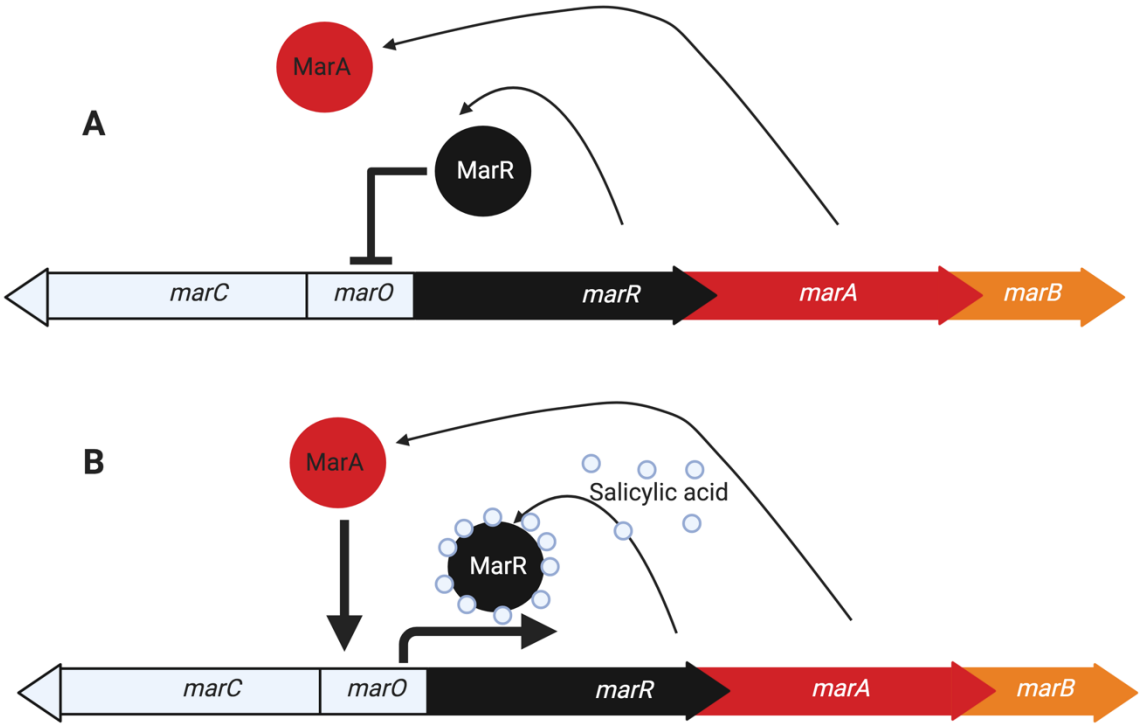
Figure 1.



Created with BioRender.com

Figure 1: *Salmonella* successfully evades challenges in the human intestine. It crosses the intestinal epithelial cell layer through M-cells using T3SS. In the lamina propia, they are phagocytosed by the immune cells. Intracellular *Salmonella* activates the immune cells. Activated immune cells enter the lumen, secrete cytokines and nitric oxide that trigger inflammation and catalyze the formation of tetrathionate, respectively. *Salmonella* uses tetrathionate efficiently than any other species of the microbiota as an electron acceptor and flourish in the intestine.

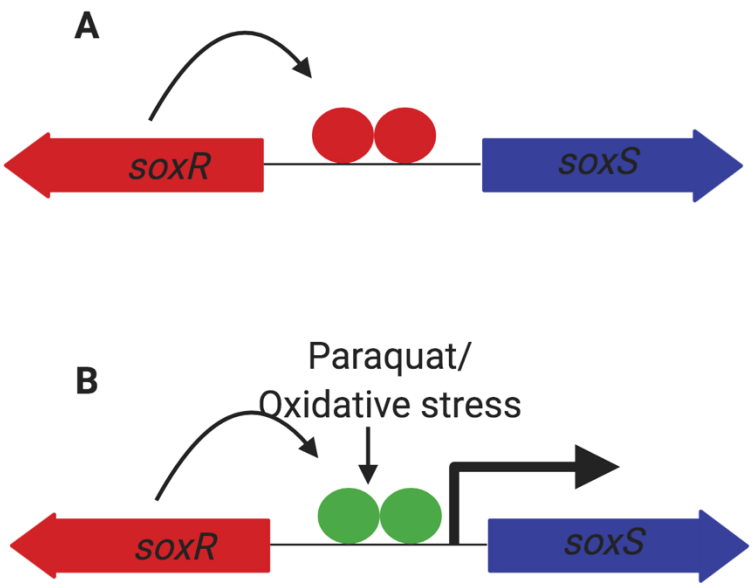
Figure 2.



Created with BioRender.com

Figure 2: Regulation of *marRAB* operon. **A)** *marR* is constitutively transcribed. MarR represses the *marRAB* operon by binding to *marO* region. **B)** Aromatic compounds like salicylic acid bind MarR and inhibit its binding to *marO*. The basal levels of MarA produced by expression of *marA* from an internal promoter binds the *marbox* in the *marO* and activates the *marRAB* operon.

Figure 3.



Created with BioRender.com

Figure 3: Regulation of *soxRS* genes. **A)** SoxR is constitutively produced. Under normal conditions, the iron-sulfur cluster of SoxR is in reduced state. Though SoxR dimer bind the *soxS* promoter in the reduced state, *soxS* is not activated. The red colored solid circles represent reduced SoxR dimers **B)** Under oxidative stress or in the presence of redox compounds like paraquat, the iron-sulfur cluster in SoxR dimer oxidizes. In the oxidized state, SoxR dimer activates transcription of *soxS*. The green solid circles represent oxidized SoxR dmers.

Figure 4.

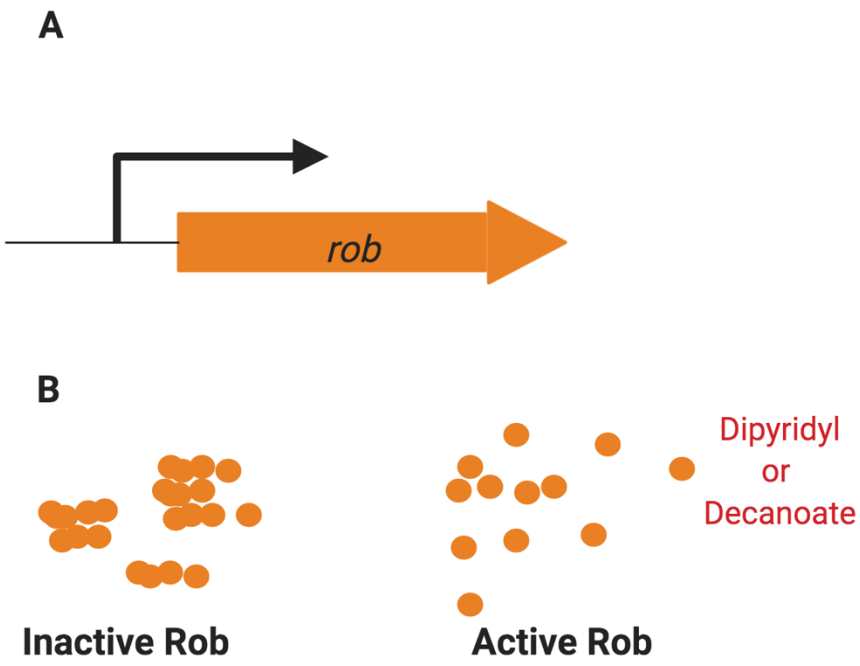


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Figure 4: Regulation of *rob*. **A)** *rob* is constitutively transcribed. **B)** Rob is post-translationally regulated. Under normal conditions, Rob forms inactive clusters. In the presence of fatty acids like dipyridyl and decanoate, Rob is dispersed into active monomers.

Figure 5.

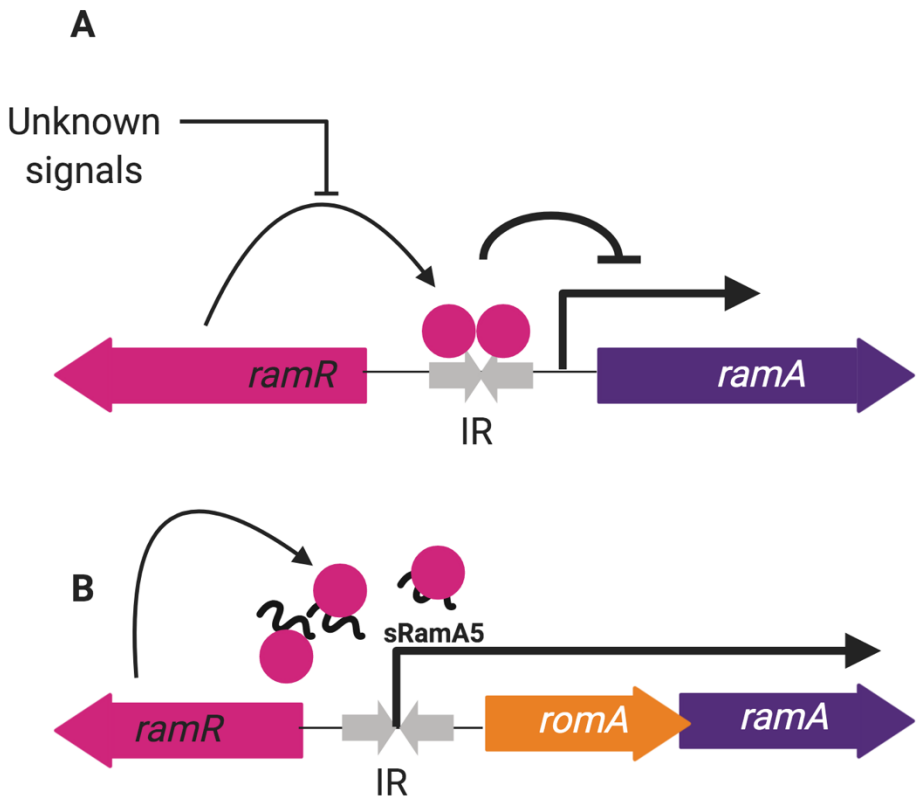

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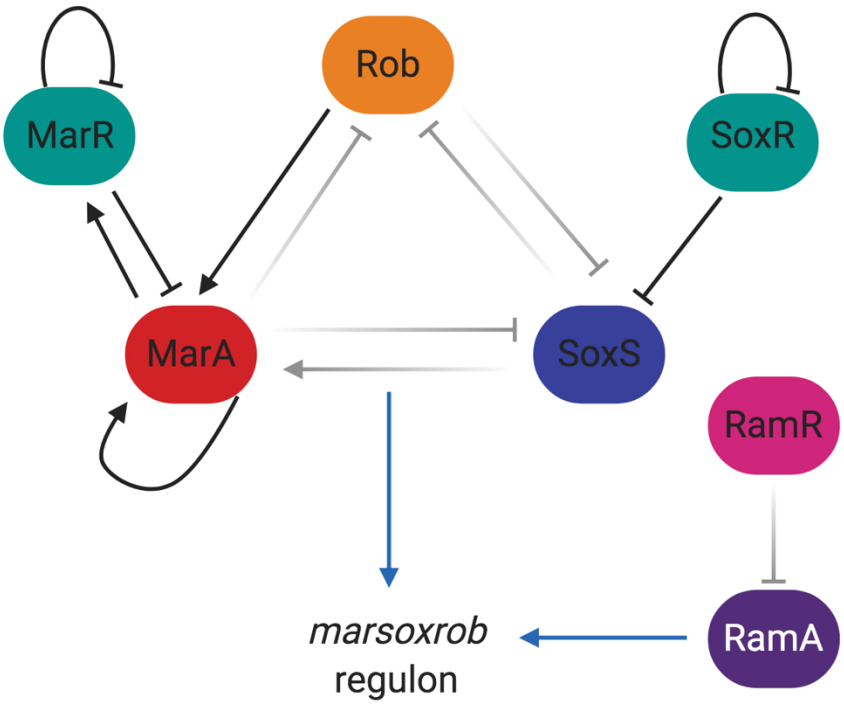
Figure 5: *ramRA* regulation in *Salmonella* (A) and *Klebsiella pneumonia* (B). **A)** RamR dimer binds to the inverted repeats in the promoter region of *ramA* and represses it. The signals involved in de-repression of ramA by RamR is not known. **B)** In *Klebsiella*, a small RNA, sRamA5 and an extra gene, *romA* are co-transcribed along with *ramA*. sRamA5 sequesters RamR dimers and de-represses *ramA*.

Figure 6.



Created with BioRender.com

Figure 6: Cross regulation between Multidrug regulators, MarA, SoxS, Rob and RamA. Solid black lines indicate presence of regulation at physiological concentrations. Fading grey lines indicate regulation present only when expressed from a plasmid.

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Chapter2: Material and methods

2.1) Media and growth Conditions

Strains were grown in Luria-Bertani (LB) broth at 37°C for plasmid isolation, genetic manipulations and propagation. To conduct SPI-1 genetic experiments, strains were grown overnight in LB without salt before seeding into fresh regular LB media and grown for 12 hours without shaking at 37°C. When harboring temperature-sensitive plasmids pKD46, pCP20, or pINT-ts; strains were grown at 30°C. Strains were grown in Tryptone media (1% tryptone, 0.8% NaCl) for all assays unless otherwise noted. Motility assays were conducted in swimming agar (0.3% Difco agar, 1% tryptone, 0.8% NaCl) at room temperature to enhance resolution. Where indicated carbenicillin (100 μg/ml), kanamycin (50 μg/ml), tetracycline (10 μg/ml), L-arabinose (Ara, 0.2 w/v%), or anhydrotetracycline (High ATc, 100 ng/ml; Low ATc, 1 ng/ml) were amended to growth medium.

2.2) Strain and plasmid construction.

Relevant genotypes and properties of strains and plasmids used in this work can be found in **Table S1**. Gene deletions and subsequent marker removal were performed using the λRed recombinase method of Datsenko and Wanner (Datsenko & Wanner, 2000) using oligonucleotides described in **Table S2**.

Single-copy promoter fusions were integrated into the chromosome using the CRIM-based system of Haldimann and Wanner (Haldimann & Wanner, 2001) or the FLP/FRT-based system of Ellermeier and coworkers (C. D. Ellermeier, Janakiraman, & Slauch, 2002). All strains were made isogenic to *S. Typhimurium* LT2 and compound genotypes were constructed by generalized transduction with phage P22 HT*int* using standard methods (Thierauf, 2009) .

Ectopic expression vectors and *yfp*(Venus) transcriptional fusions were constructed using standard molecular cloning procedures (Sambrook, 2001). Briefly, all genes, promoters, and 3xFLAG fusions were amplified by polymerase chain reaction (PCR) using Phusion DNA polymerase (New England Biolabs) with oligonucleotide primer sets with restriction endonuclease sites containing overhangs described in **Table S2**. PCR products were digested with restriction endonucleases (typically combinations of *Hin*dIII, *Kpn*I, and *Eco*RI) and ligated into the corresponding restriction sites of either pBAD30 (Guzman, Belin, Carson, & Beckwith, 1995) , pET28a (Novagen), or pVenus (Saini, Pearl, & Rao, 2009).

2.3) Motility assays.

Motility assays were conducted with logarithmic phase cultures to observe effects on matrix-associated motility. Cultures were inoculated 1:1000 from overnight cultures and grown until mid-logarithmic phase (OD_{600} = 0.6) in tryptone media followed by normalization by OD_{600}. Normalized cultures were inoculated (1µL) into the soft agar medium and plates were incubated at room

temperature overnight. Photographs are taken with a CCD camera (FOTODYNE).

2.4) Transcriptional reporter assays.

Fresh cultures were started from overnight cultures and grown to mid-log phase in tryptone media. For fluorescence-based reporter assays, 200 µL of cultures was transferred into 96-well black and transparent bottom plates (CORNING, Costar Assay Plate, 96 well) followed by fluorescence measurements (500/5 nm ex.; 520/5 nm em.). The relative fluorescent units (RFU) of cultures were corrected for background fluorescence and normalized to their corresponding $0D_{600}$ values. β-galactosidase assays were performed as described by Thibodeau and coworkers with measurements and analysis described by Slauch and Silhavy (Slauch & Silhavy, 1991; Thibodeau, Fang, & Joung, 2004). All fluorescence and absorbance measurements were made using a Cytation3 multimode microplate reader (BioTek). All statistical analyses were performed using R. The Tukey HSD test was used for multiple pairwise comparisons between samples and the Student's *t*-test were used for direct comparisons. Where both tests were used, the largest *P*-value was reported.

2.5) Immunoblotting, enzyme-linked immunosorbent assay and periplasmic proteins fractionation

For all immunoblot assays, cell pellets from 2 ml of culture were resuspended and incubated at room temperature in cell lysis buffer (Tris-buffered CellLytic B lysis buffer (Sigma-aldrich); 10 mM Tris-HCl, pH 8.0, 150 mM NaCl).

For each sample, a total of 100 µg of total protein was separated on MES/Tris-buffered 12 % Bis-tris gels at 150 V for 2.5 hours. Proteins were transferred to polyvinylidene diflouride (PVDF) membrane using a Tris/CAPS-buffered semi-dry transfer. FliC protein was detected by using a monoclonal anti-flagellin antibody (SC-69948, Santa Cruz Biotechnology) at a 1:3000 dilution. FlhC-3xFLAG was detected using anti-FLAG monoclonal antibody (M2, Sigma) at 1:3000 dilution. DnaK protein was used as a loading control in all samples and was detected using *E.coli* DnaK monoclonal antibody (8E2/2, Enzo) at a 1:10000 dilution. Anti-Mouse IgG conjugated with horseradish-peroxidase (1:30000 dilution) used as secondary antibody was detected by SuperSignal chemiluminescent substrate (Thermo Scientific).

For all enzyme-linked immunosorbent assays (ELISA), strains were started from an overnight seed culture and grown under inducing conditions for 8 hours at 37°C. Harvested cells were resuspended in 200 µl of TBS buffer (10 mM Tris-Cl, pH 8.0, 150 mM NaCl) with protease inhibitors (Roche cOmplete mini, EDTA free) and sonicated at 30% amplitude for 5 s On and 30 s Off pulses for 4 cycles. Lysed cells were centrifuged at 20,000 x*g* for 10 minutes to remove debris. Protein concentration was measured spectroscopically against a BSA standard (Biotek Cytation3) and diluted in coating buffer (100mM Sodium Bicarbonate/ Sodium Carbonate, pH 9.6) to a concentration of 500 µg/ml. Diluted lysate (50 µl) was added to wells of coated 96-well microplates (COSTAR high affinity) and incubated for 2 hours followed by washing twice with 200 µl PBS (0.137 M NaCl, 2.7 mM KCl, 10mM Na_2HPO_4, 1.8 mM KH_2PO_4). The washed

wells were incubated in blocking buffer (PBS buffer + 5% non-fat milk) for 2 hours. The wells were washed twice with 200 µl PBS. To each well, 100 µl of FliC monoclonal anti-flagellin antibody at 1:500 dilution in blocking buffer was added and incubated for 2 hours. The unbound antibody was washed off the wells twice with 200 µl PBS, followed by the addition of 100 µl of Anti-Mouse IgG (Life Technologies) conjugated with horseradish-peroxidase (1:1000 dilution in blocking buffer) was added to the wells and incubated for 2 hrs. This is followed by six washes with 200 µl PBS. Finally, 100 µl of SuperSignal chemiluminescent substrate (Thermo Scientific) was added to the wells and incubated for 30 s before measuring the luminescence in a microplate reader (Biotek Cytation3).

Periplasmic fractions of cells were separated by subjecting harvested cells to osmotic shock. Cell pellets were incubated sequentially in spheroplast buffer (0.1 M Tris-Cl, 0.5 M sucrose, 0.5 mM EDTA) and distilled water which causes the release of the periplasmic proteins into the supernatant. Pellets were further processed to obtain cytoplasmic fractions (Malherbe, Humphreys, & Davé, 2019).

2.6) Chromatin immunoprecipitation - polymerase chain reaction (ChIP-PCR) assays

Chromatin immunoprecipitation assays were performed as described by Wade and coworkers with minor modifications (Petrone, Stringer, & Wade, 2014). Briefly, 40 ml fresh cultures were inoculated 1:1000 into LB broth from overnight cultures and grown until mid-log phase (OD_{600}~0.7) at 37°C. Formaldehyde was added to fix cells at a final concentration of 1% and shaken for 5 minutes at 37°C. Cross-linking was quenched by adding glycine at a final

concentration of 200mM to the cultures with shaking for 10 minutes at room temperature. Cells were pelleted by centrifugation at 10000 x*g*, washed twice with ice cold Tris-buffer saline (TBS), and frozen at -80°C until further use.

Cells were then lysed and sonicated followed by immunoprecipitation. Cell pellets were resuspended in 2 ml of FA cell lysis buffer (50 mM Tris-Cl, pH 7, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% sodium deoxycholate, 0.1% SDS, 1 tablet of protease inhibitor cocktail (cOmplete mini, EDTA free) and 4 mg/ml lysozyme) and incubated at room temperature for 20 minutes to initiate lysis. The lysate was sonicated in a Q800R sonicator (Q-sonica) at 100% amplitude with 10 seconds ON and OFF pulses for 30 minutes to shear DNA to 200-500 bp fragments followed by centrifugation at 10000 x*g* to remove cell debris. A volume of 100 µL of the supernatant was saved as an input control and the remaining supernatant (1.9 ml) was added to pre-equilibrated 40 µL of Protein A/G beads (Pierce) in FA lysis buffer. Following incubation, 10 µL of Monoclonal M2 anti-FLAG antibody (Sigma) was added to the above mix and incubated at room temperature on a rotator for 1 hour. Protein A/G beads were separated and washed with FA lysis buffer (500 mM NaCl) followed by ChIP wash buffer (10mM Tris- Cl, pH 8, 250 mM LiCl, 1 mM EDTA, 0.5% Nonidet-P40, 0.5% sodium deoxycholate) and finally with TE (10 mM Tris-Cl, 1mM EDTA). Resulting protein-DNA complexes were eluted from the beads in ChIP elution buffer (50mM Tris-HCl, pH 7.5, 10mM EDTA, 1% SDS) and incubated at 100°C for 10 minutes. The eluate and input samples were purified and concentrated using Clean & Concentrator-5 columns (Zymo Research).

Enrichment of *flhDC* promoter fragments was measured by quantitative PCR (qPCR). To check if any of the MarA-3XFLAG, SoxS-3XFLAG, Rob-3XFLAG and RamA-3XFLAG proteins bound the *flhDC* promoter, the DNA from the above ChIP assay was used as template in a qPCR to check the enrichment of *flhDC* promoter region compared to control region (*gyrA* promoter region). Primers specific to *flhDC* (P-LCM243, P-LCM242) and *gyrA* (P-LCM110, P-LCM111) promoter regions that generate a product of approximately 200 bp were used. The enrichment of a target region is calculated according to $2^{-\Delta\Delta Ct}$ method (103).

For the ChIP-Seq experiments, the purified immunoprecipitated DNA was used to make libraries using NEB kit for Illumina sequencing (E6240) and sequenced on a MiSeq platform. The reads were analyzed using MACS software for peak calling and the bedgraphs were built using R (Y. Zhang et al., 2008).

2.7) Electromobility shift assays

A non-radioactive electrophoretic mobility shift assay (EMSA) kit was used to detect protein-DNA binding (LightShift™, Thermo Scientific). Rob protein used was purified by nickel-affinity chromatography and the tag removed as described elsewhere (Chubiz et al., 2012). Protein concentrations were calculated via Bradford assay against a BSA standard. Biotinylated and unmodified 559 bp and 135 bp DNA fragments spanning the promoter regions of *flhDC* and *pSLT026* genes, respectively were generated by PCR. The binding reactions consisting of 10 fmol probe and different molar amounts of Rob protein in binding buffer (10

mM Tris-Cl, 50 mM KCl, 1 mM DTT, 2.5% glycerol, 5 mM $MgCl_2$, 1 µg poly dI-dC) were incubated for 20 minutes at room temperature before loading on a 4% polyacrylamide gel followed by DNA transfer to a nylon membrane. Biotinylated DNA was detected by blotting with streptavidin-horseradish peroxidase conjugate followed by chemiluminescent detection (Supersignal™ ECL, Thermo Scientific).

2.8) RNA Sequencing

RNA was isolated from *Salmonella enterica* 14028 strains harboring pBAD30, pMarA, pSoxS, pRob and pRamA. All the strains were induced by adding 0.2% arabinose to the medium. RNA was isolated using Direct-zol RNA isolation kit (Zymogen, R2071). rRNA was depleted using Ribozero kit (E6350S). 1 µG of rRNA depleted RNA was used to generate libraries using NEB Ultra Directional RNA Library kit (E7420). Libraries were quantified and size checked using NEBNext Library Quant kit (E7630S) and Agilent bioanalyzer kit (5067-1505), respectively. Quantified libraries were sent to University of Missouri – DNA core facility for sequencing on a HiSeq platform. Raw sequences were aligned with whole genome sequence of *Salmonella enterica* 14028 (Pubmed: NZ_CP034479.1) and mapped using Bowtie (Langmead, 2010).Number of reads per gene were counted using HTSeq (Anders, Pyl, & Huber, 2015) and the differential gene expression analysis was performed with an R package – DESeq (Anders & Huber, 2010).

2.9) β-galactosidase assay

β-galactosidase assays were performed as described by Thibodeau and coworkers (Thibodeau et al., 2004). Briefly 5 mL cultures were grown until mid-log phase (approximate OD_{600} = 0.6) and permeabilized by adding 100 uL chloroform and 50 uL 0.1% SDS and vortexing for 30 seconds. The reaction mixtures consisting of 135 uL Z-buffer (0.06 M $Na_2HPO_4.7H_2O$, 0.04 M $NaH_2PO_4.H_2O$, 0.01 M KCl, 0.001 M $MgSO_4$, 0.05 M β-mercaptoethanol, pH 7.0), 15 µL permeabilized cells, 30 µL ortho-Nitrophenyl-β-galactoside (4 mg/mL) are transferred to a 96-well plate and OD_{420} is measured every 5 minutes over 1 hour time period in a BioTek Cytation-3 plate reader. The relative LacZ activity was calculated using the following formula: 1000 (V_{mean}/OD_{600}**).**

Table S1. Bacterial strains and plasmids used in this study.

Strains	Genotype	Source or reference[a]
LCM1930	WT[b]	
LCM1959	WT[c]	
LCM2131	*ΔmarRAB::kan*	
LCM2366	*ΔmarRAB::*FRT	
LCM1961	*ΔmarRAB, ΔsoxRS, Δrob:kan*	
LCM2368	*Δrob::*FRT	
LCM2371	*ΔmarRAB::*FRT*, Δrob::*FRT	
LCM2380	*ΔmarRAB::*FRT *ΔsoxS::*FRT *Δrob::*FRT *ΔramRA:*FRT	
LCM1970	*marA::3xFLAG*	
LCM1971	*soxS::3xFLAG*	
LCM1972	*rob::3xFLAG*	
LCM1973	*ramA:3xFLAG*	
LCM2324	*att*λ::[*kan flhDC'-yfp oriR6K*]	
LCM2325	*att*λ::[*kan flhB'-yfp oriR6K*]	
LCM2326	*att*λ::[*kan fliC'-yfp oriR6K*]	
LCM2399	*ΔmarRAB*::FRT *att*λ::[*kan flhDC'-yfp oriR6K*]	
LCM2417	*ΔmarRAB*::FRT *att*λ::[*kan flhB'-yfp oriR6K*]	
LCM2432	*ΔmarRAB*::FRT *att*λ::[*kan fliC'-yfp oriR6K*]	
LCM2401	*Δrob*::FRT *att*λ::[*kan flhDC'-yfp oriR6K*]	
LCM2419	*Δrob*::FRT *att*λ::[*kan flhB'-yfp oriR6K*]	
LCM2434	*Δrob*::FRT *att*λ::[*kan fliC'-yfp oriR6K*]	
LCM2407	*ΔmarRAB*::FRT, *Δrob*::FRT *att*λ::[*kan flhDC'-yfp oriR6K*]	

LCM2422	*ΔmarRAB*::FRT, *Δrob*::FRT *att*λ::[*kan flhB'-yfp oriR6K*]	
LCM2437	*ΔmarRAB*::FRT, *Δrob*::FRT *att*λ::[*kan fliC'-yfp oriR6K*]	
LCM2658	*soxR^{Con} zjc*::*cat, flhC3xFLAG*	
LCM2416	*ΔmarRAB::*FRT *ΔsoxS::*FRT *Δrob::*FRT *ΔramRA:*FRT, *att*λ::[*kan flhDC'-yfp oriR6K*]	
LCM2431	*ΔmarRAB::*FRT *ΔsoxS::*FRT *Δrob::*FRT *ΔramRA:*FRT, *att*λ::[*kan flhB'-yfp oriR6K*]	
LCM2446	*ΔmarRAB::*FRT *ΔsoxS::*FRT *Δrob::*FRT *ΔramRA:*FRT, *att*λ::[*kan fliC'-yfp oriR6K*]	
LCM2449	*soxR^{Con} zjc*::*FRT*	
LCM2471	*soxR^{Con} att*λ::[*kan flhDC'-yfp oriR6K*]	
LC2472	*soxR^{Con} att*λ::[*kan flhB'-yfp oriR6K*]	
LCM2473	*soxR^{Con} att*λ::[*kan fliC'-yfp oriR6K*]	
LCM2678	ϕ(*tetRA-flhDC)*	
LCM2687	*soxR^{Con}* ϕ(*tetRA-flhDC*)	
LCM2701	ϕ(*tetRA-flhDC*) *att*λ::[*kan fliC'-yfp oriR6K*]	
LCM2716	*soxR^{Con} ::cat* ϕ(*tetRA-flhDC*) *att*λ::[*kan fliC'-yfp oriR6K*]	
LCM2696	*flhC3xFLAG*	
LCM2697	*soxR^{Con} flhC3xFLAG*	
LCM2712	ϕ(*tetRA-flhDC*) *flhC3xFLAG*	
LCM2713	*soxR^{Con}* ϕ(*tetRA-flhDC*) *flhC3xFLAG*	
LCM2597	*Δhfq*::*cat*	
LCM2598	*Δhfq*::*cat soxR^{Con}*	
LCM2714	*Δhfq* - FRT *cat* FRT *att*λ::[*kan fliC'-yfp oriR6K*]	

LCM2715	*Δhfq* - FRT *cat* FRT *soxR*^{Con} *att*λ::[*kan fliC'-yfp oriR6K*]	
LCM2700	FRT *ΔfljBA-lacZY kan*	
LCM2669	*att*λ::[*kan soxS'-yfp oriR6K*]	
LCM2672	*soxR*^{Con} *att*λ::[*kan soxS'-yfp oriR6K*]	
JS575	*att*λ::[pDX1::*hilA'-lacZ*]	(J. R. Ellermeier & Slauch, 2008)
LCM2250	FRT *ΔhilD-lacZY kan*	
LCM2249	FRT *ΔhilD-lacZY kan*	
LCM2720	*att*λ::[*kan marRAB'-yfp oriR6K*]	
LCM1972	*3xFLAG-rob*	
LCM2087	FRT *ΔpSLT26-lacZY ApraR*	
LCM2088	FRT *ΔpSLT26-lacZY ApraR Δrob*	
LCM2027	*pSLT026::3XFLAG*, *Δrob*	
Plasmids		
pKD46	*bla* P_{BAD} *gam bet exo* pSC101 *ori*(Ts)	(Datsenko & Wanner, 2000)
pCP20	*bla cat* cI857 λP_R*'-flp* pSC101 *ori*(Ts)	(Datsenko & Wanner, 2000)
pINT-ts	*bla, int, oriR6K*	(Datsenko & Wanner, 2000)
pKD13	*bla rgnB* FRT *kan* FRT *oriR6K*	(Datsenko & Wanner, 2000)
pKD32	*bla rgnB* FRT *cat* FRT *oriR6K*	(Datsenko & Wanner, 2000)
pSUB11	*bla 3xFlag* FRT *kan* FRT *oriR6K*	(Uzzau, Figueroa-Bossi, Rubino, & Bossi, 2001)

pBAD30	P_{BAD}, *araC*, *bla*, pACYC184 *ori*	(Guzman et al., 1995)
pMarA	pBAD30::*RBS-marA*	
pSoxS	pBAD30::*RBS-soxS*	
pRob	pBAD30::*RBS-rob*	
pRamA	pBAD30::*RBS-ramA*	
pMarA-3XFLAG	pBAD30::*RBS-marA3xflag*	
pSoxS-3XFLAG	pBAD30::*RBS-soxS3xflag*	
pRob-3XFLAG	pBAD30::*RBS-rob3xflag*	
pRamA-3XFLAG	pBAD30::*RBS-ramA3xflag*	
pST2178	pET28a::*6xHis-rob*	
pVenus	*kan MCS yfp*(*venus*) *t0 att*λ *oriR6K*	(Saini et al., 2009)
pVenus-FlhDC	*kan MCS flhDC'-yfp t0 att*λ *oriR6K*	
pVenus-FlgB	*kan MCS flhB'-yfp t0 att*λ *oriR6K*	
pVenus-FliC	*kan MCS fliC'-yfp t0 att*λ *oriR6K*	
pVenus-SoxS	*kan MCS soxS'-yfp t0 att*λ *oriR6K*	
pKG136	*kan* FRT *lacZY* t_{his} *oriR6K*	(C. D. Ellermeier et al., 2002)

a. All the strains described here are made in this study except for those that are referenced.
b. Wild-type used in this study is *Salmonella enterica* serovar Typhimurium strain LT2.
c. Wild-type used to perform transcriptomics in chapter 3 is *Salmonella enterica* serovar Typhimurium strain 14028.

Table S2. Primers used in this study

Primer	Sequence[a]	Description
P-LCM221	ATA **GAA TTC** TTT ATA AGG AGG AAA AAC ATT TGA GTA TTT GCT CAA GAA A	Forward primer to amplify *marA* with an EcoRI site to clone into pBAD30
P-LCM244	ATA **AAG CTT** CTA GTA GTT GCC ATG GTT CAG C	Reverse primer to amplify *marA* with a HindIII site to clone into pBAD30
P-LCM222	ATA **GAA TTC** TTT ATA AGG AGG AAA AAC ATA TGT CGC ATC AGC AGA TAA TT	Forward primer to amplify *soxs* with an EcoRI site to clone into pBAD30
P-LCM245	ATA **AAG CTT** CTA CAG GCG GTG ACG GTA AT	Reverse primer to amplify *soxS* with a HindIII site to clone into pBAD30
P-LCM223	ATA **GAA TTC** TTT ATA AGG AGG AAA AAC ATA TGG ATC AGG CTG GCA TAA TT	Forward primer to amplify *rob* with an EcoRI site to clone into pBAD30
P-LCM246	ATA **AAG CTT** TTA ACG GCG AAT CGG GAT CAG AAA TTC	Reverse primer to amplify *rob* with a HindIII site to clone into pBAD30
P-LCM224	ATA **GAA TTC** TTT ATA AGG AGG AAA AAC ATA TGA CCA TTT CCG CTC AGG TT	Forward primer to amplify *ramA* with an EcoRI site to clone into pBAD30
P-LCM247	ATA **AAG CTT** TCA ATG CGT ACG GCC ATG CT	Reverse primer to amplify *ramA* with a HindIII site to clone into pBAD30
P-LCM79	CGA ATC ACG GTA TAT GCT GCC GCT GAA CCA TGG CAA CTA CGA CTA CAA AGA CCA TGA CGG	Forward primer to amplify *3x Flag* region of pSUB11 to insert it at 3' end of *marA*
P-LCM80	CAG CAT TTT CAT GGT GCT CTT CGC GTG GCG CAT AAA CAA ACA TAT GAA TAT CCT CCT TAG	Reverse primer to amplify *3x Flag* region of pSUB11 to insert it at 3' end of *marA*
P-LCM81	CGA GTT CGA TCG CAC TCC CAG CGA TTA CCG TCA CCG CCT GGA CTA CAA	Forward primer to amplify *3x Flag* region of pSUB11

	AGA CCA TGA CGG	to insert it at 3' end of *soxS*
P-LCM82	AAC AAA CGC CCG CGC CTC TGA CGA TAC GCG GGC AGA CGC CCA TAT GAA TAT CCT CCT TAG	Reverse primer to amplify *3x Flag* region of pSUB11 to insert it at 3' end of *soxS*
P-LCM83	TAT CAA CCT GCG CTG CGA ATT TCT GAT CCC GAT TCG CCG TGA CTA CAA AGA CCA TGA CGG	Forward primer to amplify *3x Flag* region of pSUB11 to insert it at 3' end of *rob*
P-LCM84	TTT CGC ATC TGG ACG CCC CTG CAT TGG ATG AGC TAC AGC GCA TAT GAA TAT CCT CCT TAG	Reverse primer to amplify *3x Flag* region of pSUB11 to insert it at 3' end of *rob*
P-LCM85	GCC AGG CGC TTA TCG TAA AGA AAA GCA TGG CCG TAC GCA TGA CTA CAA AGA CCA TGA CGG	Forward primer to amplify *3x Flag* region of pSUB11 to insert it at 3' end of *ramA*
P-LCM86	ATC TGG CGG CGC TGG TTT TCG CTG GCC GAT TAA ACA TTT CCA TAT GAA TAT CCT CCT TAG	Reverse primer to amplify *3x Flag* region of pSUB11 to insert it at 3' end of *ramA*
P-LCM220	ATA **AAG CTT** CTA TTT ATC GTC GTC ATC TTT	Reverse primer with a HindIII site to amplify *marA-3xFlag, soxS-3xFlag, rob-3xFlag, ramA-3xFlag* and clone into pBAD30
P-LCM366	GAT CAG GTA CCA ACT CGC TCC TTG ATT GCA AG	Forward primer with a KpnI site to amplify *flhDC* promoter to clone into pVenus
P-LCM367	GAT CAG AAT TCT AGC AAC TCG GAT GTA TGC ATT G	Reverse primer with EcoRI site to amplify *flhDC* promoter to clone into pVenus
P-LCM368	GAT CAG GTA CCA GGT GGA TAC CTC GAA AGC TG	Forward primer with a KpnI site to amplify *flhB* promoter to clone into pVenus
P-LCM369	GAT CAG AAT TCT GTT TTG TCG TCG TCG CTC T	Reverse primer with EcoRI site to amplify *flhB* promoter to clone into

		pVenus
P-LCM370	GAT CAG GTA CCG AAA TTG AAG CCA TGC CTT CTT CC	Forward primer with a KpnI site to amplify *fliC* promoter to clone into pVenus
P-LCM371	GAT CAG AAT TCC AGG CTG TTT GTA TTA ATG ACT TGT GC	Reverse primer with EcoRI site to amplify *fliC* promoter to clone into pVenus
P-LCM455	ATA GGT ACC CAG GGC GGA CAC AGC AAC	Forward primer with a KpnI site to amplify *soxS* promoter to clone into pVenus
P-LCM456	ATA GAA TTC AAG GGT CTG AAT TAT CTG CTG ATG C	Reverse primer with EcoRI site to amplify *soxS* promoter to clone into pVenus
P-LCM63	GGC AAC CAT TTT GAA AAG CAC CAG TGA TCT GTT CAA TGA ATG TAG GCT GGA GCT GCT TC	Forward primer to amplify a antibiotic marker region from pKD13/32 plasmid to insert into the chromosome resulting in a *ΔmarRAB* deletion.
P-LCM64	ACG GTA CTA AAA AAA TGC CCC GCA AAA CGG GGC AAA GAG GCT GTC AAA CAT GAG AAT TAA	Reverse primer to amplify a antibiotic marker region from pKD13/32 to insert into the chromosome resulting in a *ΔmarRAB* deletion.
P-LCM67	CTA CAG GCG GTG ACG GTA ATC GCT GGG AGT GCG ATC GAA CTG TAG GCT GGA GCT GCT TC	Forward primer to amplify a antibiotic marker region from pKD13/32 plasmid to insert into the chromosome resulting in a *ΔsoxRS* deletion.
P-LCM68	TTA ATC ATC TTC AAG CAG CCG GGC GCC CGT CCC GTG TTC GCT GTC AAA CAT GAG AAT TAA	Reverse primer to amplify a antibiotic marker region from pKD13/32 to insert into the chromosome resulting in a *ΔsoxRS* deletion.

P-LCM71	TTA ACG GCG AAT CGG GAT CAG AAA TTC GCA GCG CAG GTT GTG TAG GCT GGA GCT GCT TC	Forward primer to amplify a antibiotic marker region from pKD13/32 to insert into the chromosome resulting in a *Δrob* deletion.
P-LCM72	ATG GAT CAG GCT GGC ATA ATT CGC GAC CTG TTA ATC TGG CCT GTC AAA CAT GAG AAT TAA	Reverse primer to amplify a antibiotic marker region from pKD13/32 to insert into the chromosome resulting in a *Δrob* deletion.
P-LCM75	TTA TTG CTC CTC GCG AGT CAG CGC GCG CCA CAT GGC TTC GTG TAG GCT GGA GCT GCT TC	Forward primer to amplify a antibiotic marker region from pKD13/32 to insert into the chromosome resulting in a *ΔramRA* deletion.
P-LCM76	TCA ATG CGT ACG GCC ATG CTT TTC TTT ACG ATA AGC GCC TCT GTC AAA CAT GAG AAT TAA	Reverse primer to amplify a antibiotic marker region from pKD13/32 to insert into the chromosome resulting in a *ΔramRA* deletion.
P-LCM388	GCG TAG CGA CTG TCC GCT GCG AAA TCC AGG CGA CAG GTA ATG TAG GCT GGA GCT GCT TC	Forward primer to amplify a DNA fragment with stop codon followed by antibiotic marker from pKD13 to generate a truncated *soxR*
P-LCM389	AAT CAT CTT CAA GCA GCC GGG CGC CCG TCC CGT GTT CGC CCT GTC AAA CAT GAG AAT TAA	Reverse primer to amplify a DNA fragment with stop codon followed by antibiotic marker from pKD13 to generate a truncated *soxR*
P-LCM414	AAG TAC AAA TAA GCA TAT AAG GAA AAG AGA ATG GCT AAG GGT GTA GGC TGG AGC TGC TTC	Forward primer to amplify a antibiotic marker from pKD32 to insert into the chromosome resulting in a *Δhfq* deletion

P-LCM415	AGA GAC TGA ATA AGT TCA CGC GCT GTT TAT CCA TGT CGG GCT GTC AAA CAT GAG AAT TAA	Reverse primer to amplify a antibiotic marker from pKD32 to insert into the chromosome resulting in a *Δhfq* deletion
P-LCM490	GAG GAT TGC TTT ATC AAA AAC CTT CCA AAA GGA AAA TTT TGT GTA GGC TGG AGC TGC TTC	Forward primer to amplify a antibiotic marker from pKD32 to insert into the chromosome resulting in a *ΔfljBA* deletion
P-LCM491	AGT TTT ACT TTT CTC ACG GAA TTT TTT ATT ACC GTA GGC GCT GTC AAA CAT GAG AAT TAA	Reverse primer to amplify a antibiotic marker from pKD32 to insert into the chromosome resulting in a *ΔfljBA* deletion
P-LCM343	GTG AAC AAG GAA AGC TAA AAG TTA AAT CAA ATG AGC TTA TTT AAG ACC CAC TTT CAC ATT TAA GTT	Forward primer to amplify *tetRA* operon from Tn10d transposon to insert at 203 bp upstream of *flhD* start codon
P-LCM474	TAC ATC AAT TTT TAC AAA TGC CTA AGA TTT TTC CTA ATT CCT AAG CAC TTG TCT CCT G	Reverse primer to amplify *tetRA* operon from Tn10d transposon to insert at 203 bp upstream of *flhD* start codon
P-LCM402	TAT TCC ACA ACT GCT GGA TGA ACA GAT CGA ACA GGC TGT TGA CTA CAA AGA CCA TGA CGG	Forward primer to generate a *3xFLAG* DNA fragment from pSUB11 to insert at 3' end of *flhC*
P-LCM403	TGA CTT ACC GCT GCT GGA GTG TTT GTC CAC ACC GTT TCG GCA TAT GAA TAT CCT CCT TAG	Reverse primer to generate a *3xFLAG* DNA fragment from pSUB11 to insert at 3' end of *flhC*
P-LCM243	TCG TAA GTA TTC CGT TAA AAT ATG TG	Forward qPCR primer for *flhDC* promoter
P-LCM242	GGG AAG GCC CGG TAA AAG	Reverse qPCR primer for *flhDC* promoter
P-LCM110	TCC GCA GTG TAT GAC ACC AT	Forward qPCR primer for

		gyrA
P-LCM111	CAC GAA ATC CAC CGT CTC TT	Reverse qPCR primer for *gyrA*
P-LCM336	TTT TAA CAG CGG AGG GCG TA	5’ Biotin labeled primer to amplify the *flhDC* promoter region for EMSA
P-LCM337	CAG AAT AAC CAA CTT TAT TTT TGT GC	Reverse primer to amplify the *flhDC* promoter region for EMSA
P-LCM338	TTT TAA CAG CGG AGG GCG TA	Forward primer to amplify the *flhDC* promoter that is used as a specific competitor in EMSA
P-LCM229	TGT GGA CGA CGG ATG AAA TA	5’ Biotin labeled primer to amplify the *pSLT026* promoter region for EMSA
P-LCM228	TGT GGA CGA CGG ATG AAA TA	Forward primer to amplify the *pSLT026* promoter region that is used as a specific competitor in EMSA
P-LCM154	GGT GAA AGG GGA AGA CAC AA	Reverse primer to amplify the *pSLT026* promoter region for EMSA
P-LCM152	CAG AAA TCG CCG TCT GCA AAC TGG ATC AAC TAC CTG TCC CGA CTA CAA AGA CCA TGA CGG	Forward primer to amplify *3x Flag* region of pSUB11 to insert it at 3’ end of *pSLT026*
P-LCM153	TTT TAA TAC CGG TTA TAT ATT TAC GTT TAC CTG TCC CCT CCA TAT GAA TAT CCT CCT TAG	Reverse primer to amplify *3x Flag* region of pSUB11 to insert it at 3’ end of *pSLT026*

a. Nucleotides in bold letters are the restriction endonuclease enzyme sites.

Chapter 3: Transcriptomics of *Salmonella enterica* serovar Typhimurium strains over-expressing MarA homologs

3.1) Introduction

Information about *mar-sox-rob* regulon available to date has been obtained mostly from RNA sequencing and micro-array experiments conducted in a non-pathogenic *E.coli* (Duval, 2013). Enteric pathogen *S.* Typhimurium has genes and regulatory networks similar to its close relative *E.coli*, but information regarding the *mar-sox-rob* regulon in this bacterium is not available. *S.* Typhimurium has another MarA homolog called RamA, which is absent in *E.coli*, which is not well studied (A. M. Bailey et al., 2010). In spite of sharing similar genes as *E.coli*, *S.* Typhimurium harbors several virulence genes that are necessary for successful infection. There is substantial evidence showing MarA homologs have a role in virulence and biofilm formation in few species of *Enterobacteriaceae* (Vila & Soto, 2012). The overlap of intestinal chemical compounds that regulate MarA homologs and virulence phenotype motivated me to speculate the possibility of direct regulation of virulence genes by MarA homologs in *S.* Typhimurium. An initial genome wide study is a good starting point to identify the new candidates of the *mar-sox-rob* regulon.

The MarA homologs bind their promoters with different affinities. Deleting the repressors of MarA homologs – MarR, SoxR and RamA – may increase the expression of MarA homologs but this increase is equivalent to their endogenous levels which may not be enough to activate or repress their weak targets. To

bypass this limitation, MarA homologs need to be expressed more than endogenous levels. In strain lacking their repressors, over expression of transcription factors may result in false positive identification of targets in transcriptomic experiments. These false targets may be eliminated in confirmatory downstream experiments.

Simultaneous transcriptomic analysis of all four MarA homologs will elucidate the *mar-sox-rob* regulon and also identify genes and pathways that are specifically regulated by each homolog. Here, I expressed MarA homologs from medium copy plasmid pBAD30 that is induced by arabinose. I later extracted RNA from these strains, synthesized cDNA, generated libraries and performed directional RNA sequencing. From my data, I found several novel genes that are regulated by MarA homologs, especially those involved in virulence.

3.2) Results

Expression of MarA homologs from pBAD30 vector

As mentioned in Chapter 1, *marRAB*, *soxRS* and *ramRA* operons are not active under normal conditions. They are expressed only under chemical stress. Chemical inducers have pleiotropic effects and may turn on various genes that are independent of MarA homologs. So, chemical inducers cannot be used to identify genes regulated specifically by MarA homologs. To overcome this, I decided to express MarA homologs from a medium copy number plasmid that is tightly regulated. pBAD30 is a vector where a gene of interest can be expressed from the *araBAD* promoter. This plasmid also harbors the *araC* gene, whose

product is both a repressor and an activator of the *araBAD* promoter in the absence and presence of arabinose, respectively (Guzman et al., 1995). 0.2% arabinose was added to the cultures to activate *marA* homolog genes that are under the control of the *araBAD* promoter.

To test the expression of MarA homologs from the pBAD30 vector, I performed a western blot. Since antibodies for MarA homologs are not commercially available, I fused 3XFLAG peptides to the C-terminal end of MarA homologs and used anti-FLAG antibodies to detect them. Cultures expressing each of the MarA homologs were grown to the mid-log phase (OD_{600} 0.6) in the absence and presence of 0.2% arabinose, respectively and the cells were harvested. Cells were lysed,100 μG of total protein of each sample was loaded on to the gel, and a western blot was performed (See Chapter 2: Materials and Methods). We detected MarA homologs only in the cultures that were induced by 0.2 % arabinose (**Figure 1**). This suggested that MarA homologs expressed under the control of the *araBAD* promoter in the pBAD30 vector are tightly regulated since no leaky expression was observed.

RNA-Sequencing of strains over-expressing MarA homologs

MarA, SoxS, Rob and RamA were expressed from pBAD30 in the *Salmonella enterica* strain 14028. Directional RNA libraries were constructed using a NEB kit for Illumina (E7420S) and sequenced on a HiSeq Illumina sequencer. The fold changes in expression of transcripts were calculated by comparing with the gene expression in control strain with empty vector. Each of

the MarA homologs up-regulated and down-regulated several genes, respectively (**Tables 1-8** in this chapter). As mentioned in Chapter 1, MarA homologs have similarity in their DNA binding domains. It is logical to expect that these homologs might have common targets. As expected, I observed many common targets for MarA homologs in *Salmonella*. The overlap of up-regulated and down regulated genes by MarA homologs are presented in **Figure 3 and Figure 4,** respectively.

Of all the down regulated genes by MarA homologs, my attention was drawn more towards flagellar, fimbrial and SPI-1 needle complex group of genes due to their role in virulence (**Figure 2**).

3.3) Discussion

Transcriptomic data revealed numerous genes that are both up-regulated and down-regulated by MarA homologs. Of all the genes that were expressed differently, I focused on flagellar, fimbrial and type 3 secretion system genes more due to their role in virulence of *Salmonella*. I observed the genes that coded for these virulence structures were repressed by several fold in strains over-expressing MarA homologs, as compared to a strain harboring an empty vector. The genes that code for these three virulence traits are hierarchical. They have a master regulator that regulates several other structural and functional genes. Although RNA sequencing is a power tool that gives information about the differential gene expression of the whole genome, it does not give information about which genes are regulated directly by transcription factors. Only genetic

and biochemical assays reveal such information. The mechanism of MarA homologs regulating flagellar and type 3 secretion system genes are further elucidated in the following chapters.

Figure 1.

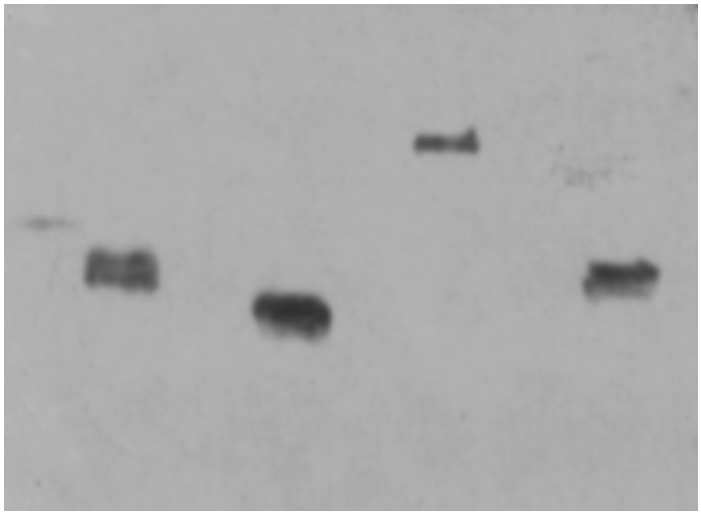

Figure 1. Expression of 3XFLAG tagged MarA homologs from pBAD30 vector in the presence and absence of 0.2 % arabinose. Lanes 1,2 – Soluble fraction from strain expressing MarA-3XFLAG (20.2 kDa) from the pBAD30 vector in the absence and presence of 0.2% arabinose, respectively. Lanes 3,4 – Soluble fraction from the strain expressing SoxS-3XFLAG (16 kDa) from the pBAD30 vector in the absence and presence of 0.2% arabinose, respectively. Lanes 5,6 – Soluble fractions from strains expressing Rob-3XFLAG (36.2 kDa) from the pBAD30 vector in absence and presence of arabinose, respectively. Lanes 7,8 – Soluble fractions from strains expressing RamA-3XFLAG (16.4 kDa) from pBAD30 vector in absence and presence of arabinose, respectively.

Figure 2

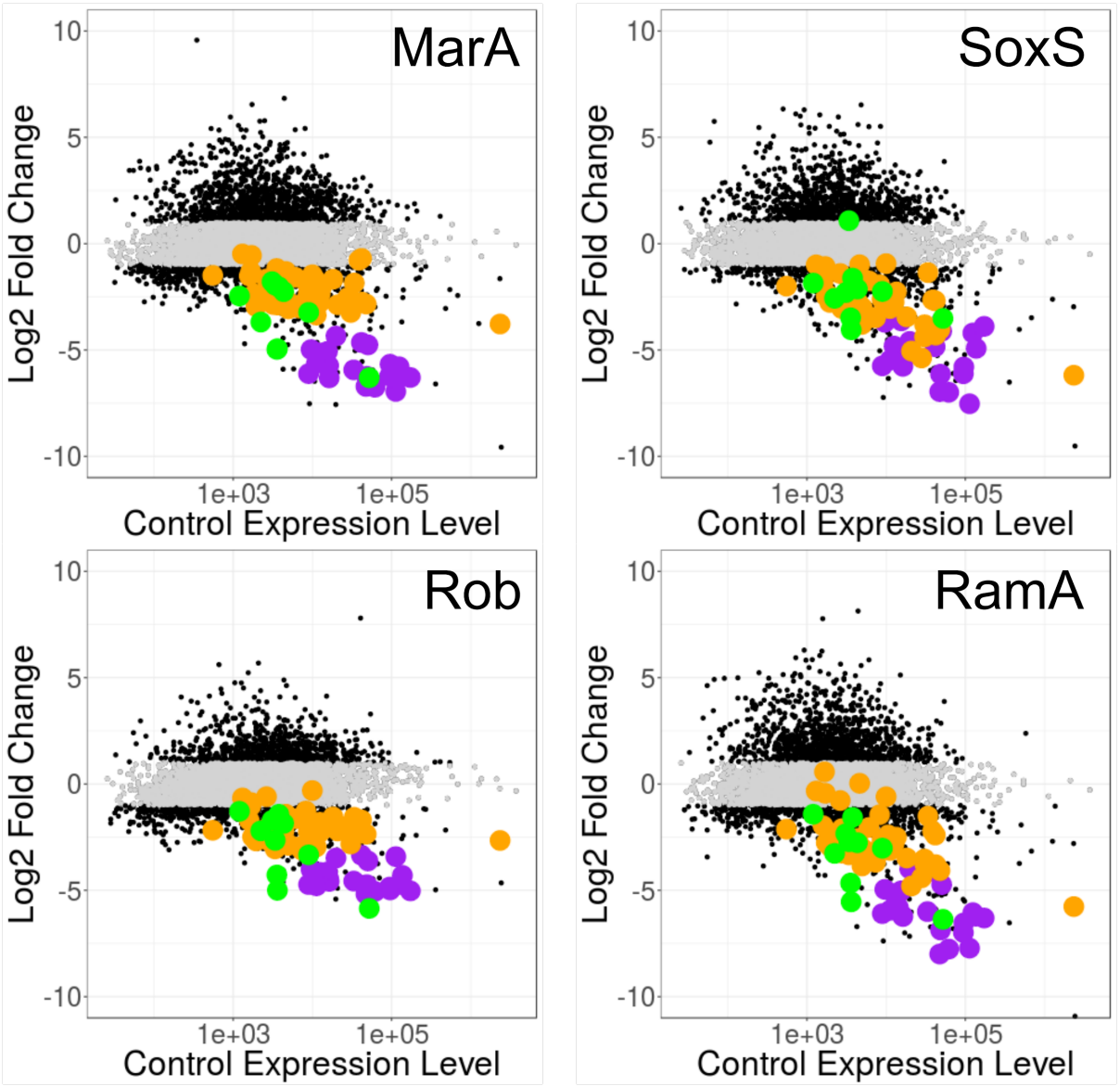
MarA
SoxS
Rob
RamA
Log2 Fold Change
Control Expression Level
10
5
0
-5
-10
1e+03
1e+05
Flagellar genes
Fimbrial genes
SPI-1 Needle complex genes

Figure 2. Transcriptomics in strains over-expressing MarA, SoxS, Rob and RamA. MarA homologs are expressed in WT *Salmonella enterica* 14028 background (LCM1959) from pMarA, pSoxS, pRob, and pRamA vectors constructed from pBAD30 vector. All the four strains have severely repressed genes coding for virulence traits – flagella, fimbria and SPI-1 needle complex.

Figure 3.

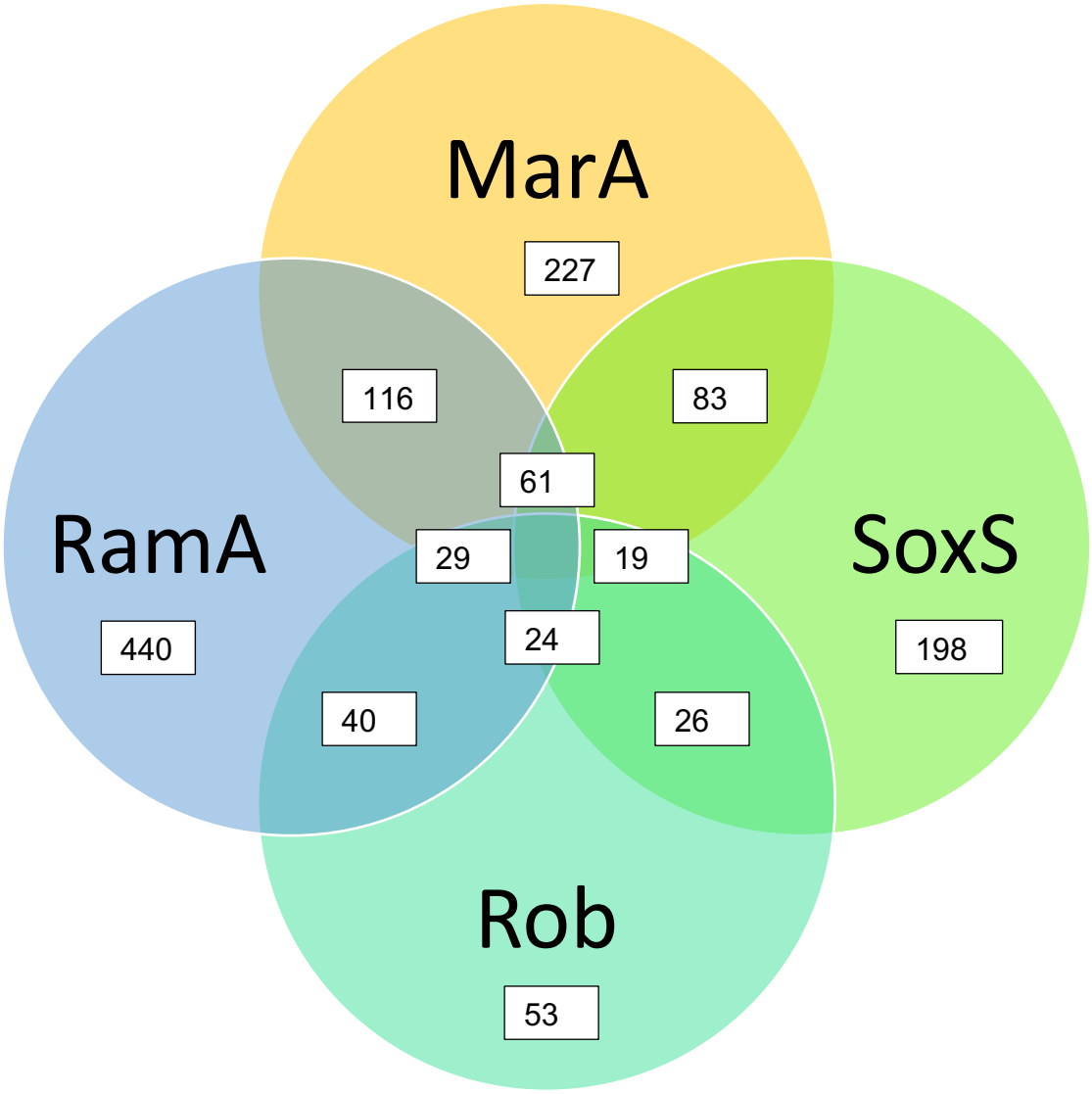

Figure 3. Venn diagram showing overlap of up-regulated genes by MarA homologs. Interestingly, we did not observe any genes that are upregulated by all MarA homologs.

Figure 4.

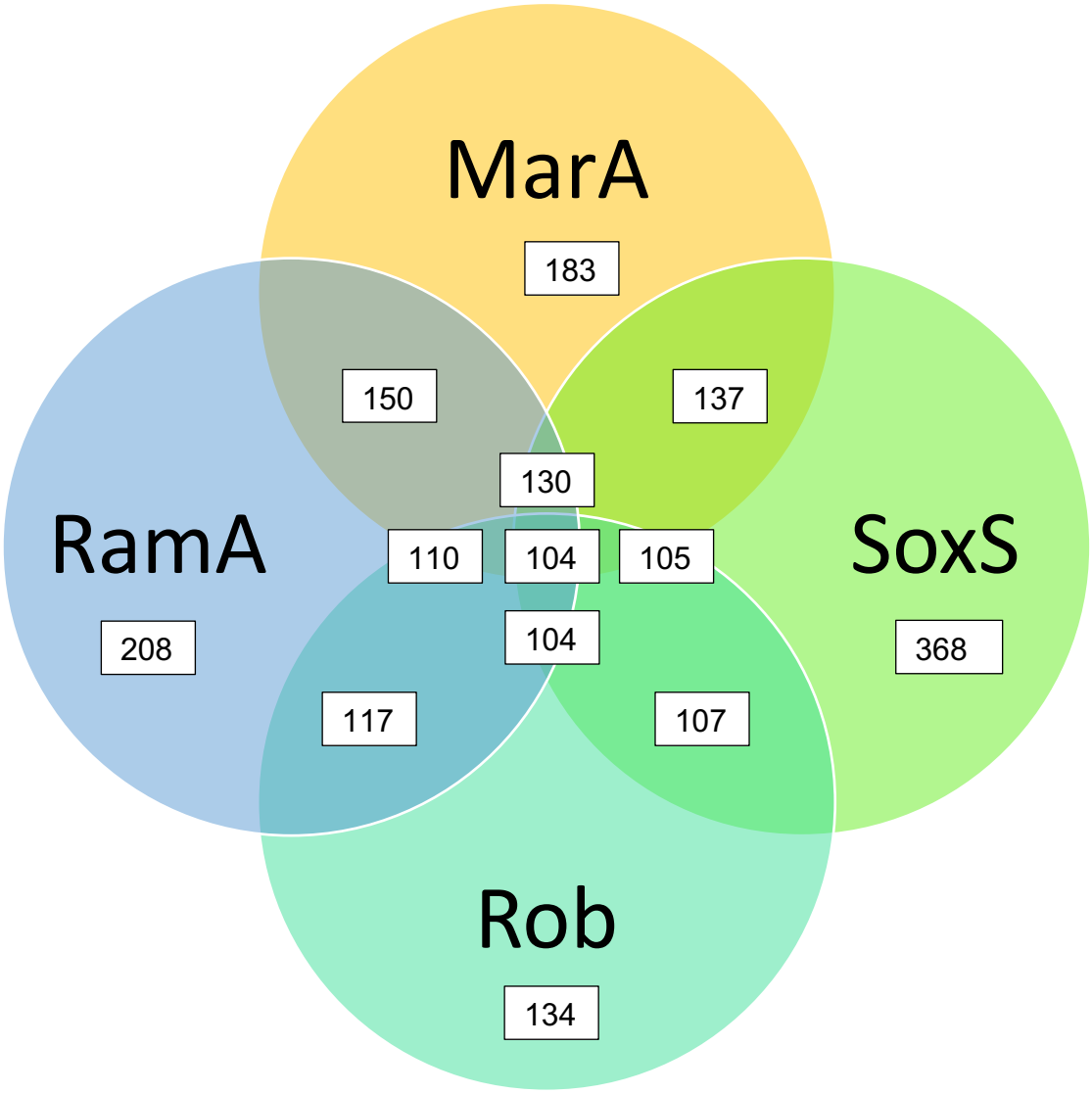

Figure 4. Venn diagram showing overlap of down-regulated genes by MarA homologs.

Table 1.

Genes that are up-regulated when MarA expression is induced from pBAD30 by adding 0.2% arabinose to the culture. Only the genes whose fold change compared the control strain (pBAD30) are significant are shown here ($P<0.05$).

Genes	Fold change
marA;locus_tag=STM14_1837	3154.5
ID=STM14_1838;Name=marR;locus_tag=STM14_1838	760.8
ID=STM14_4878;Name=sodA;locus_tag=STM14_4878	114.2
ID=STM14_1607;Name=yniB;locus_tag=STM14_1607	93.5
ID=STM14_1024;Name=ybjC;locus_tag=STM14_1024	62.1
ID=STM14_1635;Name=aroH;locus_tag=STM14_1635	55.1
ID=STM14_0204;Name=yadG;locus_tag=STM14_0204	54.2
ID=STM14_3958;Name=yhbW;locus_tag=STM14_3958	47.0
ID=STM14_3671;Name=idi;locus_tag=STM14_3671	42.7
ID=STM14_1514;Name=STM14_1514	42.6
ID=STM14_3852;Name=mdaB;locus_tag=STM14_3852	41.4
ID=STM14_3853;Name=ygiN;locus_tag=STM14_3853	41.1
ID=STM14_4368;Name=STM14_4368	40.5
ID=STM14_1025;Name=mdaA;locus_tag=STM14_1025	40.5
ID=STM14_1949;Name=rimL;locus_tag=STM14_1949	37.5
ID=STM14_3098;Name=ndk;locus_tag=STM14_3098	32.6
ID=STM14_0205;Name=yadH;locus_tag=STM14_0205	32.2
ID=STM14_4743;Name=STM14_4743	30.2
ID=STM14_4517;Name=selC;locus_tag=STM14_4517	29.3
ID=STM14_3604;Name=metZ;locus_tag=STM14_3604	29.2
ID=STM14_3214;Name=STM14_3214	27.4
ID=STM14_3605;Name=metW;locus_tag=STM14_3605	27.1
ID=STM14_1995;Name=nifJ;locus_tag=STM14_1995	27.0
ID=STM14_2819;Name=glpA;locus_tag=STM14_2819	25.9
ID=STM14_0579;Name=ybaL;locus_tag=STM14_0579	25.8
ID=STM14_3738;Name=yggJ;locus_tag=STM14_3738	25.2
ID=STM14_0479;Name=STM14_0479	25.2
ID=STM14_4058;Name=yhcP;locus_tag=STM14_4058	25.0
ID=STM14_4555;Name=nepI;gene_synonym=yicM	24.6
ID=STM14_1628;Name=btuE;locus_tag=STM14_1628	24.3
ID=STM14_1963;Name=STM14_1963	23.4
ID=STM14_1447;Name=STM14_1447	23.1
ID=STM14_3959;Name=STM14_3959	22.7
ID=STM14_4369;Name=yhjV;locus_tag=STM14_4369	22.6

ID=STM14_4744;Name=cyaY;locus_tag=STM14_4744	22.5
ID=STM14_1027;Name=ybjN;locus_tag=STM14_1027	22.4
ID=STM14_3500;Name=STM14_3500	22.1
ID=STM14_0373;Name=gpt;locus_tag=STM14_0373	22.1
ID=STM14_2023;Name=STM14_2023	22.0
ID=STM14_2886;Name=STM14_2886	21.4
ID=STM14_0189.gene;Alias=STM14_0189;Name=yacH	21.3
ID=STM14_1139;Name=pncB;locus_tag=STM14_1139	21.3
ID=STM14_0804;Name=citA;locus_tag=STM14_0804	21.1
ID=STM14_1608;Name=STM14_1608	20.9
ID=STM14_4775;Name=yigN;locus_tag=STM14_4775	20.8
ID=STM14_2820;Name=glpB;locus_tag=STM14_2820	20.4
ID=STM14_0816;Name=STM14_0816	20.4
ID=STM14_1238;Name=orfX;locus_tag=STM14_1238	19.9
ID=STM14_2887;Name=ulaA_2;gene_synonym=sgaT	19.6
ID=STM14_0819;Name=STM14_0819	19.0
ID=STM14_4685;Name=yieP;locus_tag=STM14_4685	18.7
ID=STM14_4910;Name=fpr;locus_tag=STM14_4910	18.6
ID=STM14_0820;Name=STM14_0820	18.5
ID=STM14_1185;Name=STM14_1185	18.5
ID=STM14_1603;Name=cedA;locus_tag=STM14_1603	18.3
ID=STM14_4001;Name=yrbF;locus_tag=STM14_4001	18.2
ID=STM14_3099;Name=STM14_3099	17.9
ID=STM14_2070;Name=acnA;locus_tag=STM14_2070	17.3
ID=STM14_0817;Name=potE;locus_tag=STM14_0817	17.2
ID=STM14_1108;Name=STM14_1108	17.1
ID=STM14_0435;Name=hemB;locus_tag=STM14_0435	17.1
ID=STM14_3739;Name=gshB;locus_tag=STM14_3739	17.0
ID=STM14_0818;Name=speF;locus_tag=STM14_0818	17.0
ID=STM14_1448;Name=STM14_1448	16.8
ID=STM14_3851;Name=STM14_3851	16.6
ID=STM14_1072;Name=STM14_1072	16.6
ID=STM14_0193;Name=kdgT;locus_tag=STM14_0193	16.5
ID=STM14_4518;Name=STM14_4518	16.3
ID=STM14_1073;Name=STM14_1073	16.2
ID=STM14_2137;Name=narL;locus_tag=STM14_2137	16.2
ID=STM14_0206;Name=stiH;locus_tag=STM14_0206	16.0
ID=STM14_1109;Name=cmk;locus_tag=STM14_1109	15.2
ID=STM14_3581;Name=sdaC;locus_tag=STM14_3581	14.8
ID=STM14_2822;Name=STM14_2822	14.7
ID=STM14_1239;Name=STM14_1239	14.7
ID=STM14_3156;Name=yfhL;locus_tag=STM14_3156	14.7
ID=STM14_1026;Name=rimK;locus_tag=STM14_1026	14.5
ID=STM14_5486;Name=STM14_5486	14.5
ID=STM14_2553;Name=phsC;locus_tag=STM14_2553	14.4

ID=STM14_1016;Name=mdfA;locus_tag=STM14_1016	14.3
ID=STM14_1323;Name=htrB;locus_tag=STM14_1323	14.2
ID=STM14_2215;Name=STM14_2215	14.1
ID=STM14_3146;Name=STM14_3146	14.0
ID=STM14_0188;Name=STM14_0188	13.7
ID=STM14_1948;Name=ydcK;locus_tag=STM14_1948	13.7
ID=STM14_3502;Name=STM14_3502	13.7
ID=STM14_2790;Name=napF;locus_tag=STM14_2790	13.6
ID=STM14_0187;Name=STM14_0187	13.4
ID=STM14_2609;Name=wcaB;locus_tag=STM14_2609	13.4
ID=STM14_1402;Name=potB;locus_tag=STM14_1402	13.3
ID=STM14_4887;Name=sbp;locus_tag=STM14_4887	13.2
ID=STM14_1882;Name=STM14_1882	13.2
ID=STM14_4886;Name=STM14_4886	13.0
ID=STM14_2821;Name=glpC;locus_tag=STM14_2821	13.0
ID=STM14_2022;Name=STM14_2022	12.8
ID=STM14_3501;Name=STM14_3501	12.7
ID=STM14_1629;Name=btuD;locus_tag=STM14_1629	12.6
ID=STM14_1992;Name=hslJ;locus_tag=STM14_1992	12.5
ID=STM14_0878;Name=lysZ;locus_tag=STM14_0878	12.4
ID=STM14_0450;Name=STM14_0450	12.2
ID=STM14_0434;Name=prpE;locus_tag=STM14_0434	12.0
ID=STM14_4684;Name=yieO;locus_tag=STM14_4684	11.9
ID=STM14_1536;Name=STM14_1536	11.8
ID=STM14_3582;Name=sdaB;locus_tag=STM14_3582	11.7
ID=STM14_2213;Name=manY;locus_tag=STM14_2213	11.7
ID=STM14_0350;Name=STM14_0350	11.6
ID=STM14_1071;Name=serW;locus_tag=STM14_1071	11.5
ID=STM14_2523;Name=cbiA;locus_tag=STM14_2523	11.5
ID=STM14_2827;Name=yfaV;locus_tag=STM14_2827	11.4
ID=STM14_2486;Name=asnT_1;locus_tag=STM14_2486	11.1
ID=STM14_2136;Name=narX;locus_tag=STM14_2136	11.0
ID=STM14_4073;Name=STM14_4073	10.9
ID=STM14_2697;Name=STM14_2697	10.9
ID=STM14_2885;Name=STM14_2885	10.9
ID=STM14_2024;Name=STM14_2024	10.9
ID=STM14_0877;Name=lysY;locus_tag=STM14_0877	10.8
ID=STM14_2521;Name=cbiC;locus_tag=STM14_2521	10.8
ID=STM14_0757;Name=STM14_0757	10.7
ID=STM14_1322;Name=STM14_1322	10.6
ID=STM14_0361;Name=STM14_0361	10.5
ID=STM14_5158;Name=phnB;locus_tag=STM14_5158	10.5
ID=STM14_0895;Name=STM14_0895	10.4
ID=STM14_2212;Name=manX;locus_tag=STM14_2212	10.4
ID=STM14_3764;Name=yqgA;locus_tag=STM14_3764	10.4

ID=STM14_1515;Name=STM14_1515	10.3
ID=STM14_4866;Name=rhaB;locus_tag=STM14_4866	10.3
ID=STM14_0525;Name=yajG;locus_tag=STM14_0525	10.2
ID=STM14_1602;Name=STM14_1602	10.2
ID=STM14_3147;Name=yfhD;locus_tag=STM14_3147	10.2
ID=STM14_2996;Name=yfeK;locus_tag=STM14_2996	10.2
ID=STM14_3406;Name=STM14_3406	10.1
ID=STM14_0568;Name=apt;locus_tag=STM14_0568	10.1
ID=STM14_2888;Name=STM14_2888	10.0
ID=STM14_4317;Name=yhiN;locus_tag=STM14_4317	10.0
ID=STM14_2524;Name=STM14_2524	9.9
ID=STM14_2520;Name=cbiD;locus_tag=STM14_2520	9.8
ID=STM14_2385;Name=yedE;locus_tag=STM14_2385	9.8
ID=STM14_2513;Name=cbiK;locus_tag=STM14_2513	9.8
ID=STM14_1537;Name=STM14_1537	9.7
ID=STM14_0512;Name=thiJ;locus_tag=STM14_0512	9.7
ID=STM14_1842;Name=yneI;locus_tag=STM14_1842	9.7
ID=STM14_1836;Name=marB;locus_tag=STM14_1836	9.7
ID=STM14_2889;Name=STM14_2889	9.6
ID=STM14_0601;Name=sfbB;locus_tag=STM14_0601	9.6
ID=STM14_1205;Name=uup;locus_tag=STM14_1205	9.5
ID=STM14_0876;Name=lysW;locus_tag=STM14_0876	9.4
ID=STM14_0050;Name=STM14_0050	9.4
ID=STM14_2519;Name=cbiE;locus_tag=STM14_2519	9.1
ID=STM14_1095;Name=ycaM;locus_tag=STM14_1095	9.1
ID=STM14_1028;Name=potF;locus_tag=STM14_1028	9.0
ID=STM14_2727;Name=setB;locus_tag=STM14_2727	8.9
ID=STM14_2071;Name=STM14_2071	8.8
ID=STM14_0875;Name=valT;locus_tag=STM14_0875	8.8
ID=STM14_2518;Name=cbiT;locus_tag=STM14_2518	8.6
ID=STM14_2386;Name=yedF;locus_tag=STM14_2386	8.6
ID=STM14_0578;Name=gsk;locus_tag=STM14_0578	8.5
ID=STM14_3975;Name=leuU;locus_tag=STM14_3975	8.4
ID=STM14_2094;Name=STM14_2094	8.4
ID=STM14_4000;Name=yrbE;locus_tag=STM14_4000	8.4
ID=STM14_4499;Name=STM14_4499	8.2
ID=STM14_1993;Name=STM14_1993	8.0
ID=STM14_2872;Name=STM14_2872	8.0
ID=STM14_1961;Name=STM14_1961	8.0
ID=STM14_2211;Name=STM14_2211	7.9
ID=STM14_944;Name=ybhQ;locus_tag=STM14_944	7.9
ID=STM14_1900;Name=smvA;locus_tag=STM14_1900	7.9
ID=STM14_3407;Name=STM14_3407	7.8
ID=STM14_1561;Name=STM14_1561	7.8
ID=STM14_2516;Name=cbiG;locus_tag=STM14_2516	7.7

ID=STM14_0380;Name=thrW;locus_tag=STM14_0380	7.6
ID=STM14_0171;Name=hofB;locus_tag=STM14_0171	7.5
ID=STM14_2698;Name=mglB;locus_tag=STM14_2698	7.4
ID=STM14_2535;Name=pduK;locus_tag=STM14_2535	7.4
ID=STM14_1029;Name=potG;locus_tag=STM14_1029	7.3
ID=STM14_5462;Name=STM14_5462	7.2
ID=STM14_0758;Name=STM14_0758	7.2
ID=STM14_1962;Name=mdoD;gene_synonym=opgD	7.2
ID=STM14_1446;Name=STM14_1446	7.1
ID=STM14_2973;Name=lysV;locus_tag=STM14_2973	7.1
ID=STM14_2785;Name=napB;locus_tag=STM14_2785	7.1
ID=STM14_3447;Name=STM14_3447	7.0
ID=STM14_2965;Name=alaX;locus_tag=STM14_2965	7.0
ID=STM14_0216;Name=panB;locus_tag=STM14_0216	6.9
ID=STM14_1843;Name=yneH;locus_tag=STM14_1843	6.7
ID=STM14_1020;Name=STM14_1020	6.7
ID=STM14_0511;Name=phnX;locus_tag=STM14_0511	6.6
ID=STM14_2076;Name=STM14_2076	6.6
ID=STM14_2890;Name=STM14_2890	6.6
ID=STM14_2787;Name=napG;locus_tag=STM14_2787	6.5
ID=STM14_1398;Name=potC;locus_tag=STM14_1398	6.5
ID=STM14_4074;Name=STM14_4074	6.5
ID=STM14_2514;Name=cbiJ;gene_synonym=cobK	6.5
ID=STM14_5305;Name=STM14_5305	6.4
ID=STM14_3712;Name=STM14_3712	6.4
ID=STM14_1816;Name=STM14_1816	6.4
ID=STM14_3860;Name=STM14_3860	6.4
ID=STM14_3088;Name=yfgJ;locus_tag=STM14_3088	6.3
ID=STM14_1445;Name=STM14_1445	6.3
ID=STM14_3230;Name=STM14_3230	6.3
ID=STM14_3736;Name=sprT;locus_tag=STM14_3736	6.3
ID=STM14_4554;Name=STM14_4554	6.3
ID=STM14_2972;Name=valY;locus_tag=STM14_2972	6.2
ID=STM14_3446;Name=hycB;locus_tag=STM14_3446	6.2
ID=STM14_5274;Name=STM14_5274	6.2
ID=STM14_1399;Name=STM14_1399	6.1
ID=STM14_2789;Name=napD;locus_tag=STM14_2789	6.1
ID=STM14_2784;Name=napC;locus_tag=STM14_2784	6.0
ID=STM14_3996;Name=STM14_3996	5.9
ID=STM14_0546;Name=glnK;locus_tag=STM14_0546	5.8
ID=STM14_2515;Name=cbiH;locus_tag=STM14_2515	5.8
ID=STM14_4497;Name=STM14_4497	5.7
ID=STM14_1721;Name=valV;locus_tag=STM14_1721	5.7
ID=STM14_4085;Name=yhdU;locus_tag=STM14_4085	5.7
ID=STM14_999;Name=STM14_999	5.7

ID=STM14_3583;Name=STM14_3583	5.6
ID=STM14_1510;Name=STM14_1510	5.5
ID=STM14_2510;Name=cbiN;locus_tag=STM14_2510	5.4
ID=STM14_3642;Name=STM14_3642	5.0
ID=STM14_3503;Name=STM14_3503	4.9
ID=STM14_0599;Name=STM14_0599	4.8
ID=STM14_2200;Name=STM14_2200	4.7
ID=STM14_1952;Name=STM14_1952	4.7
ID=STM14_1631;Name=STM14_1631	4.5
ID=STM14_2726;Name=STM14_2726	4.4
ID=STM14_2684;Name=STM14_2684	4.4
ID=STM14_2676;Name=STM14_2676	4.2
ID=STM14_1297;Name=serX;locus_tag=STM14_1297	3.9

Table 2.

Genes that are down-regulated when MarA expression is induced from pBAD30 by adding 0.2% arabinose to the culture. Only the genes whose fold change compared the control strain (pBAD30) are significant are shown here ($P<0.05$).

Genes	Fold Change
ID=STM14_0621;Name=ylbF;locus_tag=STM14_0621	3.8
ID=STM14_2224;Name=STM14_2224	3.8
ID=STM14_5416;Name=STM14_5416	3.8
ID=STM14_0633;Name=STM14_0633	3.9
ID=STM14_5415;Name=STM14_5415	3.9
ID=STM14_2265;Name=pagK;locus_tag=STM14_2265	3.9
ID=STM14_0399;Name=STM14_0399	3.9
ID=STM14_3801;Name=STM14_3801	4.1
ID=STM14_2223;Name=STM14_2223	4.1
ID=STM14_1405.gene;Alias=STM14_1405;Name=STM14_1405	4.2
ID=STM14_3800;Name=STM14_3800	4.2
ID=STM14_1497;Name=pagD;locus_tag=STM14_1497	4.2
ID=STM14_2680;Name=STM14_2680	4.2
ID=STM14_2061;Name=STM14_2061	4.2
ID=STM14_3028;Name=eutS;locus_tag=STM14_3028	4.3
ID=STM14_1937;Name=STM14_1937	4.3
ID=STM14_2270;Name=STM14_2270	4.3
ID=STM14_4339;Name=STM14_4339	4.4
ID=STM14_1480;Name=STM14_1480	4.6
ID=STM14_0720;Name=citG;locus_tag=STM14_0720	4.7
ID=STM14_1261;Name=STM14_1261	4.7
ID=STM14_1438;Name=STM14_1438	4.7
ID=STM14_5044;Name=STM14_5044	4.8
ID=STM14_1198;Name=STM14_1198	4.8
ID=STM14_0654.gene;Alias=STM14_0654;Name=STM14_0654	4.8
ID=STM14_3343;Name=STM14_3343	4.9
ID=STM14_0402;Name=STM14_0402	5.0
ID=STM14_3342;Name=STM14_3342	5.0
ID=STM14_2169;Name=STM14_2169	5.1
ID=STM14_5190;Name=STM14_5190	5.2
ID=STM14_1008;Name=STM14_1008	5.2
ID=STM14_1346;Name=flgC;locus_tag=STM14_1346	5.2
ID=STM14_3209;Name=STM14_3209	5.2

ID=STM14_2399;Name=fliO;locus_tag=STM14_2399	5.4
ID=STM14_1345;Name=flgB;locus_tag=STM14_1345	5.4
ID=STM14_0644;Name=fimW;locus_tag=STM14_0644	5.5
ID=STM14_0685;Name=ybdZ;locus_tag=STM14_0685	5.5
ID=STM14_1613;Name=STM14_1613	5.5
ID=STM14_0428;Name=yahO;locus_tag=STM14_0428	5.6
ID=STM14_3198;Name=STM14_3198	5.6
ID=STM14_5119;Name=STM14_5119	5.6
ID=STM14_5125;Name=STM14_5125	5.7
ID=STM14_1009;Name=STM14_1009	5.8
ID=STM14_2333;Name=cheR;locus_tag=STM14_2333	5.8
ID=STM14_3353;Name=STM14_3353	5.8
ID=STM14_0665;Name=STM14_0665	5.9
ID=STM14_2331;Name=cheY;locus_tag=STM14_2331	6.0
ID=STM14_0656;Name=STM14_0656	6.1
ID=STM14_2368;Name=sdiA;locus_tag=STM14_2368	6.1
ID=STM14_5184;Name=STM14_5184	6.1
ID=STM14_5045;Name=STM14_5045	6.2
ID=STM14_2359;Name=STM14_2359	6.3
ID=STM14_1503;Name=STM14_1503	6.3
ID=STM14_4768;Name=STM14_4768	6.3
ID=STM14_2329;Name=STM14_2329	6.4
ID=STM14_0723;Name=citE;locus_tag=STM14_0723	6.4
ID=STM14_0643;Name=STM14_0643	6.4
ID=STM14_3354;Name=mig-14;locus_tag=STM14_3354	6.4
ID=STM14_2689;Name=yohK;locus_tag=STM14_2689	6.5
ID=STM14_3340;Name=STM14_3340	6.5
ID=STM14_1312;Name=ymdA;locus_tag=STM14_1312	6.6
ID=STM14_5323;Name=STM14_5323	6.7
ID=STM14_3341;Name=STM14_3341	6.7
ID=STM14_3461;Name=sitD;locus_tag=STM14_3461	6.7
ID=STM14_2394;Name=fliJ;locus_tag=STM14_2394	6.7
ID=STM14_1349;Name=flgF;locus_tag=STM14_1349	6.8
ID=STM14_2373;Name=fliZ;locus_tag=STM14_2373	6.8
ID=STM14_4534;Name=cigR;locus_tag=STM14_4534	6.9
ID=STM14_1347;Name=flgD;locus_tag=STM14_1347	7.0
ID=STM14_2798;Name=STM14_2798	7.0
ID=STM14_5189;Name=STM14_5189	7.0
ID=STM14_0724;Name=citD;locus_tag=STM14_0724	7.1
ID=STM14_2393;Name=fliI;locus_tag=STM14_2393	7.1
ID=STM14_0655;Name=STM14_0655	7.1
ID=STM14_2330;Name=cheZ;locus_tag=STM14_2330	7.2
ID=STM14_0722;Name=citF;locus_tag=STM14_0722	7.2
ID=STM14_1352;Name=flgI;locus_tag=STM14_1352	7.3
ID=STM14_4769;Name=metE;locus_tag=STM14_4769	7.3

ID=STM14_1342;Name=flgM;locus_tag=STM14_1342	7.3
ID=STM14_1353;Name=flgJ;locus_tag=STM14_1353	7.4
ID=STM14_2352;Name=STM14_2352	7.5
ID=STM14_0135;Name=leuL;locus_tag=STM14_0135	7.6
ID=STM14_1351;Name=flgH;locus_tag=STM14_1351	7.8
ID=STM14_4532;Name=slsA;locus_tag=STM14_4532	7.9
ID=STM14_0725;Name=citC;locus_tag=STM14_0725	7.9
ID=STM14_2335;Name=cheW;locus_tag=STM14_2335	8.0
ID=STM14_2395;Name=fliK;locus_tag=STM14_2395	8.0
ID=STM14_2392;Name=fliH;locus_tag=STM14_2392	8.2
ID=STM14_2398;Name=fliN;locus_tag=STM14_2398	8.4
ID=STM14_2387;Name=STM14_2387	8.5
ID=STM14_4531;Name=STM14_4531	8.6
ID=STM14_0721;Name=citX;locus_tag=STM14_0721	8.6
ID=STM14_1439;Name=STM14_1439	9.1
ID=STM14_3821;Name=STM14_3821	9.2
ID=STM14_2374;Name=fliA;locus_tag=STM14_2374	9.4
ID=STM14_0638;Name=fimD;locus_tag=STM14_0638	9.4
ID=STM14_3352;Name=virK;locus_tag=STM14_3352	9.4
ID=STM14_3822;Name=STM14_3822	9.6
ID=STM14_3127;Name=asrB;locus_tag=STM14_3127	9.7
ID=STM14_2379;Name=STM14_2379	10.0
ID=STM14_1348;Name=flgE;locus_tag=STM14_1348	10.2
ID=STM14_1891;Name=adhP;gene_synonym=adhA;locus_tag=STM14_1891	10.4
ID=STM14_3820;Name=STM14_3820	11.7
ID=STM14_5188;Name=STM14_5188	12.2
ID=STM14_4935;Name=metF;locus_tag=STM14_4935	12.3
ID=STM14_3126;Name=asrA;locus_tag=STM14_3126	12.4
ID=STM14_966;Name=dps;locus_tag=STM14_966	12.8
ID=STM14_0641;Name=fimZ;locus_tag=STM14_0641	12.9
ID=STM14_2168;Name=STM14_2168	13.3
ID=STM14_4934;Name=STM14_4934	13.5
ID=STM14_5167;Name=adiY;locus_tag=STM14_5167	14.0
ID=STM14_2342;Name=STM14_2342	14.4
ID=STM14_1283;Name=STM14_1283	14.6
ID=STM14_2167;Name=STM14_2167	14.7
ID=STM14_1282;Name=STM14_1282	15.1
ID=STM14_4305;Name=tcp;locus_tag=STM14_4305	15.2
ID=STM14_3466;Name=STM14_3466	15.3
ID=STM14_3477;Name=sptP;locus_tag=STM14_3477	16.2
ID=STM14_1884;Name=STM14_1884	16.6
ID=STM14_1612;Name=STM14_1612	17.5
ID=STM14_5120;Name=STM14_5120	18.3
ID=STM14_5117;Name=STM14_5117	18.6

ID=STM14_5118;Name=STM14_5118	18.9
ID=STM14_1237;Name=sopB;locus_tag=STM14_1237	19.5
ID=STM14_3467;Name=orgC;locus_tag=STM14_3467	20.3
ID=STM14_1235;Name=STM14_1235	21.6
ID=STM14_1236;Name=pipC;locus_tag=STM14_1236	24.7
ID=STM14_3479;Name=STM14_3479	24.9
ID=STM14_3468;Name=orgB;locus_tag=STM14_3468	25.0
ID=STM14_5187;Name=STM14_5187	26.1
ID=STM14_1887;Name=yddX;locus_tag=STM14_1887	26.5
ID=STM14_5185;Name=STM14_5185	26.9
ID=STM14_3469;Name=orgA;locus_tag=STM14_3469	27.0
ID=STM14_2227;Name=STM14_2227	29.2
ID=STM14_2162;Name=STM14_2162	29.2
ID=STM14_2166;Name=STM14_2166	29.7
ID=STM14_3499;Name=invH;locus_tag=STM14_3499	29.9
ID=STM14_0637;Name=fimC;locus_tag=STM14_0637	30.6
ID=STM14_0636;Name=fimI;locus_tag=STM14_0636	31.4
ID=STM14_3478;Name=sicP;locus_tag=STM14_3478	31.5
ID=STM14_3462;Name=avrA;locus_tag=STM14_3462	32.7
ID=STM14_3475;Name=hilA;locus_tag=STM14_3475	33.5
ID=STM14_3486;Name=spaS;locus_tag=STM14_3486	35.3
ID=STM14_3480;Name=iacP;locus_tag=STM14_3480	43.1
ID=STM14_3487;Name=spaR;locus_tag=STM14_3487	43.7
ID=STM14_3493;Name=invC;locus_tag=STM14_3493	46.7
ID=STM14_3893;Name=STM14_3893	48.4
ID=STM14_3476;Name=iagB;locus_tag=STM14_3476	49.0
ID=STM14_3485;Name=sicA;locus_tag=STM14_3485	49.7
ID=STM14_2161;Name=STM14_2161	49.9
ID=STM14_3484;Name=sipB;locus_tag=STM14_3484	50.8
ID=STM14_3490;Name=spaO;locus_tag=STM14_3490	53.2
ID=STM14_5166;Name=yjdE;locus_tag=STM14_5166	54.2
ID=STM14_3481;Name=sipA;locus_tag=STM14_3481	55.2
ID=STM14_3494;Name=invB;locus_tag=STM14_3494	56.2
ID=STM14_5186;Name=STM14_5186	59.3
ID=STM14_2160;Name=STM14_2160	59.7
ID=STM14_3482;Name=sipD;locus_tag=STM14_3482	61.1
ID=STM14_3799;Name=STM14_3799	61.6
ID=STM14_2244;Name=sopE2;locus_tag=STM14_2244	66.5
ID=STM14_3492;Name=invI;locus_tag=STM14_3492	67.5
ID=STM14_3488;Name=spaQ;locus_tag=STM14_3488	69.1
ID=STM14_3491;Name=invJ;locus_tag=STM14_3491	69.5
ID=STM14_3474;Name=hilD;locus_tag=STM14_3474	72.6
ID=STM14_2164;Name=STM14_2164	73.2
ID=STM14_3473;Name=prgH;locus_tag=STM14_3473	74.8
ID=STM14_3470;Name=prgK;locus_tag=STM14_3470	75.4

ID=STM14_3483;Name=sipC;locus_tag=STM14_3483	78.3
ID=STM14_0635;Name=fimA;locus_tag=STM14_0635	78.9
ID=STM14_3489;Name=spaP;locus_tag=STM14_3489	79.2
ID=STM14_3495;Name=invA;locus_tag=STM14_3495	83.4
ID=STM14_3497;Name=invG;locus_tag=STM14_3497	84.2
ID=STM14_3496;Name=invE;locus_tag=STM14_3496	85.5
ID=STM14_2165;Name=STM14_2165	88.6
ID=STM14_5169;Name=adi;locus_tag=STM14_5169	96.9
ID=STM14_3463;Name=sprB;locus_tag=STM14_3463	97.7
ID=STM14_3472;Name=prgI;locus_tag=STM14_3472	104.7
ID=STM14_3471;Name=prgJ;locus_tag=STM14_3471	107.4
ID=STM14_3498;Name=invF;locus_tag=STM14_3498	109.9
ID=STM14_3465;Name=hilC;locus_tag=STM14_3465	123.4
ID=STM14_3464;Name=STM14_3464	184.0
ID=STM14_5168;Name=STM14_5168	189.1
ID=STM14_1885;Name=hdeB;locus_tag=STM14_1885	759.5

Table 3.

Genes that are up-regulated when SoxS expression is induced from pBAD30 by adding 0.2% arabinose to the culture. Only the genes whose fold change compared the control strain (pBAD30) are significant are shown here (P<0.05).

Genes	Fold change
ID=STM14_5127;Name=soxS;locus_tag=STM14_5127	2359.0
ID=STM14_1995;Name=nifJ;locus_tag=STM14_1995	92.3
ID=STM14_1628;Name=btuE;locus_tag=STM14_1628	80.7
ID=STM14_1024;Name=ybjC;locus_tag=STM14_1024	78.5
ID=STM14_0034;Name=bcfG;locus_tag=STM14_0034	68.6
ID=STM14_1025;Name=mdaA;locus_tag=STM14_1025	66.6
ID=STM14_1629;Name=btuD;locus_tag=STM14_1629	63.9
ID=STM14_5126;Name=yjcC;locus_tag=STM14_5126	62.2
ID=STM14_1536;Name=STM14_1536	53.8
ID=STM14_4878;Name=sodA;locus_tag=STM14_4878	48.5
ID=STM14_1537;Name=STM14_1537	48.0
ID=STM14_0450;Name=STM14_0450	45.8
ID=STM14_3804;Name=yghU;locus_tag=STM14_3804	45.8
ID=STM14_3853;Name=ygiN;locus_tag=STM14_3853	45.4
ID=STM14_1185;Name=STM14_1185	44.2
ID=STM14_0361;Name=STM14_0361	44.1
ID=STM14_4446;Name=STM14_4446	43.2
ID=STM14_3395;Name=STM14_3395	39.2
ID=STM14_2979;Name=yfeR;locus_tag=STM14_2979	36.6
ID=STM14_3214;Name=STM14_3214	35.6
ID=STM14_4599;Name=ibpB;locus_tag=STM14_4599	35.0
ID=STM14_4433;Name=sgbU;locus_tag=STM14_4433	33.3
ID=STM14_2720;Name=nfo;locus_tag=STM14_2720	27.8
ID=STM14_1631;Name=STM14_1631	27.4
ID=STM14_4910;Name=fpr;locus_tag=STM14_4910	26.7
ID=STM14_1834;Name=ydeE;locus_tag=STM14_1834	26.4
ID=STM14_1838;Name=marR;locus_tag=STM14_1838	26.1
ID=STM14_5486;Name=STM14_5486	26.1
ID=STM14_3805;Name=STM14_3805	25.1
ID=STM14_0579;Name=ybaL;locus_tag=STM14_0579	24.5
ID=STM14_2020;Name=STM14_2020	24.1
ID=STM14_4598;Name=STM14_4598	23.7
ID=STM14_3431;Name=STM14_3431	23.6

ID=STM14_4361;Name=STM14_4361	23.0
ID=STM14_2819;Name=glpA;locus_tag=STM14_2819	22.0
ID=STM14_4368;Name=STM14_4368	21.7
ID=STM14_1632;Name=ydiV;locus_tag=STM14_1632	21.2
ID=STM14_1630;Name=nlpC;locus_tag=STM14_1630	20.9
ID=STM14_0479;Name=STM14_0479	20.4
ID=STM14_1538;Name=STM14_1538	19.5
ID=STM14_4445;Name=mtlR;locus_tag=STM14_4445	19.4
ID=STM14_4600;Name=ibpA;locus_tag=STM14_4600	19.2
ID=STM14_1026;Name=rimK;locus_tag=STM14_1026	18.9
ID=STM14_4227;Name=yhgH;locus_tag=STM14_4227	18.1
ID=STM14_4383;Name=lpfE;locus_tag=STM14_4383	17.5
ID=STM14_4685;Name=yieP;locus_tag=STM14_4685	17.2
ID=STM14_3764;Name=yqgA;locus_tag=STM14_3764	17.0
ID=STM14_4517;Name=selC;locus_tag=STM14_4517	17.0
ID=STM14_1139;Name=pncB;locus_tag=STM14_1139	16.8
ID=STM14_1607;Name=yniB;locus_tag=STM14_1607	16.6
ID=STM14_3146;Name=STM14_3146	16.6
ID=STM14_1027;Name=ybjN;locus_tag=STM14_1027	16.4
ID=STM14_2076;Name=STM14_2076	16.2
ID=STM14_3739;Name=gshB;locus_tag=STM14_3739	15.9
ID=STM14_1447;Name=STM14_1447	15.1
ID=STM14_5348;Name=treR;locus_tag=STM14_5348	15.0
ID=STM14_2733;Name=yeiP;locus_tag=STM14_2733	14.8
ID=STM14_2886;Name=STM14_2886	14.7
ID=STM14_0910;Name=ybhA;locus_tag=STM14_0910	14.3
ID=STM14_3147;Name=yfhD;locus_tag=STM14_3147	14.2
ID=STM14_0513;Name=apbA;locus_tag=STM14_0513	14.0
ID=STM14_1044;Name=ybjP;locus_tag=STM14_1044	13.8
ID=STM14_4684;Name=yieO;locus_tag=STM14_4684	13.5
ID=STM14_4911;Name=glpX;locus_tag=STM14_4911	13.5
ID=STM14_0817;Name=potE;locus_tag=STM14_0817	13.2
ID=STM14_2820;Name=glpB;locus_tag=STM14_2820	13.0
ID=STM14_0818;Name=speF;locus_tag=STM14_0818	12.9
ID=STM14_2727;Name=setB;locus_tag=STM14_2727	12.9
ID=STM14_1321;Name=yceE;locus_tag=STM14_1321	12.7
ID=STM14_0050;Name=STM14_0050	12.6
ID=STM14_1448;Name=STM14_1448	12.4
ID=STM14_3044;Name=acrD;locus_tag=STM14_3044	12.3
ID=STM14_0546;Name=glnK;locus_tag=STM14_0546	12.2
ID=STM14_0512;Name=thiJ;locus_tag=STM14_0512	12.1
ID=STM14_3111;Name=pepB;locus_tag=STM14_3111	11.9
ID=STM14_0188;Name=STM14_0188	11.9
ID=STM14_1836;Name=marB;locus_tag=STM14_1836	11.9
ID=STM14_4369;Name=yhjV;locus_tag=STM14_4369	11.8

ID=STM14_3841;Name=STM14_3841	11.8
ID=STM14_0816;Name=STM14_0816	11.8
ID=STM14_2283;Name=exoX;locus_tag=STM14_2283	11.8
ID=STM14_4030;Name=nanA;locus_tag=STM14_4030	11.7
ID=STM14_1603;Name=cedA;locus_tag=STM14_1603	11.7
ID=STM14_3110;Name=sseB;locus_tag=STM14_3110	11.7
ID=STM14_4920;Name=hslV;locus_tag=STM14_4920	11.6
ID=STM14_1108;Name=STM14_1108	11.5
ID=STM14_1932;Name=STM14_1932	11.5
ID=STM14_0451;Name=STM14_0451	11.4
ID=STM14_0598;Name=STM14_0598	11.2
ID=STM14_1207;Name=pqiB;locus_tag=STM14_1207	11.1
ID=STM14_3723;Name=STM14_3723	11.1
ID=STM14_1109;Name=cmk;locus_tag=STM14_1109	11.0
ID=STM14_2887;Name=ulaA_2;gene_synonym=sgaT;locus_tag=STM14_2887	11.0
ID=STM14_4153;Name=yheL;locus_tag=STM14_4153	11.0
ID=STM14_1945;Name=STM14_1945	11.0
ID=STM14_2827;Name=yfaV;locus_tag=STM14_2827	10.8
ID=STM14_3958;Name=yhbW;locus_tag=STM14_3958	10.7
ID=STM14_3425;Name=srlD;locus_tag=STM14_3425	10.7
ID=STM14_0687;Name=fepE;locus_tag=STM14_0687	10.6
ID=STM14_3581;Name=sdaC;locus_tag=STM14_3581	10.6
ID=STM14_1206;Name=pqiA;locus_tag=STM14_1206	10.5
ID=STM14_1933;Name=STM14_1933	10.5
ID=STM14_0819;Name=STM14_0819	10.4
ID=STM14_0572;Name=htpG;locus_tag=STM14_0572	10.3
ID=STM14_2209;Name=STM14_2209	10.0
ID=STM14_5204;Name=fxsA;locus_tag=STM14_5204	9.9
ID=STM14_4518;Name=STM14_4518	9.8
ID=STM14_0820;Name=STM14_0820	9.8
ID=STM14_4334;Name=treF;locus_tag=STM14_4334	9.7
ID=STM14_1205;Name=uup;locus_tag=STM14_1205	9.5
ID=STM14_3582;Name=sdaB;locus_tag=STM14_3582	9.5
ID=STM14_4564;Name=STM14_4564	9.4
ID=STM14_3604;Name=metZ;locus_tag=STM14_3604	9.4
ID=STM14_2816;Name=STM14_2816	9.4
ID=STM14_1136;Name=STM14_1136	9.2
ID=STM14_3782;Name=STM14_3782	9.2
ID=STM14_4381;Name=proK;locus_tag=STM14_4381	9.2
ID=STM14_1077;Name=aat;locus_tag=STM14_1077	9.1
ID=STM14_3745;Name=yggT;locus_tag=STM14_3745	9.1
ID=STM14_3389;Name=nrdE;locus_tag=STM14_3389	8.8
ID=STM14_4360;Name=yhjR;locus_tag=STM14_4360	8.8
ID=STM14_4362;Name=yhjS;locus_tag=STM14_4362	8.7

ID=STM14_2838;Name=STM14_2838	8.5
ID=STM14_4488;Name=mutM;locus_tag=STM14_4488	8.5
ID=STM14_4563;Name=STM14_4563	8.5
ID=STM14_2978;Name=yfeN;locus_tag=STM14_2978	8.5
ID=STM14_1320;Name=msyB;locus_tag=STM14_1320	8.5
ID=STM14_5477;Name=yjjG;locus_tag=STM14_5477	8.4
ID=STM14_0014;Name=dnaJ;locus_tag=STM14_0014	8.3
ID=STM14_3816;Name=yghW;locus_tag=STM14_3816	8.3
ID=STM14_0601;Name=sfbB;locus_tag=STM14_0601	8.3
ID=STM14_4621;Name=STM14_4621	8.2
ID=STM14_2022;Name=STM14_2022	8.2
ID=STM14_2905;Name=STM14_2905	8.2
ID=STM14_2885;Name=STM14_2885	8.1
ID=STM14_3605;Name=metW;locus_tag=STM14_3605	8.0
ID=STM14_0511;Name=phnX;locus_tag=STM14_0511	7.8
ID=STM14_3043;Name=STM14_3043	7.7
ID=STM14_0434;Name=prpE;locus_tag=STM14_0434	7.7
ID=STM14_2675;Name=yohC;locus_tag=STM14_2675	7.7
ID=STM14_1319;Name=yceK;locus_tag=STM14_1319	7.7
ID=STM14_3635;Name=STM14_3635	7.5
ID=STM14_0599;Name=STM14_0599	7.5
ID=STM14_3724;Name=STM14_3724	7.4
ID=STM14_2245;Name=STM14_2245	7.4
ID=STM14_2486;Name=asnT_1;locus_tag=STM14_2486	7.2
ID=STM14_4620;Name=dgoA;locus_tag=STM14_4620	7.2
ID=STM14_4669;Name=STM14_4669	7.2
ID=STM14_2128;Name=STM14_2128	7.1
ID=STM14_3100;Name=STM14_3100	7.1
ID=STM14_0809;Name=STM14_0809	7.0
ID=STM14_4359;Name=yhjQ;locus_tag=STM14_4359	6.9
ID=STM14_3899;Name=ygjO;locus_tag=STM14_3899	6.9
ID=STM14_4886;Name=STM14_4886	6.9
ID=STM14_1399;Name=STM14_1399	6.9
ID=STM14_2790;Name=napF;locus_tag=STM14_2790	6.8
ID=STM14_5124;Name=STM14_5124	6.8
ID=STM14_5327;Name=STM14_5327	6.8
ID=STM14_4847;Name=STM14_4847	6.8
ID=STM14_1602;Name=STM14_1602	6.7
ID=STM14_2908;Name=STM14_2908	6.6
ID=STM14_4317;Name=yhiN;locus_tag=STM14_4317	6.5
ID=STM14_3026;Name=eutQ;locus_tag=STM14_3026	6.5
ID=STM14_4386;Name=lpfB;locus_tag=STM14_4386	6.5
ID=STM14_4419;Name=STM14_4419	6.4
ID=STM14_1398;Name=potC;locus_tag=STM14_1398	6.4
ID=STM14_1242;Name=copR;locus_tag=STM14_1242	6.3

ID=STM14_2156;Name=pth;locus_tag=STM14_2156	6.3
ID=STM14_4622;Name=dgoK;locus_tag=STM14_4622	6.3
ID=STM14_1551;Name=STM14_1551	6.3
ID=STM14_0453;Name=STM14_0453	6.2
ID=STM14_2818;Name=glpT;locus_tag=STM14_2818	6.1
ID=STM14_1540;Name=STM14_1540	6.1
ID=STM14_4233;Name=STM14_4233	6.0
ID=STM14_2785;Name=napB;locus_tag=STM14_2785	5.9
ID=STM14_5462;Name=STM14_5462	5.9
ID=STM14_1072;Name=STM14_1072	5.9
ID=STM14_2534;Name=pduJ;locus_tag=STM14_2534	5.8
ID=STM14_0878;Name=lysZ;locus_tag=STM14_0878	5.8
ID=STM14_3858;Name=nudF;locus_tag=STM14_3858	5.8
ID=STM14_2726;Name=STM14_2726	5.6
ID=STM14_4567;Name=STM14_4567	5.5
ID=STM14_3783;Name=STM14_3783	5.2
ID=STM14_1541;Name=STM14_1541	5.1
ID=STM14_1510;Name=STM14_1510	5.0
ID=STM14_1071;Name=serW;locus_tag=STM14_1071	5.0
ID=STM14_5347;Name=STM14_5347	4.7
ID=STM14_3273;Name=STM14_3273	4.7
ID=STM14_2924;Name=STM14_2924	4.7
ID=STM14_2115;Name=STM14_2115	4.7
ID=STM14_3272;Name=STM14_3272	4.6
ID=STM14_2822;Name=STM14_2822	4.6
ID=STM14_2015;Name=STM14_2015	4.6
ID=STM14_1328;Name=yceO;locus_tag=STM14_1328	4.5
ID=STM14_1969;Name=STM14_1969	4.3
ID=STM14_1996;Name=STM14_1996	4.2
ID=STM14_0430;Name=STM14_0430	4.2
ID=STM14_0350;Name=STM14_0350	4.1

Table 4.

Genes that are down-regulated when SoxS expression is induced from pBAD30 by adding 0.2% arabinose to the culture. Only the genes whose fold change compared the control strain (pBAD30) are significant are shown here (P<0.05).

Genes	Fold change
ID=STM14_0135;Name=leuL;locus_tag=STM14_0135	3.7
ID=STM14_1613;Name=STM14_1613	3.7
ID=STM14_5190;Name=STM14_5190	3.7
ID=STM14_1261;Name=STM14_1261	3.7
ID=STM14_5447;Name=STM14_5447	4.0
ID=STM14_2388;Name=fliE;locus_tag=STM14_2388	4.0
ID=STM14_2091;Name=yciG;locus_tag=STM14_2091	4.0
ID=STM14_1356;Name=STM14_1356	4.1
ID=STM14_1497;Name=pagD;locus_tag=STM14_1497	4.3
ID=STM14_2477;Name=STM14_2477	4.3
ID=STM14_5134;Name=STM14_5134	4.3
ID=STM14_0399;Name=STM14_0399	4.4
ID=STM14_2460;Name=STM14_2460	4.4
ID=STM14_0723;Name=citE;locus_tag=STM14_0723	4.5
ID=STM14_2680;Name=STM14_2680	4.5
ID=STM14_0428;Name=yahO;locus_tag=STM14_0428	4.5
ID=STM14_2451;Name=STM14_2451	4.8
ID=STM14_1508;Name=STM14_1508	4.8
ID=STM14_1312;Name=ymdA;locus_tag=STM14_1312	5.2
ID=STM14_2428;Name=STM14_2428	5.2
ID=STM14_2449;Name=STM14_2449	5.4
ID=STM14_1346;Name=flgC;locus_tag=STM14_1346	5.4
ID=STM14_1345;Name=flgB;locus_tag=STM14_1345	5.6
ID=STM14_1198;Name=STM14_1198	5.6
ID=STM14_2402;Name=fliR;locus_tag=STM14_2402	5.7
ID=STM14_2458;Name=STM14_2458	5.7
ID=STM14_2429;Name=STM14_2429	5.8
ID=STM14_0641;Name=fimZ;locus_tag=STM14_0641	5.9
ID=STM14_2061;Name=STM14_2061	6.0
ID=STM14_2443;Name=STM14_2443	6.0
ID=STM14_2174;Name=ycgR;locus_tag=STM14_2174	6.0
ID=STM14_5119;Name=STM14_5119	6.0
ID=STM14_2391;Name=fliG;locus_tag=STM14_2391	6.2
ID=STM14_3892;Name=yqjI;locus_tag=STM14_3892	6.2

ID=STM14_0721;Name=citX;locus_tag=STM14_0721	6.2
ID=STM14_2062;Name=osmB;locus_tag=STM14_2062	6.2
ID=STM14_1579;Name=STM14_1579	6.2
ID=STM14_1507;Name=STM14_1507	6.2
ID=STM14_2689;Name=yohK;locus_tag=STM14_2689	6.3
ID=STM14_3734;Name=STM14_3734	6.3
ID=STM14_3127;Name=asrB;locus_tag=STM14_3127	6.3
ID=STM14_2442;Name=STM14_2442	6.4
ID=STM14_2688;Name=yohJ;locus_tag=STM14_2688	6.4
ID=STM14_0724;Name=citD;locus_tag=STM14_0724	6.5
ID=STM14_5518;Name=STM14_5518	6.5
ID=STM14_2169;Name=STM14_2169	6.5
ID=STM14_2400;Name=fliP;locus_tag=STM14_2400	6.7
ID=STM14_2397;Name=fliM;locus_tag=STM14_2397	6.7
ID=STM14_1347;Name=flgD;locus_tag=STM14_1347	6.7
ID=STM14_1351;Name=flgH;locus_tag=STM14_1351	6.8
ID=STM14_1352;Name=flgI;locus_tag=STM14_1352	7.0
ID=STM14_1353;Name=flgJ;locus_tag=STM14_1353	7.1
ID=STM14_2834;Name=STM14_2834	7.1
ID=STM14_4532;Name=slsA;locus_tag=STM14_4532	7.1
ID=STM14_5189;Name=STM14_5189	7.1
ID=STM14_1503;Name=STM14_1503	7.2
ID=STM14_1504.gene;Alias=STM14_1504;Name=STM14_1504	7.2
ID=STM14_2167;Name=STM14_2167	7.4
ID=STM14_2444;Name=STM14_2444	7.5
ID=STM14_3354;Name=mig-14;locus_tag=STM14_3354	7.6
ID=STM14_3353;Name=STM14_3353	7.6
ID=STM14_3340;Name=STM14_3340	7.7
ID=STM14_2395;Name=fliK;locus_tag=STM14_2395	7.8
ID=STM14_1349;Name=flgF;locus_tag=STM14_1349	8.0
ID=STM14_1884;Name=STM14_1884	8.1
ID=STM14_3820;Name=STM14_3820	8.2
ID=STM14_1578;Name=STM14_1578	8.2
ID=STM14_2399;Name=fliO;locus_tag=STM14_2399	8.2
ID=STM14_1348;Name=flgE;locus_tag=STM14_1348	8.3
ID=STM14_2574;Name=udg;locus_tag=STM14_2574	8.4
ID=STM14_2448;Name=STM14_2448	8.4
ID=STM14_3126;Name=asrA;locus_tag=STM14_3126	8.4
ID=STM14_4769;Name=metE;locus_tag=STM14_4769	8.6
ID=STM14_4674;Name=asnA;locus_tag=STM14_4674	8.6
ID=STM14_3821;Name=STM14_3821	8.6
ID=STM14_4531;Name=STM14_4531	8.8
ID=STM14_2835;Name=yfbE;locus_tag=STM14_2835	8.9
ID=STM14_1350;Name=flgG;locus_tag=STM14_1350	9.0
ID=STM14_2394;Name=fliJ;locus_tag=STM14_2394	9.5

ID=STM14_965;Name=STM14_965	9.7
ID=STM14_3822;Name=STM14_3822	9.9
ID=STM14_2338;Name=motA;locus_tag=STM14_2338	10.9
ID=STM14_0549;Name=ybaY;locus_tag=STM14_0549	10.9
ID=STM14_2392;Name=fliH;locus_tag=STM14_2392	11.0
ID=STM14_2798;Name=STM14_2798	11.1
ID=STM14_2393;Name=fliI;locus_tag=STM14_2393	11.2
ID=STM14_0636;Name=fimI;locus_tag=STM14_0636	11.2
ID=STM14_1282;Name=STM14_1282	11.4
ID=STM14_3479;Name=STM14_3479	11.4
ID=STM14_0635;Name=fimA;locus_tag=STM14_0635	11.4
ID=STM14_1283;Name=STM14_1283	11.4
ID=STM14_1612;Name=STM14_1612	11.7
ID=STM14_3352;Name=virK;locus_tag=STM14_3352	12.0
ID=STM14_5446;Name=tsr;locus_tag=STM14_5446	12.0
ID=STM14_3475;Name=hilA;locus_tag=STM14_3475	12.4
ID=STM14_5188;Name=STM14_5188	12.7
ID=STM14_2244;Name=sopE2;locus_tag=STM14_2244	12.7
ID=STM14_3478;Name=sicP;locus_tag=STM14_3478	13.0
ID=STM14_2332;Name=cheB;locus_tag=STM14_2332	13.2
ID=STM14_0702;Name=ybdL;locus_tag=STM14_0702	13.5
ID=STM14_3343;Name=STM14_3343	13.5
ID=STM14_2331;Name=cheY;locus_tag=STM14_2331	13.6
ID=STM14_2387;Name=STM14_2387	13.8
ID=STM14_2398;Name=fliN;locus_tag=STM14_2398	13.9
ID=STM14_3342;Name=STM14_3342	14.0
ID=STM14_1341;Name=flgN;locus_tag=STM14_1341	14.0
ID=STM14_3466;Name=STM14_3466	14.2
ID=STM14_2329;Name=STM14_2329	14.3
ID=STM14_3483;Name=sipC;locus_tag=STM14_3483	14.8
ID=STM14_2333;Name=cheR;locus_tag=STM14_2333	15.1
ID=STM14_2339;Name=STM14_2339	15.5
ID=STM14_3482;Name=sipD;locus_tag=STM14_3482	16.2
ID=STM14_1354;Name=flgK;locus_tag=STM14_1354	16.3
ID=STM14_2797;Name=ompC;locus_tag=STM14_2797	16.3
ID=STM14_5187;Name=STM14_5187	16.4
ID=STM14_0637;Name=fimC;locus_tag=STM14_0637	16.5
ID=STM14_3469;Name=orgA;locus_tag=STM14_3469	17.3
ID=STM14_5120;Name=STM14_5120	17.5
ID=STM14_2330;Name=cheZ;locus_tag=STM14_2330	17.7
ID=STM14_5118;Name=STM14_5118	17.9
ID=STM14_3481;Name=sipA;locus_tag=STM14_3481	18.4
ID=STM14_2852;Name=STM14_2852	18.8
ID=STM14_1891;Name=adhP;gene_synonym=adhA;locus_tag=STM14_1891	19.1

ID=STM14_1237;Name=sopB;locus_tag=STM14_1237	19.2
ID=STM14_3480;Name=iacP;locus_tag=STM14_3480	19.4
ID=STM14_4768;Name=STM14_4768	19.5
ID=STM14_1355;Name=flgL;locus_tag=STM14_1355	19.7
ID=STM14_5117;Name=STM14_5117	19.9
ID=STM14_1236;Name=pipC;locus_tag=STM14_1236	20.3
ID=STM14_2374;Name=fliA;locus_tag=STM14_2374	20.9
ID=STM14_2334;Name=cheM;locus_tag=STM14_2334	21.1
ID=STM14_4935;Name=metF;locus_tag=STM14_4935	21.5
ID=STM14_2168;Name=STM14_2168	21.6
ID=STM14_3341;Name=STM14_3341	23.0
ID=STM14_5185;Name=STM14_5185	23.6
ID=STM14_1235;Name=STM14_1235	24.0
ID=STM14_966;Name=dps;locus_tag=STM14_966	24.1
ID=STM14_3467;Name=orgC;locus_tag=STM14_3467	24.2
ID=STM14_3486;Name=spaS;locus_tag=STM14_3486	28.1
ID=STM14_2166;Name=STM14_2166	28.6
ID=STM14_3468;Name=orgB;locus_tag=STM14_3468	28.7
ID=STM14_3474;Name=hilD;locus_tag=STM14_3474	30.3
ID=STM14_2336;Name=cheA;locus_tag=STM14_2336	30.6
ID=STM14_4305;Name=tcp;locus_tag=STM14_4305	31.3
ID=STM14_3462;Name=avrA;locus_tag=STM14_3462	31.6
ID=STM14_2227;Name=STM14_2227	32.6
ID=STM14_4934;Name=STM14_4934	32.9
ID=STM14_1342;Name=flgM;locus_tag=STM14_1342	33.1
ID=STM14_3476;Name=iagB;locus_tag=STM14_3476	34.5
ID=STM14_3487;Name=spaR;locus_tag=STM14_3487	35.2
ID=STM14_5166;Name=yjdE;locus_tag=STM14_5166	37.4
ID=STM14_2342;Name=STM14_2342	37.7
ID=STM14_2162;Name=STM14_2162	38.3
ID=STM14_2335;Name=cheW;locus_tag=STM14_2335	39.5
ID=STM14_2337;Name=motB;locus_tag=STM14_2337	41.4
ID=STM14_5186;Name=STM14_5186	43.2
ID=STM14_3485;Name=sicA;locus_tag=STM14_3485	44.4
ID=STM14_3494;Name=invB;locus_tag=STM14_3494	45.5
ID=STM14_2161;Name=STM14_2161	45.6
ID=STM14_1887;Name=yddX;locus_tag=STM14_1887	45.7
ID=STM14_3490;Name=spaO;locus_tag=STM14_3490	49.8
ID=STM14_3799;Name=STM14_3799	49.9
ID=STM14_3488;Name=spaQ;locus_tag=STM14_3488	53.6
ID=STM14_3489;Name=spaP;locus_tag=STM14_3489	54.5
ID=STM14_3484;Name=sipB;locus_tag=STM14_3484	55.6
ID=STM14_2164;Name=STM14_2164	56.1
ID=STM14_3493;Name=invC;locus_tag=STM14_3493	57.3
ID=STM14_2160;Name=STM14_2160	58.1

ID=STM14_3499;Name=invH;locus_tag=STM14_3499	58.1
ID=STM14_3491;Name=invJ;locus_tag=STM14_3491	62.1
ID=STM14_4346;Name=yhjH;locus_tag=STM14_4346	62.6
ID=STM14_3893;Name=STM14_3893	62.8
ID=STM14_3492;Name=invI;locus_tag=STM14_3492	63.9
ID=STM14_2379;Name=STM14_2379	67.1
ID=STM14_3495;Name=invA;locus_tag=STM14_3495	68.8
ID=STM14_3473;Name=prgH;locus_tag=STM14_3473	69.4
ID=STM14_3470;Name=prgK;locus_tag=STM14_3470	70.4
ID=STM14_2378;Name=fliC;locus_tag=STM14_2378	72.8
ID=STM14_2165;Name=STM14_2165	80.8
ID=STM14_3496;Name=invE;locus_tag=STM14_3496	84.2
ID=STM14_3497;Name=invG;locus_tag=STM14_3497	86.5
ID=STM14_5169;Name=adi;locus_tag=STM14_5169	91.3
ID=STM14_5168;Name=STM14_5168	101.2
ID=STM14_3472;Name=prgI;locus_tag=STM14_3472	124.2
ID=STM14_3471;Name=prgJ;locus_tag=STM14_3471	126.9
ID=STM14_3463;Name=sprB;locus_tag=STM14_3463	144.5
ID=STM14_3464;Name=STM14_3464	150.1
ID=STM14_3465;Name=hilC;locus_tag=STM14_3465	184.4
ID=STM14_3498;Name=invF;locus_tag=STM14_3498	207.1
ID=STM14_1885;Name=hdeB;locus_tag=STM14_1885	731.4
ID=STM14_0399;Name=STM14_0399	4.4
ID=STM14_2460;Name=STM14_2460	4.4
ID=STM14_0723;Name=citE;locus_tag=STM14_0723	4.5
ID=STM14_2680;Name=STM14_2680	4.5
ID=STM14_0428;Name=yahO;locus_tag=STM14_0428	4.5
ID=STM14_2451;Name=STM14_2451	4.8
ID=STM14_1508;Name=STM14_1508	4.8
ID=STM14_1312;Name=ymdA;locus_tag=STM14_1312	5.2
ID=STM14_2428;Name=STM14_2428	5.2
ID=STM14_2449;Name=STM14_2449	5.4
ID=STM14_1346;Name=flgC;locus_tag=STM14_1346	5.4
ID=STM14_1345;Name=flgB;locus_tag=STM14_1345	5.6
ID=STM14_1198;Name=STM14_1198	5.6
ID=STM14_2402;Name=fliR;locus_tag=STM14_2402	5.7
ID=STM14_2458;Name=STM14_2458	5.7
ID=STM14_2429;Name=STM14_2429	5.8
ID=STM14_0641;Name=fimZ;locus_tag=STM14_0641	5.9
ID=STM14_2061;Name=STM14_2061	6.0
ID=STM14_2443;Name=STM14_2443	6.0
ID=STM14_2174;Name=ycgR;locus_tag=STM14_2174	6.0
ID=STM14_5119;Name=STM14_5119	6.0
ID=STM14_2391;Name=fliG;locus_tag=STM14_2391	6.2
ID=STM14_3892;Name=yqjI;locus_tag=STM14_3892	6.2

ID=STM14_0721;Name=citX;locus_tag=STM14_0721	6.2
ID=STM14_2062;Name=osmB;locus_tag=STM14_2062	6.2
ID=STM14_1579;Name=STM14_1579	6.2
ID=STM14_1507;Name=STM14_1507	6.2
ID=STM14_2689;Name=yohK;locus_tag=STM14_2689	6.3
ID=STM14_3734;Name=STM14_3734	6.3
ID=STM14_3127;Name=asrB;locus_tag=STM14_3127	6.3
ID=STM14_2442;Name=STM14_2442	6.4
ID=STM14_2688;Name=yohJ;locus_tag=STM14_2688	6.4
ID=STM14_0724;Name=citD;locus_tag=STM14_0724	6.5
ID=STM14_5518;Name=STM14_5518	6.5
ID=STM14_2169;Name=STM14_2169	6.5
ID=STM14_2400;Name=fliP;locus_tag=STM14_2400	6.7
ID=STM14_2397;Name=fliM;locus_tag=STM14_2397	6.7
ID=STM14_1347;Name=flgD;locus_tag=STM14_1347	6.7
ID=STM14_1351;Name=flgH;locus_tag=STM14_1351	6.8
ID=STM14_1352;Name=flgI;locus_tag=STM14_1352	7.0
ID=STM14_1353;Name=flgJ;locus_tag=STM14_1353	7.1
ID=STM14_2834;Name=STM14_2834	7.1
ID=STM14_4532;Name=slsA;locus_tag=STM14_4532	7.1
ID=STM14_5189;Name=STM14_5189	7.1
ID=STM14_1503;Name=STM14_1503	7.2
ID=STM14_1504.gene;Alias=STM14_1504;Name=STM14_1504	7.2
ID=STM14_2167;Name=STM14_2167	7.4
ID=STM14_2444;Name=STM14_2444	7.5
ID=STM14_3354;Name=mig-14;locus_tag=STM14_3354	7.6
ID=STM14_3353;Name=STM14_3353	7.6
ID=STM14_3340;Name=STM14_3340	7.7
ID=STM14_2395;Name=fliK;locus_tag=STM14_2395	7.8
ID=STM14_1349;Name=flgF;locus_tag=STM14_1349	8.0
ID=STM14_1884;Name=STM14_1884	8.1
ID=STM14_3820;Name=STM14_3820	8.2
ID=STM14_1578;Name=STM14_1578	8.2
ID=STM14_2399;Name=fliO;locus_tag=STM14_2399	8.2
ID=STM14_1348;Name=flgE;locus_tag=STM14_1348	8.3
ID=STM14_2574;Name=udg;locus_tag=STM14_2574	8.4
ID=STM14_2448;Name=STM14_2448	8.4
ID=STM14_3126;Name=asrA;locus_tag=STM14_3126	8.4
ID=STM14_4769;Name=metE;locus_tag=STM14_4769	8.6
ID=STM14_4674;Name=asnA;locus_tag=STM14_4674	8.6
ID=STM14_3821;Name=STM14_3821	8.6
ID=STM14_4531;Name=STM14_4531	8.8
ID=STM14_2835;Name=yfbE;locus_tag=STM14_2835	8.9
ID=STM14_1350;Name=flgG;locus_tag=STM14_1350	9.0
ID=STM14_2394;Name=fliJ;locus_tag=STM14_2394	9.5

ID=STM14_965;Name=STM14_965	9.7
ID=STM14_3822;Name=STM14_3822	9.9
ID=STM14_2338;Name=motA;locus_tag=STM14_2338	10.9
ID=STM14_0549;Name=ybaY;locus_tag=STM14_0549	10.9
ID=STM14_2392;Name=fliH;locus_tag=STM14_2392	11.0
ID=STM14_2798;Name=STM14_2798	11.1
ID=STM14_2393;Name=fliI;locus_tag=STM14_2393	11.2
ID=STM14_0636;Name=fimI;locus_tag=STM14_0636	11.2
ID=STM14_1282;Name=STM14_1282	11.4
ID=STM14_3479;Name=STM14_3479	11.4
ID=STM14_0635;Name=fimA;locus_tag=STM14_0635	11.4
ID=STM14_1283;Name=STM14_1283	11.4
ID=STM14_1612;Name=STM14_1612	11.7
ID=STM14_3352;Name=virK;locus_tag=STM14_3352	12.0
ID=STM14_5446;Name=tsr;locus_tag=STM14_5446	12.0
ID=STM14_3475;Name=hilA;locus_tag=STM14_3475	12.4
ID=STM14_5188;Name=STM14_5188	12.7
ID=STM14_2244;Name=sopE2;locus_tag=STM14_2244	12.7
ID=STM14_3478;Name=sicP;locus_tag=STM14_3478	13.0
ID=STM14_2332;Name=cheB;locus_tag=STM14_2332	13.2
ID=STM14_0702;Name=ybdL;locus_tag=STM14_0702	13.5
ID=STM14_3343;Name=STM14_3343	13.5
ID=STM14_2331;Name=cheY;locus_tag=STM14_2331	13.6
ID=STM14_2387;Name=STM14_2387	13.8
ID=STM14_2398;Name=fliN;locus_tag=STM14_2398	13.9
ID=STM14_3342;Name=STM14_3342	14.0
ID=STM14_1341;Name=flgN;locus_tag=STM14_1341	14.0
ID=STM14_3466;Name=STM14_3466	14.2
ID=STM14_2329;Name=STM14_2329	14.3
ID=STM14_3483;Name=sipC;locus_tag=STM14_3483	14.8
ID=STM14_2333;Name=cheR;locus_tag=STM14_2333	15.1
ID=STM14_2339;Name=STM14_2339	15.5
ID=STM14_3482;Name=sipD;locus_tag=STM14_3482	16.2
ID=STM14_1354;Name=flgK;locus_tag=STM14_1354	16.3
ID=STM14_2797;Name=ompC;locus_tag=STM14_2797	16.3
ID=STM14_5187;Name=STM14_5187	16.4
ID=STM14_0637;Name=fimC;locus_tag=STM14_0637	16.5
ID=STM14_3469;Name=orgA;locus_tag=STM14_3469	17.3
ID=STM14_5120;Name=STM14_5120	17.5
ID=STM14_2330;Name=cheZ;locus_tag=STM14_2330	17.7
ID=STM14_5118;Name=STM14_5118	17.9
ID=STM14_3481;Name=sipA;locus_tag=STM14_3481	18.4
ID=STM14_2852;Name=STM14_2852	18.8
ID=STM14_1891;Name=adhP;gene_synonym=adhA;locus_tag=STM14_1891	19.1

ID=STM14_1237;Name=sopB;locus_tag=STM14_1237	19.2
ID=STM14_3480;Name=iacP;locus_tag=STM14_3480	19.4
ID=STM14_4768;Name=STM14_4768	19.5
ID=STM14_1355;Name=flgL;locus_tag=STM14_1355	19.7
ID=STM14_5117;Name=STM14_5117	19.9
ID=STM14_1236;Name=pipC;locus_tag=STM14_1236	20.3
ID=STM14_2374;Name=fliA;locus_tag=STM14_2374	20.9
ID=STM14_2334;Name=cheM;locus_tag=STM14_2334	21.1
ID=STM14_4935;Name=metF;locus_tag=STM14_4935	21.5
ID=STM14_2168;Name=STM14_2168	21.6
ID=STM14_3341;Name=STM14_3341	23.0
ID=STM14_5185;Name=STM14_5185	23.6
ID=STM14_1235;Name=STM14_1235	24.0
ID=STM14_966;Name=dps;locus_tag=STM14_966	24.1
ID=STM14_3467;Name=orgC;locus_tag=STM14_3467	24.2
ID=STM14_3486;Name=spaS;locus_tag=STM14_3486	28.1
ID=STM14_2166;Name=STM14_2166	28.6
ID=STM14_3468;Name=orgB;locus_tag=STM14_3468	28.7
ID=STM14_3474;Name=hilD;locus_tag=STM14_3474	30.3
ID=STM14_2336;Name=cheA;locus_tag=STM14_2336	30.6
ID=STM14_4305;Name=tcp;locus_tag=STM14_4305	31.3
ID=STM14_3462;Name=avrA;locus_tag=STM14_3462	31.6
ID=STM14_2227;Name=STM14_2227	32.6
ID=STM14_4934;Name=STM14_4934	32.9
ID=STM14_1342;Name=flgM;locus_tag=STM14_1342	33.1
ID=STM14_3476;Name=iagB;locus_tag=STM14_3476	34.5
ID=STM14_3487;Name=spaR;locus_tag=STM14_3487	35.2
ID=STM14_5166;Name=yjdE;locus_tag=STM14_5166	37.4
ID=STM14_2342;Name=STM14_2342	37.7
ID=STM14_2162;Name=STM14_2162	38.3
ID=STM14_2335;Name=cheW;locus_tag=STM14_2335	39.5
ID=STM14_2337;Name=motB;locus_tag=STM14_2337	41.4
ID=STM14_5186;Name=STM14_5186	43.2
ID=STM14_3485;Name=sicA;locus_tag=STM14_3485	44.4
ID=STM14_3494;Name=invB;locus_tag=STM14_3494	45.5
ID=STM14_2161;Name=STM14_2161	45.6
ID=STM14_1887;Name=yddX;locus_tag=STM14_1887	45.7
ID=STM14_3490;Name=spaO;locus_tag=STM14_3490	49.8
ID=STM14_3799;Name=STM14_3799	49.9
ID=STM14_3488;Name=spaQ;locus_tag=STM14_3488	53.6
ID=STM14_3489;Name=spaP;locus_tag=STM14_3489	54.5
ID=STM14_3484;Name=sipB;locus_tag=STM14_3484	55.6
ID=STM14_2164;Name=STM14_2164	56.1
ID=STM14_3493;Name=invC;locus_tag=STM14_3493	57.3
ID=STM14_2160;Name=STM14_2160	58.1

ID=STM14_3499;Name=invH;locus_tag=STM14_3499	58.1
ID=STM14_3491;Name=invJ;locus_tag=STM14_3491	62.1
ID=STM14_4346;Name=yhjH;locus_tag=STM14_4346	62.6
ID=STM14_3893;Name=STM14_3893	62.8
ID=STM14_3492;Name=invI;locus_tag=STM14_3492	63.9
ID=STM14_2379;Name=STM14_2379	67.1
ID=STM14_3495;Name=invA;locus_tag=STM14_3495	68.8
ID=STM14_3473;Name=prgH;locus_tag=STM14_3473	69.4
ID=STM14_3470;Name=prgK;locus_tag=STM14_3470	70.4
ID=STM14_2378;Name=fliC;locus_tag=STM14_2378	72.8
ID=STM14_2165;Name=STM14_2165	80.8
ID=STM14_3496;Name=invE;locus_tag=STM14_3496	84.2
ID=STM14_3497;Name=invG;locus_tag=STM14_3497	86.5
ID=STM14_5169;Name=adi;locus_tag=STM14_5169	91.3
ID=STM14_5168;Name=STM14_5168	101.2
ID=STM14_3472;Name=prgI;locus_tag=STM14_3472	124.2
ID=STM14_3471;Name=prgJ;locus_tag=STM14_3471	126.9
ID=STM14_3463;Name=sprB;locus_tag=STM14_3463	144.5
ID=STM14_3464;Name=STM14_3464	150.1
ID=STM14_3465;Name=hilC;locus_tag=STM14_3465	184.4
ID=STM14_3498;Name=invF;locus_tag=STM14_3498	207.1
ID=STM14_1885;Name=hdeB;locus_tag=STM14_1885	731.4

Table 5.

Genes that are up-regulated when Rob expression is induced from pBAD30 by adding 0.2% arabinose to the culture. Only the genes whose fold change compared the control strain (pBAD30) are significant are shown here (P<0.05).

Genes	Fold change
ID=STM14_5507;Name=rob;locus_tag=STM14_5507	223.0
ID=STM14_1662;Name=STM14_1662	51.5
ID=STM14_4368;Name=STM14_4368	49.0
ID=STM14_0189.gene;Alias=STM14_0189;Name=yacH;locus_tag=STM14_0189	37.6
ID=STM14_3500;Name=STM14_3500	29.7
ID=STM14_4369;Name=yhjV;locus_tag=STM14_4369	27.1
ID=STM14_4446;Name=STM14_4446	27.0
ID=STM14_0910;Name=ybhA;locus_tag=STM14_0910	24.8
ID=STM14_2693;Name=STM14_2693	22.9
ID=STM14_4387;Name=lpfA;locus_tag=STM14_4387	22.2
ID=STM14_2020;Name=STM14_2020	20.9
ID=STM14_3501;Name=STM14_3501	19.2
ID=STM14_1628;Name=btuE;locus_tag=STM14_1628	17.6
ID=STM14_0373;Name=gpt;locus_tag=STM14_0373	17.1
ID=STM14_3502;Name=STM14_3502	17.0
ID=STM14_2694;Name=yeiA;locus_tag=STM14_2694	16.9
ID=STM14_0479;Name=STM14_0479	14.9
ID=STM14_1447;Name=STM14_1447	14.7
ID=STM14_2023;Name=STM14_2023	14.5
ID=STM14_938;Name=ybhM;locus_tag=STM14_938	14.2
ID=STM14_0188;Name=STM14_0188	13.9
ID=STM14_1025;Name=mdaA;locus_tag=STM14_1025	12.6
ID=STM14_1024;Name=ybjC;locus_tag=STM14_1024	12.0
ID=STM14_4445;Name=mtlR;locus_tag=STM14_4445	11.5
ID=STM14_0450;Name=STM14_0450	11.2
ID=STM14_0187;Name=STM14_0187	10.5
ID=STM14_3395;Name=STM14_3395	10.5
ID=STM14_3026;Name=eutQ;locus_tag=STM14_3026	9.9
ID=STM14_1963;Name=STM14_1963	9.7
ID=STM14_940;Name=STM14_940	9.5
ID=STM14_1537;Name=STM14_1537	9.4
ID=STM14_1479;Name=STM14_1479	9.0
ID=STM14_1629;Name=btuD;locus_tag=STM14_1629	9.0

ID=STM14_3903;Name=ygjR;locus_tag=STM14_3903	8.6
ID=STM14_1016;Name=mdfA;locus_tag=STM14_1016	8.6
ID=STM14_2727;Name=setB;locus_tag=STM14_2727	8.4
ID=STM14_2886;Name=STM14_2886	8.3
ID=STM14_939;Name=STM14_939	8.1
ID=STM14_1536;Name=STM14_1536	7.8
ID=STM14_2887;Name=ulaA_2;gene_synonym=sgaT;locus_tag=STM14_2887	7.8
ID=STM14_1630;Name=nlpC;locus_tag=STM14_1630	7.8
ID=STM14_3440;Name=hycH;locus_tag=STM14_3440	7.5
ID=STM14_2888;Name=STM14_2888	7.4
ID=STM14_5291;Name=STM14_5291	6.8
ID=STM14_0820;Name=STM14_0820	6.7
ID=STM14_0819;Name=STM14_0819	6.6
ID=STM14_4370;Name=dppF;locus_tag=STM14_4370	6.6
ID=STM14_3441;Name=hycG;locus_tag=STM14_3441	6.4
ID=STM14_2022;Name=STM14_2022	6.1
ID=STM14_0143;Name=STM14_0143	5.7
ID=STM14_4497;Name=STM14_4497	5.5
ID=STM14_2024;Name=STM14_2024	5.3
ID=STM14_2726;Name=STM14_2726	4.8

Table 6.

Genes that are down-regulated when Rob expression is induced from pBAD30 by adding 0.2% arabinose to the culture. Only the genes whose fold change compared the control strain (pBAD30) are significant are shown here (P<0.05).

Genes	Fold change
ID=STM14_5183;Name=STM14_5183	3.8
ID=STM14_4339;Name=STM14_4339	4.0
ID=STM14_0621;Name=ylbF;locus_tag=STM14_0621	4.0
ID=STM14_4045;Name=oadG;locus_tag=STM14_4045	4.3
ID=STM14_0076;Name=STM14_0076	4.3
ID=STM14_1198;Name=STM14_1198	4.3
ID=STM14_1312;Name=ymdA;locus_tag=STM14_1312	4.4
ID=STM14_0428;Name=yahO;locus_tag=STM14_0428	4.4
ID=STM14_3773;Name=STM14_3773	4.5
ID=STM14_2388;Name=fliE;locus_tag=STM14_2388	4.5
ID=STM14_1261;Name=STM14_1261	4.5
ID=STM14_0641;Name=fimZ;locus_tag=STM14_0641	4.6
ID=STM14_3801;Name=STM14_3801	4.7
ID=STM14_5479;Name=STM14_5479	4.7
ID=STM14_0611;Name=ybbV;locus_tag=STM14_0611	4.7
ID=STM14_3774;Name=STM14_3774	4.7
ID=STM14_4531;Name=STM14_4531	4.8
ID=STM14_0619;Name=fdrA;locus_tag=STM14_0619	4.8
ID=STM14_5184;Name=STM14_5184	4.8
ID=STM14_0436;Name=STM14_0436	4.9
ID=STM14_4147;Name=bfd;locus_tag=STM14_4147	4.9
ID=STM14_1304;Name=csgF;locus_tag=STM14_1304	4.9
ID=STM14_0661;Name=STM14_0661	4.9
ID=STM14_5315;Name=STM14_5315	5.0
ID=STM14_4768;Name=STM14_4768	5.1
ID=STM14_0437;Name=STM14_0437	5.2
ID=STM14_3821;Name=STM14_3821	5.3
ID=STM14_0643;Name=STM14_0643	5.6
ID=STM14_1345;Name=flgB;locus_tag=STM14_1345	5.6
ID=STM14_1346;Name=flgC;locus_tag=STM14_1346	5.6
ID=STM14_1347;Name=flgD;locus_tag=STM14_1347	5.8
ID=STM14_1353;Name=flgJ;locus_tag=STM14_1353	6.1
ID=STM14_1352;Name=flgI;locus_tag=STM14_1352	6.2
ID=STM14_0639;Name=fimH;locus_tag=STM14_0639	6.3

ID=STM14_2399;Name=fliO;locus_tag=STM14_2399	6.3
ID=STM14_1351;Name=flgH;locus_tag=STM14_1351	6.4
ID=STM14_0665;Name=STM14_0665	6.4
ID=STM14_3028;Name=eutS;locus_tag=STM14_3028	6.5
ID=STM14_3340;Name=STM14_3340	6.6
ID=STM14_5189;Name=STM14_5189	6.6
ID=STM14_0633;Name=STM14_0633	6.7
ID=STM14_1613;Name=STM14_1613	6.7
ID=STM14_2374;Name=fliA;locus_tag=STM14_2374	7.1
ID=STM14_4769;Name=metE;locus_tag=STM14_4769	7.1
ID=STM14_2342;Name=STM14_2342	7.1
ID=STM14_1349;Name=flgF;locus_tag=STM14_1349	7.2
ID=STM14_4934;Name=STM14_4934	7.2
ID=STM14_0634;Name=STM14_0634	7.2
ID=STM14_2398;Name=fliN;locus_tag=STM14_2398	7.4
ID=STM14_1283;Name=STM14_1283	7.5
ID=STM14_3341;Name=STM14_3341	7.6
ID=STM14_1348;Name=flgE;locus_tag=STM14_1348	7.7
ID=STM14_2392;Name=fliH;locus_tag=STM14_2392	7.8
ID=STM14_1282;Name=STM14_1282	7.8
ID=STM14_1350;Name=flgG;locus_tag=STM14_1350	7.9
ID=STM14_3342;Name=STM14_3342	8.2
ID=STM14_3343;Name=STM14_3343	8.2
ID=STM14_3466;Name=STM14_3466	8.3
ID=STM14_2394;Name=fliJ;locus_tag=STM14_2394	8.3
ID=STM14_2243;Name=STM14_2243	8.5
ID=STM14_1891;Name=adhP;gene_synonym=adhA	8.6
ID=STM14_2393;Name=fliI;locus_tag=STM14_2393	8.6
ID=STM14_2227;Name=STM14_2227	9.1
ID=STM14_3820;Name=STM14_3820	9.1
ID=STM14_4305;Name=tcp;locus_tag=STM14_4305	9.6
ID=STM14_2387;Name=STM14_2387	9.7
ID=STM14_3477;Name=sptP;locus_tag=STM14_3477	10.0
ID=STM14_0638;Name=fimD;locus_tag=STM14_0638	10.1
ID=STM14_3468;Name=orgB;locus_tag=STM14_3468	10.2
ID=STM14_2168;Name=STM14_2168	10.4
ID=STM14_5188;Name=STM14_5188	10.5
ID=STM14_1884;Name=STM14_1884	10.5
ID=STM14_2167;Name=STM14_2167	10.6
ID=STM14_3465;Name=hilC;locus_tag=STM14_3465	10.7
ID=STM14_1887;Name=yddX;locus_tag=STM14_1887	10.7
ID=STM14_3467;Name=orgC;locus_tag=STM14_3467	11.2
ID=STM14_3462;Name=avrA;locus_tag=STM14_3462	11.3
ID=STM14_5120;Name=STM14_5120	11.9
ID=STM14_5185;Name=STM14_5185	12.1

ID=STM14_3469;Name=orgA;locus_tag=STM14_3469	12.3
ID=STM14_5166;Name=yjdE;locus_tag=STM14_5166	12.6
ID=STM14_3464;Name=STM14_3464	12.6
ID=STM14_1612;Name=STM14_1612	12.7
ID=STM14_5187;Name=STM14_5187	13.1
ID=STM14_2162;Name=STM14_2162	13.2
ID=STM14_3479;Name=STM14_3479	13.4
ID=STM14_3463;Name=sprB;locus_tag=STM14_3463	13.5
ID=STM14_5117;Name=STM14_5117	13.6
ID=STM14_2166;Name=STM14_2166	14.4
ID=STM14_5118;Name=STM14_5118	15.3
ID=STM14_5186;Name=STM14_5186	15.8
ID=STM14_3478;Name=sicP;locus_tag=STM14_3478	16.3
ID=STM14_1235;Name=STM14_1235	16.6
ID=STM14_2161;Name=STM14_2161	17.4
ID=STM14_1236;Name=pipC;locus_tag=STM14_1236	17.5
ID=STM14_1237;Name=sopB;locus_tag=STM14_1237	17.6
ID=STM14_3475;Name=hilA;locus_tag=STM14_3475	17.6
ID=STM14_3486;Name=spaS;locus_tag=STM14_3486	17.8
ID=STM14_2165;Name=STM14_2165	18.0
ID=STM14_2164;Name=STM14_2164	18.8
ID=STM14_3494;Name=invB;locus_tag=STM14_3494	18.8
ID=STM14_0636;Name=fimI;locus_tag=STM14_0636	19.4
ID=STM14_3474;Name=hilD;locus_tag=STM14_3474	19.7
ID=STM14_3487;Name=spaR;locus_tag=STM14_3487	19.7
ID=STM14_3499;Name=invH;locus_tag=STM14_3499	20.7
ID=STM14_3480;Name=iacP;locus_tag=STM14_3480	21.1
ID=STM14_3495;Name=invA;locus_tag=STM14_3495	21.3
ID=STM14_3493;Name=invC;locus_tag=STM14_3493	21.5
ID=STM14_3490;Name=spaO;locus_tag=STM14_3490	21.8
ID=STM14_3476;Name=iagB;locus_tag=STM14_3476	21.8
ID=STM14_3799;Name=STM14_3799	22.1
ID=STM14_3491;Name=invJ;locus_tag=STM14_3491	23.6
ID=STM14_3482;Name=sipD;locus_tag=STM14_3482	23.6
ID=STM14_3489;Name=spaP;locus_tag=STM14_3489	23.9
ID=STM14_2160;Name=STM14_2160	24.5
ID=STM14_3893;Name=STM14_3893	26.0
ID=STM14_3492;Name=invI;locus_tag=STM14_3492	26.2
ID=STM14_3496;Name=invE;locus_tag=STM14_3496	26.3
ID=STM14_3488;Name=spaQ;locus_tag=STM14_3488	26.5
ID=STM14_3481;Name=sipA;locus_tag=STM14_3481	26.5
ID=STM14_3470;Name=prgK;locus_tag=STM14_3470	26.6
ID=STM14_3497;Name=invG;locus_tag=STM14_3497	27.4
ID=STM14_3485;Name=sicA;locus_tag=STM14_3485	27.6
ID=STM14_3473;Name=prgH;locus_tag=STM14_3473	28.8

ID=STM14_2244;Name=sopE2;locus_tag=STM14_2244	29.8
ID=STM14_3498;Name=invF;locus_tag=STM14_3498	30.1
ID=STM14_5169;Name=adi;locus_tag=STM14_5169	31.5
ID=STM14_3484;Name=sipB;locus_tag=STM14_3484	31.6
ID=STM14_0637;Name=fimC;locus_tag=STM14_0637	31.9
ID=STM14_3483;Name=sipC;locus_tag=STM14_3483	32.5
ID=STM14_3471;Name=prgJ;locus_tag=STM14_3471	33.1
ID=STM14_3472;Name=prgI;locus_tag=STM14_3472	36.2
ID=STM14_5168;Name=STM14_5168	42.5
ID=STM14_0635;Name=fimA;locus_tag=STM14_0635	57.6

Table 7.

Genes that are up-regulated when RamA expression is induced from pBAD30 by adding 0.2% arabinose to the culture. Only the genes whose fold change compared the control strain (pBAD30) are significant are shown here (P<0.05).

Genes	Fold change
ID=STM14_4878;Name=sodA;locus_tag=STM14_4878	280.8
ID=STM14_0678;Name=STM14_0678	219.2
ID=STM14_1024;Name=ybjC;locus_tag=STM14_1024	78.4
ID=STM14_0189.gene;Alias=STM14_0189;Name=yacH	75.9
ID=STM14_1025;Name=mdaA;locus_tag=STM14_1025	65.8
ID=STM14_0034;Name=bcfG;locus_tag=STM14_0034	61.2
ID=STM14_3958;Name=yhbW;locus_tag=STM14_3958	57.6
ID=STM14_3853;Name=ygiN;locus_tag=STM14_3853	57.2
ID=STM14_2819;Name=glpA;locus_tag=STM14_2819	56.6
ID=STM14_2023;Name=STM14_2023	49.9
ID=STM14_1995;Name=nifJ;locus_tag=STM14_1995	47.5
ID=STM14_1185;Name=STM14_1185	44.5
ID=STM14_3795;Name=STM14_3795	39.2
ID=STM14_0361;Name=STM14_0361	37.7
ID=STM14_2720;Name=nfo;locus_tag=STM14_2720	36.7
ID=STM14_2820;Name=glpB;locus_tag=STM14_2820	36.6
ID=STM14_3764;Name=yqgA;locus_tag=STM14_3764	36.1
ID=STM14_1628;Name=btuE;locus_tag=STM14_1628	35.4
ID=STM14_0188;Name=STM14_0188	35.1
ID=STM14_0674;Name=nfnB;locus_tag=STM14_0674	34.9
ID=STM14_2137;Name=narL;locus_tag=STM14_2137	34.1
ID=STM14_4368;Name=STM14_4368	33.6
ID=STM14_1537;Name=STM14_1537	32.9
ID=STM14_0143;Name=STM14_0143	31.8
ID=STM14_2790;Name=napF;locus_tag=STM14_2790	31.5
ID=STM14_3214;Name=STM14_3214	31.4
ID=STM14_3792;Name=STM14_3792	31.3
ID=STM14_1536;Name=STM14_1536	30.3
ID=STM14_5348;Name=treR;locus_tag=STM14_5348	29.9
ID=STM14_3098;Name=ndk;locus_tag=STM14_3098	29.8
ID=STM14_1361;Name=STM14_1361	28.6
ID=STM14_0204;Name=yadG;locus_tag=STM14_0204	28.0
ID=STM14_3796;Name=STM14_3796	27.0
ID=STM14_1949;Name=rimL;locus_tag=STM14_1949	26.9

ID=STM14_4369;Name=yhjV;locus_tag=STM14_4369	26.5
ID=STM14_3804;Name=yghU;locus_tag=STM14_3804	26.3
ID=STM14_938;Name=ybhM;locus_tag=STM14_938	25.6
ID=STM14_0187;Name=STM14_0187	24.9
ID=STM14_3794;Name=STM14_3794	24.8
ID=STM14_2024;Name=STM14_2024	24.7
ID=STM14_1366;Name=fabH;locus_tag=STM14_1366	24.6
ID=STM14_1819;Name=rspB;locus_tag=STM14_1819	24.1
ID=STM14_4912;Name=glpK;locus_tag=STM14_4912	24.0
ID=STM14_3852;Name=mdaB;locus_tag=STM14_3852	23.4
ID=STM14_0579;Name=ybaL;locus_tag=STM14_0579	22.5
ID=STM14_0205;Name=yadH;locus_tag=STM14_0205	22.4
ID=STM14_1945;Name=STM14_1945	22.4
ID=STM14_3500;Name=STM14_3500	22.3
ID=STM14_4001;Name=yrbF;locus_tag=STM14_4001	21.8
ID=STM14_3959;Name=STM14_3959	21.4
ID=STM14_3671;Name=idi;locus_tag=STM14_3671	21.3
ID=STM14_0479;Name=STM14_0479	21.3
ID=STM14_1552;Name=yeaK;locus_tag=STM14_1552	21.2
ID=STM14_1629;Name=btuD;locus_tag=STM14_1629	21.0
ID=STM14_2022;Name=STM14_2022	20.9
ID=STM14_1607;Name=yniB;locus_tag=STM14_1607	20.7
ID=STM14_0044;Name=STM14_0044	20.3
ID=STM14_1515;Name=STM14_1515	19.8
ID=STM14_2136;Name=narX;locus_tag=STM14_2136	19.8
ID=STM14_1027;Name=ybjN;locus_tag=STM14_1027	19.3
ID=STM14_4446;Name=STM14_4446	19.2
ID=STM14_3791;Name=STM14_3791	19.0
ID=STM14_1139;Name=pncB;locus_tag=STM14_1139	18.7
ID=STM14_1026;Name=rimK;locus_tag=STM14_1026	18.7
ID=STM14_1016;Name=mdfA;locus_tag=STM14_1016	18.5
ID=STM14_2787;Name=napG;locus_tag=STM14_2787	18.2
ID=STM14_2821;Name=glpC;locus_tag=STM14_2821	18.1
ID=STM14_4246;Name=glpD;locus_tag=STM14_4246	18.1
ID=STM14_0373;Name=gpt;locus_tag=STM14_0373	18.0
ID=STM14_2886;Name=STM14_2886	17.9
ID=STM14_2020;Name=STM14_2020	17.8
ID=STM14_2785;Name=napB;locus_tag=STM14_2785	17.6
ID=STM14_0201;Name=gcd;locus_tag=STM14_0201	16.9
ID=STM14_2887;Name=ulaA_2;gene_synonym=sgaT	16.7
ID=STM14_3793;Name=STM14_3793	16.5
ID=STM14_2789;Name=napD;locus_tag=STM14_2789	16.4
ID=STM14_940;Name=STM14_940	16.4
ID=STM14_0818;Name=speF;locus_tag=STM14_0818	15.9
ID=STM14_2827;Name=yfaV;locus_tag=STM14_2827	15.5

ID=STM14_4911;Name=glpX;locus_tag=STM14_4911	15.5
ID=STM14_4910;Name=fpr;locus_tag=STM14_4910	15.5
ID=STM14_0820;Name=STM14_0820	15.4
ID=STM14_1551;Name=STM14_1551	15.4
ID=STM14_0819;Name=STM14_0819	15.3
ID=STM14_2283;Name=exoX;locus_tag=STM14_2283	15.1
ID=STM14_3099;Name=STM14_3099	15.0
ID=STM14_3501;Name=STM14_3501	15.0
ID=STM14_2076;Name=STM14_2076	14.8
ID=STM14_4743;Name=STM14_4743	14.7
ID=STM14_0193;Name=kdgT;locus_tag=STM14_0193	14.7
ID=STM14_5477;Name=yjjG;locus_tag=STM14_5477	14.5
ID=STM14_939;Name=STM14_939	14.4
ID=STM14_2784;Name=napC;locus_tag=STM14_2784	14.4
ID=STM14_3395;Name=STM14_3395	14.1
ID=STM14_2786;Name=napH;locus_tag=STM14_2786	14.0
ID=STM14_4775;Name=yigN;locus_tag=STM14_4775	14.0
ID=STM14_1447;Name=STM14_1447	13.9
ID=STM14_2888;Name=STM14_2888	13.8
ID=STM14_3026;Name=eutQ;locus_tag=STM14_3026	13.7
ID=STM14_2727;Name=setB;locus_tag=STM14_2727	13.7
ID=STM14_1838;Name=marR;locus_tag=STM14_1838	13.5
ID=STM14_3502;Name=STM14_3502	13.3
ID=STM14_1044;Name=ybjP;locus_tag=STM14_1044	13.3
ID=STM14_3739;Name=gshB;locus_tag=STM14_3739	13.0
ID=STM14_1608;Name=STM14_1608	13.0
ID=STM14_0910;Name=ybhA;locus_tag=STM14_0910	13.0
ID=STM14_0817;Name=potE;locus_tag=STM14_0817	12.9
ID=STM14_4913;Name=glpF;locus_tag=STM14_4913	12.8
ID=STM14_1603;Name=cedA;locus_tag=STM14_1603	12.8
ID=STM14_4382;Name=yhjW;locus_tag=STM14_4382	12.6
ID=STM14_1538;Name=STM14_1538	12.6
ID=STM14_4517;Name=selC;locus_tag=STM14_4517	12.4
ID=STM14_4000;Name=yrbE;locus_tag=STM14_4000	12.4
ID=STM14_4433;Name=sgbU;locus_tag=STM14_4433	12.4
ID=STM14_1206;Name=pqiA;locus_tag=STM14_1206	12.3
ID=STM14_2957;Name=STM14_2957	12.3
ID=STM14_2070;Name=acnA;locus_tag=STM14_2070	12.1
ID=STM14_0359;Name=STM14_0359	12.1
ID=STM14_1367;Name=fabD;locus_tag=STM14_1367	11.9
ID=STM14_4183;Name=nirB;locus_tag=STM14_4183	11.7
ID=STM14_2213;Name=manY;locus_tag=STM14_2213	11.7
ID=STM14_2733;Name=yeiP;locus_tag=STM14_2733	11.6
ID=STM14_1205;Name=uup;locus_tag=STM14_1205	11.5
ID=STM14_4381;Name=proK;locus_tag=STM14_4381	11.5

ID=STM14_4555;Name=nepI;gene_synonym=yicM	11.4
ID=STM14_1513;Name=STM14_1513	11.3
ID=STM14_2979;Name=yfeR;locus_tag=STM14_2979	11.2
ID=STM14_2627;Name=yegB;locus_tag=STM14_2627	11.2
ID=STM14_4518;Name=STM14_4518	11.2
ID=STM14_3581;Name=sdaC;locus_tag=STM14_3581	11.0
ID=STM14_3797;Name=STM14_3797	11.0
ID=STM14_1321;Name=yceE;locus_tag=STM14_1321	11.0
ID=STM14_4182;Name=STM14_4182	11.0
ID=STM14_2788;Name=napA;locus_tag=STM14_2788	10.9
ID=STM14_3057;Name=perM;locus_tag=STM14_3057	10.9
ID=STM14_2822;Name=STM14_2822	10.9
ID=STM14_1432;Name=STM14_1432	10.9
ID=STM14_1561;Name=STM14_1561	10.8
ID=STM14_1697;Name=sseD;locus_tag=STM14_1697	10.8
ID=STM14_4744;Name=cyaY;locus_tag=STM14_4744	10.7
ID=STM14_4332;Name=STM14_4332	10.4
ID=STM14_961;Name=STM14_961	10.2
ID=STM14_0816;Name=STM14_0816	10.1
ID=STM14_0757;Name=STM14_0757	10.0
ID=STM14_1514;Name=STM14_1514	10.0
ID=STM14_2885;Name=STM14_2885	9.9
ID=STM14_1948;Name=ydcK;locus_tag=STM14_1948	9.8
ID=STM14_1516;Name=STM14_1516	9.5
ID=STM14_4445;Name=mtlR;locus_tag=STM14_4445	9.4
ID=STM14_2889;Name=STM14_2889	9.2
ID=STM14_3551;Name=cysH;locus_tag=STM14_3551	9.1
ID=STM14_0216;Name=panB;locus_tag=STM14_0216	9.0
ID=STM14_0829;Name=ybgH;locus_tag=STM14_0829	9.0
ID=STM14_4387;Name=lpfA;locus_tag=STM14_4387	8.8
ID=STM14_1518;Name=STM14_1518	8.8
ID=STM14_1175;Name=STM14_1175	8.7
ID=STM14_2290;Name=edd;locus_tag=STM14_2290	8.7
ID=STM14_3999;Name=yrbD;locus_tag=STM14_3999	8.7
ID=STM14_2626;Name=yegO;locus_tag=STM14_2626	8.6
ID=STM14_1116;Name=ycaR;locus_tag=STM14_1116	8.6
ID=STM14_2697;Name=STM14_2697	8.6
ID=STM14_3921;Name=yhaO;locus_tag=STM14_3921	8.5
ID=STM14_0206;Name=stiH;locus_tag=STM14_0206	8.5
ID=STM14_1448;Name=STM14_1448	8.4
ID=STM14_2535;Name=pduK;locus_tag=STM14_2535	8.3
ID=STM14_4184;Name=nirD;locus_tag=STM14_4184	8.3
ID=STM14_3582;Name=sdaB;locus_tag=STM14_3582	8.3
ID=STM14_3719;Name=yggG;locus_tag=STM14_3719	8.3
ID=STM14_2130;Name=narJ;locus_tag=STM14_2130	8.2

ID=STM14_2289;Name=eda;locus_tag=STM14_2289	8.2
ID=STM14_0300;Name=dkgB;locus_tag=STM14_0300	8.2
ID=STM14_1510;Name=STM14_1510	8.1
ID=STM14_3805;Name=STM14_3805	8.1
ID=STM14_1882;Name=STM14_1882	8.0
ID=STM14_4889;Name=STM14_4889	8.0
ID=STM14_989;Name=STM14_989	7.9
ID=STM14_4300;Name=yhhF;locus_tag=STM14_4300	7.9
ID=STM14_0758;Name=STM14_0758	7.8
ID=STM14_2215;Name=STM14_2215	7.8
ID=STM14_5347;Name=STM14_5347	7.7
ID=STM14_2370;Name=yecS;locus_tag=STM14_2370	7.6
ID=STM14_962;Name=glnQ;locus_tag=STM14_962	7.5
ID=STM14_1050;Name=poxB;locus_tag=STM14_1050	7.3
ID=STM14_2698;Name=mglB;locus_tag=STM14_2698	7.2
ID=STM14_2132;Name=narG;locus_tag=STM14_2132	7.1
ID=STM14_4902;Name=yneA;locus_tag=STM14_4902	7.1
ID=STM14_4886;Name=STM14_4886	7.1
ID=STM14_0878;Name=lysZ;locus_tag=STM14_0878	7.0
ID=STM14_2131;Name=narH;locus_tag=STM14_2131	7.0
ID=STM14_0215;Name=STM14_0215	7.0
ID=STM14_2976;Name=xapB;locus_tag=STM14_2976	6.9
ID=STM14_0434;Name=prpE;locus_tag=STM14_0434	6.9
ID=STM14_0358;Name=sinR;locus_tag=STM14_0358	6.9
ID=STM14_5441;Name=STM14_5441	6.9
ID=STM14_3146;Name=STM14_3146	6.8
ID=STM14_0171;Name=hofB;locus_tag=STM14_0171	6.8
ID=STM14_3851;Name=STM14_3851	6.8
ID=STM14_0877;Name=lysY;locus_tag=STM14_0877	6.7
ID=STM14_5039;Name=STM14_5039	6.7
ID=STM14_3300;Name=STM14_3300	6.7
ID=STM14_2950;Name=pgtP;locus_tag=STM14_2950	6.6
ID=STM14_2709;Name=STM14_2709	6.5
ID=STM14_2275;Name=STM14_2275	6.4
ID=STM14_4497;Name=STM14_4497	6.3
ID=STM14_0186;Name=STM14_0186	6.3
ID=STM14_1324;Name=yceA;locus_tag=STM14_1324	6.2
ID=STM14_1842;Name=ynel;locus_tag=STM14_1842	6.2
ID=STM14_3043;Name=STM14_3043	6.1
ID=STM14_4496;Name=rph;locus_tag=STM14_4496	6.1
ID=STM14_3798;Name=STM14_3798	6.1
ID=STM14_3996;Name=STM14_3996	6.1
ID=STM14_5038;Name=STM14_5038	6.0
ID=STM14_2726;Name=STM14_2726	5.9
ID=STM14_4185;Name=STM14_4185	5.9

ID=STM14_4904;Name=yneC;locus_tag=STM14_4904	5.7
ID=STM14_0521;Name=cyoC;locus_tag=STM14_0521	5.7
ID=STM14_2486;Name=asnT_1;locus_tag=STM14_2486	5.7
ID=STM14_1550;Name=STM14_1550	5.7
ID=STM14_1602;Name=STM14_1602	5.6
ID=STM14_2012;Name=STM14_2012	5.6
ID=STM14_3723;Name=STM14_3723	5.4
ID=STM14_2632;Name=STM14_2632	5.0
ID=STM14_2631;Name=STM14_2631	5.0
ID=STM14_2890;Name=STM14_2890	5.0
ID=STM14_0350;Name=STM14_0350	4.9
ID=STM14_2536;Name=pduL;locus_tag=STM14_2536	4.9
ID=STM14_4337;Name=STM14_4337	4.8
ID=STM14_2630.gene;Alias=STM14_2630;Name=STM14_2630	4.7
ID=STM14_1631;Name=STM14_1631	4.5
ID=STM14_4336;Name=STM14_4336	4.0
ID=STM14_1821;Name=STM14_1821	3.8

Table 8.

Genes that are down-regulated when RamA expression is induced from pBAD30 by adding 0.2% arabinose to the culture. Only the genes whose fold change compared the control strain (pBAD30) are significant are shown here (P<0.05).

Genes	Fold change
ID=STM14_1499;Name=STM14_1499	3.7
ID=STM14_3221;Name=STM14_3221	3.8
ID=STM14_1497;Name=pagD;locus_tag=STM14_1497	3.9
ID=STM14_1261;Name=STM14_1261	3.9
ID=STM14_5211;Name=STM14_5211	4.0
ID=STM14_5189;Name=STM14_5189	4.0
ID=STM14_1311;Name=csgC;locus_tag=STM14_1311	4.1
ID=STM14_5190;Name=STM14_5190	4.2
ID=STM14_2359;Name=STM14_2359	4.2
ID=STM14_5416;Name=STM14_5416	4.3
ID=STM14_4339;Name=STM14_4339	4.3
ID=STM14_1312;Name=ymdA;locus_tag=STM14_1312	4.3
ID=STM14_2442;Name=STM14_2442	4.3
ID=STM14_2388;Name=fliE;locus_tag=STM14_2388	4.3
ID=STM14_2247;Name=STM14_2247	4.4
ID=STM14_1503;Name=STM14_1503	4.4
ID=STM14_0720;Name=citG;locus_tag=STM14_0720	4.4
ID=STM14_5044;Name=STM14_5044	4.5
ID=STM14_0661;Name=STM14_0661	4.5
ID=STM14_1613;Name=STM14_1613	4.5
ID=STM14_5125;Name=STM14_5125	4.5
ID=STM14_5415;Name=STM14_5415	4.7
ID=STM14_0398;Name=STM14_0398	4.7
ID=STM14_4147;Name=bfd;locus_tag=STM14_4147	4.7
ID=STM14_2449;Name=STM14_2449	4.7
ID=STM14_3353;Name=STM14_3353	4.8
ID=STM14_0399;Name=STM14_0399	4.8
ID=STM14_3706;Name=yggA;locus_tag=STM14_3706	4.9
ID=STM14_0634;Name=STM14_0634	4.9
ID=STM14_5184;Name=STM14_5184	4.9
ID=STM14_2346;Name=otsB;locus_tag=STM14_2346	4.9
ID=STM14_3801;Name=STM14_3801	5.0
ID=STM14_2680;Name=STM14_2680	5.1
ID=STM14_1198;Name=STM14_1198	5.3

ID=STM14_0705;Name=ybdO;locus_tag=STM14_0705	5.4
ID=STM14_5045;Name=STM14_5045	5.4
ID=STM14_5119;Name=STM14_5119	5.5
ID=STM14_0633;Name=STM14_0633	5.5
ID=STM14_0654.gene;Alias=STM14_0654;Name=STM14_0654	5.8
ID=STM14_0652;Name=gtrA;locus_tag=STM14_0652	5.9
ID=STM14_2400;Name=fliP;locus_tag=STM14_2400	5.9
ID=STM14_0135;Name=leuL;locus_tag=STM14_0135	6.0
ID=STM14_3892;Name=yqjI;locus_tag=STM14_3892	6.0
ID=STM14_0774;Name=STM14_0774	6.0
ID=STM14_2373;Name=fliZ;locus_tag=STM14_2373	6.0
ID=STM14_0655;Name=STM14_0655	6.0
ID=STM14_2391;Name=fliG;locus_tag=STM14_2391	6.1
ID=STM14_2428;Name=STM14_2428	6.2
ID=STM14_0643;Name=STM14_0643	6.2
ID=STM14_1346;Name=flgC;locus_tag=STM14_1346	6.2
ID=STM14_2688;Name=yohJ;locus_tag=STM14_2688	6.3
ID=STM14_1356;Name=STM14_1356	6.3
ID=STM14_1347;Name=flgD;locus_tag=STM14_1347	6.3
ID=STM14_0639;Name=fimH;locus_tag=STM14_0639	6.5
ID=STM14_3371;Name=ygaU;locus_tag=STM14_3371	6.6
ID=STM14_0722;Name=citF;locus_tag=STM14_0722	6.6
ID=STM14_2395;Name=fliK;locus_tag=STM14_2395	6.6
ID=STM14_4398;Name=yiaG;locus_tag=STM14_4398	6.7
ID=STM14_2444;Name=STM14_2444	6.7
ID=STM14_0642;Name=fimY;locus_tag=STM14_0642	6.8
ID=STM14_1345;Name=flgB;locus_tag=STM14_1345	6.8
ID=STM14_2329;Name=STM14_2329	7.0
ID=STM14_2397;Name=fliM;locus_tag=STM14_2397	7.0
ID=STM14_2091;Name=yciG;locus_tag=STM14_2091	7.1
ID=STM14_5518;Name=STM14_5518	7.2
ID=STM14_2443;Name=STM14_2443	7.2
ID=STM14_0428;Name=yahO;locus_tag=STM14_0428	7.3
ID=STM14_0723;Name=citE;locus_tag=STM14_0723	7.3
ID=STM14_1353;Name=flgJ;locus_tag=STM14_1353	7.3
ID=STM14_1352;Name=flgI;locus_tag=STM14_1352	7.3
ID=STM14_5122;Name=STM14_5122	7.5
ID=STM14_2330;Name=cheZ;locus_tag=STM14_2330	7.5
ID=STM14_2331;Name=cheY;locus_tag=STM14_2331	7.5
ID=STM14_1351;Name=flgH;locus_tag=STM14_1351	7.6
ID=STM14_0454;Name=psiF;locus_tag=STM14_0454	7.7
ID=STM14_0702;Name=ybdL;locus_tag=STM14_0702	7.7
ID=STM14_4769;Name=metE;locus_tag=STM14_4769	7.8
ID=STM14_4532;Name=slsA;locus_tag=STM14_4532	7.9
ID=STM14_4579;Name=ivbL;locus_tag=STM14_4579	7.9

ID=STM14_4578;Name=STM14_4578	8.0
ID=STM14_1349;Name=flgF;locus_tag=STM14_1349	8.0
ID=STM14_2333;Name=cheR;locus_tag=STM14_2333	8.1
ID=STM14_0638;Name=fimD;locus_tag=STM14_0638	8.1
ID=STM14_2429;Name=STM14_2429	8.3
ID=STM14_1554;Name=STM14_1554	8.5
ID=STM14_1350;Name=flgG;locus_tag=STM14_1350	8.6
ID=STM14_2332;Name=cheB;locus_tag=STM14_2332	8.6
ID=STM14_1555;Name=STM14_1555	8.6
ID=STM14_1348;Name=flgE;locus_tag=STM14_1348	8.7
ID=STM14_4697;Name=ilvL;locus_tag=STM14_4697	8.8
ID=STM14_2392;Name=fliH;locus_tag=STM14_2392	9.1
ID=STM14_2339;Name=STM14_2339	9.4
ID=STM14_2399;Name=fliO;locus_tag=STM14_2399	9.5
ID=STM14_0724;Name=citD;locus_tag=STM14_0724	9.5
ID=STM14_0721;Name=citX;locus_tag=STM14_0721	9.5
ID=STM14_0641;Name=fimZ;locus_tag=STM14_0641	9.6
ID=STM14_4674;Name=asnA;locus_tag=STM14_4674	9.8
ID=STM14_3126;Name=asrA;locus_tag=STM14_3126	9.9
ID=STM14_3340;Name=STM14_3340	9.9
ID=STM14_2394;Name=fliJ;locus_tag=STM14_2394	10.0
ID=STM14_3734;Name=STM14_3734	10.6
ID=STM14_5167;Name=adiY;locus_tag=STM14_5167	10.6
ID=STM14_0730;Name=STM14_0730	10.8
ID=STM14_4733;Name=STM14_4733	10.8
ID=STM14_2798;Name=STM14_2798	10.8
ID=STM14_2352;Name=STM14_2352	11.0
ID=STM14_2338;Name=motA;locus_tag=STM14_2338	11.2
ID=STM14_3822;Name=STM14_3822	11.2
ID=STM14_3342;Name=STM14_3342	11.4
ID=STM14_2374;Name=fliA;locus_tag=STM14_2374	11.5
ID=STM14_3821;Name=STM14_3821	11.5
ID=STM14_3343;Name=STM14_3343	11.9
ID=STM14_3466;Name=STM14_3466	12.4
ID=STM14_2393;Name=fliI;locus_tag=STM14_2393	12.8
ID=STM14_1283;Name=STM14_1283	12.9
ID=STM14_1282;Name=STM14_1282	13.2
ID=STM14_1341;Name=flgN;locus_tag=STM14_1341	13.3
ID=STM14_2167;Name=STM14_2167	13.4
ID=STM14_5188;Name=STM14_5188	13.4
ID=STM14_2852;Name=STM14_2852	13.4
ID=STM14_0549;Name=ybaY;locus_tag=STM14_0549	13.5
ID=STM14_1612;Name=STM14_1612	13.6
ID=STM14_1355;Name=flgL;locus_tag=STM14_1355	13.9
ID=STM14_2334;Name=cheM;locus_tag=STM14_2334	14.0

ID=STM14_2398;Name=fliN;locus_tag=STM14_2398	14.4
ID=STM14_4531;Name=STM14_4531	14.5
ID=STM14_2387;Name=STM14_2387	15.0
ID=STM14_2168;Name=STM14_2168	15.8
ID=STM14_3467;Name=orgC;locus_tag=STM14_3467	15.9
ID=STM14_2336;Name=cheA;locus_tag=STM14_2336	16.0
ID=STM14_1354;Name=flgK;locus_tag=STM14_1354	16.8
ID=STM14_5118;Name=STM14_5118	17.2
ID=STM14_1884;Name=STM14_1884	17.4
ID=STM14_1891;Name=adhP;gene_synonym=adhA	17.4
ID=STM14_3468;Name=orgB;locus_tag=STM14_3468	17.6
ID=STM14_3477;Name=sptP;locus_tag=STM14_3477	17.8
ID=STM14_5120;Name=STM14_5120	17.9
ID=STM14_4935;Name=metF;locus_tag=STM14_4935	18.1
ID=STM14_5117;Name=STM14_5117	18.6
ID=STM14_5187;Name=STM14_5187	19.4
ID=STM14_4768;Name=STM14_4768	19.6
ID=STM14_3341;Name=STM14_3341	20.0
ID=STM14_966;Name=dps;locus_tag=STM14_966	20.8
ID=STM14_2337;Name=motB;locus_tag=STM14_2337	21.3
ID=STM14_3479;Name=STM14_3479	21.9
ID=STM14_2335;Name=cheW;locus_tag=STM14_2335	22.1
ID=STM14_1235;Name=STM14_1235	23.9
ID=STM14_1237;Name=sopB;locus_tag=STM14_1237	24.0
ID=STM14_2342;Name=STM14_2342	24.2
ID=STM14_0636;Name=fimI;locus_tag=STM14_0636	25.2
ID=STM14_3469;Name=orgA;locus_tag=STM14_3469	26.6
ID=STM14_1342;Name=flgM;locus_tag=STM14_1342	27.7
ID=STM14_2227;Name=STM14_2227	27.7
ID=STM14_1236;Name=pipC;locus_tag=STM14_1236	28.8
ID=STM14_5185;Name=STM14_5185	29.5
ID=STM14_4305;Name=tcp;locus_tag=STM14_4305	30.9
ID=STM14_3478;Name=sicP;locus_tag=STM14_3478	31.0
ID=STM14_2162;Name=STM14_2162	31.5
ID=STM14_3799;Name=STM14_3799	32.5
ID=STM14_3475;Name=hilA;locus_tag=STM14_3475	32.5
ID=STM14_2166;Name=STM14_2166	33.6
ID=STM14_4934;Name=STM14_4934	35.1
ID=STM14_3462;Name=avrA;locus_tag=STM14_3462	35.7
ID=STM14_2379;Name=STM14_2379	35.7
ID=STM14_3486;Name=spaS;locus_tag=STM14_3486	35.7
ID=STM14_4346;Name=yhjH;locus_tag=STM14_4346	36.4
ID=STM14_3496;Name=invE;locus_tag=STM14_3496	37.5
ID=STM14_3499;Name=invH;locus_tag=STM14_3499	38.3
ID=STM14_1887;Name=yddX;locus_tag=STM14_1887	41.1

ID=STM14_2161;Name=STM14_2161	42.4
ID=STM14_3893;Name=STM14_3893	42.9
ID=STM14_3480;Name=iacP;locus_tag=STM14_3480	44.8
ID=STM14_5166;Name=yjdE;locus_tag=STM14_5166	45.5
ID=STM14_0637;Name=fimC;locus_tag=STM14_0637	46.5
ID=STM14_5186;Name=STM14_5186	51.0
ID=STM14_2378;Name=fliC;locus_tag=STM14_2378	54.4
ID=STM14_3487;Name=spaR;locus_tag=STM14_3487	55.7
ID=STM14_2164;Name=STM14_2164	57.6
ID=STM14_3493;Name=invC;locus_tag=STM14_3493	59.3
ID=STM14_3476;Name=iagB;locus_tag=STM14_3476	59.7
ID=STM14_3485;Name=sicA;locus_tag=STM14_3485	61.3
ID=STM14_3482;Name=sipD;locus_tag=STM14_3482	63.9
ID=STM14_2244;Name=sopE2;locus_tag=STM14_2244	64.8
ID=STM14_3481;Name=sipA;locus_tag=STM14_3481	66.2
ID=STM14_3488;Name=spaQ;locus_tag=STM14_3488	68.2
ID=STM14_3494;Name=invB;locus_tag=STM14_3494	69.1
ID=STM14_3490;Name=spaO;locus_tag=STM14_3490	72.8
ID=STM14_3474;Name=hilD;locus_tag=STM14_3474	74.3
ID=STM14_3489;Name=spaP;locus_tag=STM14_3489	75.0
ID=STM14_3483;Name=sipC;locus_tag=STM14_3483	78.7
ID=STM14_2165;Name=STM14_2165	79.0
ID=STM14_0635;Name=fimA;locus_tag=STM14_0635	82.3
ID=STM14_5169;Name=adi;locus_tag=STM14_5169	92.1
ID=STM14_3484;Name=sipB;locus_tag=STM14_3484	92.6
ID=STM14_2160;Name=STM14_2160	104.3
ID=STM14_3491;Name=invJ;locus_tag=STM14_3491	105.5
ID=STM14_3495;Name=invA;locus_tag=STM14_3495	111.6
ID=STM14_3470;Name=prgK;locus_tag=STM14_3470	116.4
ID=STM14_3473;Name=prgH;locus_tag=STM14_3473	126.7
ID=STM14_3492;Name=invI;locus_tag=STM14_3492	128.0
ID=STM14_3497;Name=invG;locus_tag=STM14_3497	128.4
ID=STM14_5168;Name=STM14_5168	143.0
ID=STM14_3464;Name=STM14_3464	166.4
ID=STM14_3463;Name=sprB;locus_tag=STM14_3463	203.9
ID=STM14_3498;Name=invF;locus_tag=STM14_3498	205.0
ID=STM14_3465;Name=hilC;locus_tag=STM14_3465	210.7
ID=STM14_3471;Name=prgJ;locus_tag=STM14_3471	216.9
ID=STM14_3472;Name=prgI;locus_tag=STM14_3472	254.7
ID=STM14_1885;Name=hdeB;locus_tag=STM14_1885	1939.0

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Chapter 4: Multidrug resistance regulators MarA, SoxS, Rob, and RamA repress flagellar gene expression and motility in *Salmonella enterica* serovar Typhimurium

4.1) Introduction

Motility affords many bacteria the ability to migrate to more favorable environments. Despite its intrinsic benefits, most bacteria selectively engage in or repress motile behavior in response to specific conditions (Osterman, Dikhtyar, Bogdanov, Dontsova, & Sergiev, 2015; O. A. Soutourina & Bertin, 2003). These forms of regulation are particularly true of flagellar motility, where sizeable energetic costs are required for synthesis and function (Fontaine, Stewart, Lindner, & Taddei, 2008). Correspondingly, transcriptional and post-transcriptional regulation of flagellar and chemotaxis gene expression is common and requires integration of numerous environmental and nutritional signals. Such integration leads to optimal expression of structural and chemosensory proteins required for flagellar assembly and motility (Chevance & Hughes, 2008; Chilcott & Hughes, 2000).

For *Salmonella enterica* serovar Typhimurium and its close relatives, expression of flagellar and chemotaxis genes are transcriptionally co-regulated in a hierarchical manner (**Figure 1A**). In this arrangement, the heterohexameric transcription factor $FlhD_4C_2$ is expressed from the *flhDC* operon (Class I genes) and serves as a master regulator that activates a number of downstream flagellar

structural genes (Class II genes) including an alternative sigma factor, FliA (or σ^{28}) (Chilcott & Hughes, 2000). FliA subsequently initiates expression of late flagellar and chemotaxis genes (Class III genes) (Arnosti & Chamberlin, 1989; Chilcott & Hughes, 2000; Kutsukake, Ohya, & Iino, 1990). Beyond transcriptional regulation, several secretion-dependent feedbacks provide molecular checkpoints during flagellar assembly, often by regulating FliA and $FlhD_4C_2$ DNA binding or their stability (Gillen & Hughes, 1991; Karlinsey et al., 2000; Saini, Brown, Aldridge, & Rao, 2008; Yamamoto & Kutsukake, 2006). Based on this regulatory architecture, the majority of known global regulation occurs at the level of *flhDC* transcription or alteration of $FlhD_4C_2$ activity (Chilcott & Hughes, 2000; Osterman et al., 2015; O. A. Soutourina & Bertin, 2003).

Several transcription factors are known to activate and repress *flhDC* transcription in *S.* Typhimurium. Transcriptional activators include CRP, Fur, the nucleoid binding proteins Fis and H-NS, and SlyA; as well as autoactivation by $FlhD_4C_2$ itself (Kelly et al., 2004; Komeda, Suzuki, Ishidsu, & Iino, 1975; Kutsukake, 1997; O. Soutourina et al., 1999; Spory, Bosserhoff, von Rhein, Goebel, & Ludwig, 2002; Stojiljkovic, Bäumler, & Hantke, 1994). HilD, a key regulator of *Salmonella* pathogenicity island 1 (SPI-1) has also been shown to activate *flhDC* transcription, illustrating crosstalk between flagellar and pathogenicity-associated gene expression (Singer, Kühne, Deditius, Hughes, & Erhardt, 2014). Attenuating *flhDC* expression are several regulators including RtsB, LhrA, OmpR, SsrB, and RcsB (C. D. Ellermeier & Slauch, 2003; Ilyas et al., 2018; Lehnen et al., 2002; Shin & Park, 1995; Wang, Zhao, McClelland, &

Harshey, 2007). RcsB-mediated repression of *flhDC* is coordinated by the $FlhD_4C_2$-controlled regulator RflM (also known as EcnR) (Kühne et al., 2016; Singer, Erhardt, & Hughes, 2013). Repression of *flhDC* expression is also mediated through post-translational regulation of $FlhD_4C_2$ by FliZ and FliT, YdiV (a nutritional regulator), FimZ (a fimbrial regulator), and others (Clegg & Hughes, 2002; Saini et al., 2008; Takaya et al., 2012; Yamamoto & Kutsukake, 2006). Interactions between these proteins and $FlhD_4C_2$ results in reduced $FlhD_4C_2$-dependent activation of *flhDC* expression. Through these varied regulatory systems, numerous environmental and nutritional signals are integrated to control transcription of flagellar and chemotaxis genes.

In addition to transcriptional-level regulation of *flhDC* described above, *flhDC* mRNA is regulated post-transcriptionally. From studies in *E. coli* and *S.* Typhimurium, several small regulatory RNA (sRNA) with negative effects on *flhDC* translation have been identified including ArcZ, OmrA, OmrB, and OxyS; McaS is observed to positively influence motility through stabilization of the *flhDC* transcript (de Lay & Gottesman, 2012; Thomason, Fontaine, de Lay, & Storz, 2012). These sRNAs are conserved in *S.* Typhimurium and presumably have similar effects on *flhDC* mRNA translation. Highlighting the importance of sRNA-mediated regulation of *flhDC* translation, mutants in *hfq* (encoding the sRNA binding chaperone, Hfq) are severely impaired in motility (Sittka, Pfeiffer, Tedin, & Vogel, 2007). Apart from sRNA, *flhDC* mRNA stability is also regulated by direct binding of the carbon storage regulator, CsrA (B. L. Wei et al., 2001; Yakhnin et al., 2013).

Beyond direct genetic effects, exposure to aromatic acids, phenolic compounds, and other aromatic compounds are known to inhibit the production of flagella and motility in several enteric gammaproteobacteria (Burt et al., 2007; Kunin, Tong Hua Hua, & Bakaletz, 1995). Many aromatic acids are also well-characterized chemorepellents, such as salicylic acid (Tso & Adler, 1974). In the case of membrane-permeable aromatic acids, such as benzoic acid, effects on motility have been attributed to disruption in proton motive force by shuttling of protons across the cytoplasmic membrane (Kihara & Macnab, 1981; Repaske & Adler, 1981). Additionally, certain phenols like curcumin have been shown to bind to flagellin monomers inducing flagellar shedding and loss of motility (Amol Marathe et al., 2016). However, causes for reductions in flagellar abundance and motility in the presence of aromatic acids have not been rigorously explored.

A common response to many aromatic compounds is increased levels or activation of MarA, SoxS, RamA or Rob. The homologous, AraC-family transcription factors MarA, SoxS, and Rob are known to coordinately regulate a wide array of genes in *Escherichia coli* known as the *mar-sox-rob* regulon, resulting in large-scale changes in cellular physiology and metabolism (Barbosa & Levy, 2000; Duval, 2013; Robert G. Martin & Rosner, 2002). Regulation occurs through binding of these transcription factors to a common, degenerate *marbox* sequence in promoters of *mar-sox-rob* regulon genes (Robert G. Martin et al., 1999). The regulatory targets associated with MarA homologs in other related *Enterobacteriaceae* are largely undefined, however, many common targets in *E. coli* are conserved in related species like *S. enterica* (Hartog, Ben-Shalom,

Shachar, Matthews, & Yaron, 2008; Jiménez-Castellanos et al., 2016; Sulavik, Dazer, & Miller, 1997). Beyond *E. coli*, a number of related species like *S. enterica* and *K. pneumoniae* contain an additional MarA homolog, RamA, capable of regulating *mar-sox-rob* regulon genes (George, Hall, & Stokes, 1995; van der Straaten et al., 2004).

Expression of MarA homologs occurs in response to varied chemical cues via disparate mechanisms. For MarA, SoxS, and RamA, their respective expression is controlled by $MarR_2$, $SoxR_2$, and $RamR_2$ each responding to different compounds. For instance, $MarR_2$-dependent transcriptional repression of the *marRAB* operon is relieved via $MarR_2$ binding to aromatic acids or copper-mediated disulfide bond formation between MarR monomers (Alekshun & Levy, 1999; Alekshun, Levy, Mealy, Seaton, & Head, 2001; Chubiz & Rao, 2010; Hao et al., 2014; Robert G. Martin & Rosner, 1995; Prouty, Brodsky, Falkow, & Gunn, 2004). $SoxR_2$-dependant activation of *soxS* transcription occurs through oxidation of an iron-sulfur cluster in $SoxR_2$ by redox active compounds such as methyl viologen (paraquat) (Hidalgo, Bollinger, Bradley, Walsh, & Demple, 1995; Hidalgo & Demple, 1994; Watanabe, Kita, Kobayashi, & Miki, 2008). Similar to $MarR_2$, $RamR_2$ represses transcription of *ramA* until exposed to bile salts or other aromatic compounds like indole (E. Nikaido, Yamaguchi, & Nishino, 2008; Yamasaki et al., 2019, 2013). Unlike MarA, SoxS, and RamA, Rob is activated post-transcriptionally via a sequestration-dispersion mechanism in response to aromatic and fatty acids (Griffith, Fitzpatrick, Keen, & Wolf, 2009; Rosenberg et al., 2003). Interestingly, there exists extensive regulatory cross-talk between

these systems allowing for the formation of complex feed-forward regulatory loops depending on chemical inducers present (Chubiz et al., 2012; Jain & Saini, 2016). For instance, exposure to salicylic acid results in activation of Rob and *marA* transcription yielding stronger activation of *mar-sox-rob* targets (Chubiz et al., 2012; Robert G. Martin, Jair, Wolf, & Rosner, 1996). In this way, species containing *mar-sox-rob* regulatory networks are able to sensitively tune downstream responses to a wide variety of chemical stressors in their environment based on the intracellular concentrations of MarA, SoxS, Rob, and RamA (Robert G. Martin, Bartlett, Rosner, & Wall, 2008).

Canonically, the downstream effects of MarA homologs have been associated with inducible multidrug resistance (Duval, 2013). Apart from their role in multidrug resistance, the effects of MarA homologs on prokaryotic physiology are cryptic. However, there is growing appreciation for the potential role in transcriptionally regulating other cellular processes. For instance, RamA has been shown to attenuate expression of virulence traits and efflux pumps in *S.* Typhimurium (A. M. Bailey et al., 2010). The mechanism by which MarA homologs influence traits other than antibiotic resistance in *S.* Typhimurium or other *Enterobacteriaceae* has not been fully explored.

Here, we looked to define the role of the *mar-sox-rob* regulatory proteins MarA, SoxS, Rob, and RamA in controlling flagellar gene expression and motility in *S.* Typhimurium. We found that all four of these regulators are repressors of motility, with SoxS and RamA exhibiting the strongest phenotypic effects on swimming and transcriptional repression of the flagellar regulon. Interestingly, the

repressive effects of SoxS on motility are due to both transcriptional and post-transcriptional regulation of *flhDC* expression. Based on these findings we propose an addition to the flagellar regulatory model. Flagellar repression occurs through coordinated activation of MarA, SoxS, Rob, and RamA in the presence of diverse chemical stressors. This form of repression occurs via transcriptional repression of the *flhDC* promoter as well as activation of a post-transcriptional mechanism that inhibits *flhDC* translation (**Figure 1A**). Given that known MarA, SoxS, Rob, and RamA inducers are present in the gastrointestinal environment, and these transcription factors are expressed during various stages of *S. enterica* infections, this mechanism of flagellar gene repression may have implications in the virulence lifestyle of *S. enterica* and related enterobacterial pathogens.

4.2) Results

MarA, SoxS, Rob, and RamA inhibit motility and decrease production of flagellin.

Given their broad range of regulatory targets, we examined whether elevated MarA, SoxS, Rob, levels RamA have an impact on motility and found all four regulators are capable of inhibiting motility. To test the effect of each regulator on motility, we individually complemented *marA*, *soxS*, *rob*, or *ramA* expression from an arabinose-vector in a *marRAB soxRS rob ramRA* quadruple mutant. A genetic background lacking all native loci for these transcription factors was chosen to minimize any possible regulatory cross-talk between MarA, SoxS, Rob, and RamA, such as SoxS-dependent activation of *marRAB*, that may distort

their effects on expression of downstream genes. This approach has been used in prior studies examining the *mar-sox-rob* regulon in *E. coli* (43, 65). In the quadruple mutant background, we observed MarA, SoxS, Rob, and RamA repress swimming motility, with SoxS and RamA having the most pronounced effects (**Figure 1B**). This was also observed for swimming and surface-associated swarming motility in a wild-type background with all four native *marRAB*, *soxRS*, *rob*, and *ramRA* loci intact (**Figure 8**).

Expression of each MarA homolog in the quadruple mutant background also resulted in decreased production of flagellin. To delineate whether the effects of MarA, SoxS, Rob, and RamA were the result of reductions in flagellar protein expression or post-translational effects on flagellar function, we measured levels of FliC, the flagellar filament protein by Western blot (**Figure 1C**) and enzyme-linked immunosorbent assays (ELISA) (**Figure 1D**) when *marA*, *soxS*, *rob*, or *ramA* were expressed from pBAD30 in the quadruple mutant background. Immunoblots showed expression of *marA*, *soxS*, *rob*, or *ramA* repress FliC production (**Figure 1C**). However, quantification by ELISA revealed that while SoxS and RamA strongly inhibited FliC levels, the effects of MarA and Rob were more modest as demonstrated by the significantly higher FliC levels compared to SoxS and RamA (Tukey HSD, all $P \leq 9.5 \times 10^{-3}$) (**Figure 1D**). These patterns qualitatively correlate with observed effects on motility where MarA and Rob attenuate motility to a lesser degree than SoxS and RamA (**Figure 1B**). Differences between immunoblot and ELISA measurements likely reflect differences in detection limits of FliC protein between the assays. Additionally, we

found that the phase-variable flagellin system, *fljBA*, is also significantly down-regulated under these conditions suggesting reduced FliC levels are not a result of increased FljA levels (Student's *t*-test, all $P<1x10^{-6}$) (**Figure 9**).

All classes of flagellar promoters are downregulated by MarA, SoxS, Rob, and RamA.

Based on the effects of MarA, SoxS, Rob, and RamA on motility and expression of FliC we looked to determine whether these transcription factors repress specific classes of flagellar genes. To identify which classes of flagellar genes are subject to repression by MarA-homologs, we constructed single-copy promoter fusions of *flhDC* (Class I), *flhB* (Class II), and *fliC* (Class III) to *yfp*(Venus), akin to Koirala and coworkers (Koirala et al., 2014). Using these transcriptional fusions in our quadruple mutant background we found complementation of *marA*, *soxS*, *rob*, and *ramA* expression caused significant reductions in expression compared to a plasmid control (Tukey HSD, all $P<1x10^{-7}$) from all three classes of flagellar promoters (**Figure 1E**). Most importantly, Class I was repressed by a range of 29±2.9% to 79.9±0.8% compared to the plasmid control, bounded by Rob and SoxS, respectively. Concomitantly, these reductions in Class I promoter activity were reflected in decreased expression from Class II and Class III promoters. An exception was MarA which resulted in a roughly 30% decrease in expression across all classes of flagellar promoters tested. Interestingly, MarA and Rob had nearly identical effects on Class I transcription (Tukey HSD, $P=0.95$), yet Rob had stronger negative effects on

Class II and Class III expression (**Figure 1E**) but reduced motility less than MarA (**Figure 1B**). This is likely the result of differences in growth conditions between liquid and motility agar but may also suggest alternative modes of flagellar regulation between these two transcription factors. Considering the architecture of the flagellar regulon, reductions in *flhDC* expression are likely the principal cause for reductions in flagellar gene expression caused by MarA, SoxS, Rob, and RamA.

MarA and Rob interact directly with the *flhDC* promoter.

To test whether interactions between MarA homologs and the *flhDC* promoter were direct, we used electromobility shift assays (EMSA) and chromatin immunoprecipitation followed by quantitative PCR (ChIP-qPCR) to detect binding *in vitro* and *in vivo*, respectively. Using purified Rob protein to perform EMSA, we found Rob specifically bound the *flhDC* promoter region (**Figure 2A**). When increasing concentrations of Rob protein were incubated with labeled *flhDC* promoter, a corresponding decrease in gel migration of labeled *flhDC* promoter DNA was observed. Additionally, incubation with unlabeled *flhDC* competitor DNA eliminated binding of Rob to the labeled *flhDC* promoter DNA while a similar treatment with non-specific competitor DNA (a *gyrA* DNA fragment) had no effect on Rob binding. These results show Rob specifically binds the *flhDC* promoter *in vitro*.

Building on our *in vitro* observation with Rob, we proceeded to test whether interactions between all four MarA homologs could be detected *in vivo*

using ChIP-qPCR with FLAG epitope-tagged derivatives of MarA, SoxS, Rob, and RamA (**Figure 2B**). MarA, SoxS, Rob, and RamA are known to bind to a similar site in promoter regions (the *marbox*) (Robert G. Martin et al., 1999). For this reason, we hypothesized that MarA, SoxS, and RamA may bind *flhDC*, as well. FLAG tagged MarA, SoxS, Rob, and RamA were independently expressed from an arabinose-inducible vector in a wild-type genetic background followed by formaldehyde crosslinking, ChIP, and targeted qPCR of the *flhDC* promoter region. Of these four regulators, MarA, SoxS, and Rob were observed to significantly pull down *flhDC* promoter DNA (Student's *t*-test, P=0.0035, P=0.014, and P=0.00018, respectively), whereas RamA was not (Student's *t*-test, P=0.92). We note that the fold enrichment from SoxS is modest, and may have limited biological significance. There are possible reasons for the discrepancy in binding patterns for MarA and Rob versus SoxS and RamA. Our findings may reflect the variable affinity of each of these homologs for *marbox* derivatives, wherein MarA and Rob may have higher affinities for *flhDC* than SoxS and RamA (R. G. Martin, Gillette, & Rosner, 2000). However, these *in vivo* ChIP-qPCR data cannot confirm this hypothesis since the intracellular concentrations and efficiency of immunoprecipitation of each transcription factor are not well defined. Alternatively, our cross-linking procedure may have biased detection of SoxS and RamA as compared to MarA and Rob. Other *in vivo* ChIP studies examining MarA and SoxS binding have utilized cross-linking successfully, suggesting cross-linking is not likely a source of bias (Seo, Kim, Szubin, & Palsson, 2015; Sharma et al., 2017). Finally, SoxS and RamA are subject to rapid proteolysis

potentially limiting detection by our ChIP-based assay (Griffith, Shah, & Wolf, 2004). Based on these findings, we conclude that MarA and Rob bind to the *flhDC* promoter, while SoxS and RamA do not, under the conditions tested.

MarA homologs repress *flhDC* expression post-transcriptionally.

To genetically test MarA, SoxS, Rob, and RamA dependent control of the *flhDC* promoter, we replaced the native *flhDC* promoter (Class I) *in situ* with a tetracycline-inducible promoter cassette in an otherwise wild-type genetic background. This has been shown to remove all native transcriptional regulation of the *flhDC* promoter (Karlinsey et al., 2000; Saini et al., 2008). We induced expression of *flhDC* from this construct using anhydrotetracycline (ATc), a non-toxic tetracycline derivative, at 1 ng/ml (low ATc) and 100 ng/ml (high ATc) concentrations. This allowed for detection of possible post-transcriptional effects on *flhDC* expression since reductions in flagellar expression and motility could not occur through transcriptional repression of the native *flhDC* promoter when MarA, SoxS, Rob, or RamA were ectopically expressed. Using *fliC* expression as a flagellar regulon readout, induction of *flhDC* with low ATc together with ectopic expression of *marA*, *soxS*, *rob*, or *ramA*, resulted in all four transcription factors significantly reducing *fliC* expression levels to varying degrees (Tukey HSD, all $P<1x10^{-7}$) (**Figure 3A)**. When *flhDC* expression levels were increased by high ATc induction we still found all MarA homologs significantly reduce *fliC* expression (Tukey HSD, all $P\leq9.85x10^{-5}$) with MarA and Rob having equivalent effects. Expression of *fliC* also increased by 40.4±5.1% and 36.7±2.6% under

this condition compared to the low ATc treatment for MarA and Rob, respectively (Student's *t*-test, both $P \leq 3.9 \times 10^{-7}$). Interestingly, *fliC* levels did not show similar increases between low and high ATc treatments for SoxS and RamA. These data demonstrate all four MarA homologs are capable of activating (or repressing) a post-transcriptional regulatory pathway for *flhDC*. However, MarA and Rob regulate this putative pathway to a lesser degree than SoxS and RamA.

Examining levels of FliC and motility yielded similar trends to *fliC* transcription, particularly at high ATc levels (**Figure 3B** and **3C**). Notably, FliC was higher in high ATc compared to low ATc for all MarA homologs, matching corresponding increases in motility. An exception is for SoxS where motility remains completely impaired at low and high ATc levels (**Figure 3C**). In the case of MarA at low ATc, we observed FliC levels were higher than SoxS, Rob, and RamA yet cells were non-motile (**Figure 3B** and **3C**). This may be due to MarA-dependent activation of *acrAB*, capable of removing tetracycline (Okusu, Ma, & Nikaido, 1996), or other downstream targets affecting flagellar expression in motility agar where oxygen partial pressures are lower than aerated liquid media. We also noticed changes in the levels of DnaK, a protein chaperone used as a loading control in immunoblots, in our promoter replacement studies (**Figure 3B**). Specifically, DnaK decreases at low ATc between our plasmid control and the MarA homolog vectors. While DnaK plays a positive role in flagellar assembly, we do not suspect it plays a role here as these trends in DnaK levels were not observed at high ATc concentrations (Shi, Zhou, Wild, Adler, & Gross, 1992). Overall, these data suggest when *flhDC* transcript levels are high, post-

transcriptional repression can be overcome. This is similar to the threshold effects observed for sRNA-based regulation (Levine & Hwa, 2008; Levine, Zhang, Kuhlman, & Hwa, 2007).

Since SoxS showed the strongest effects on post-transcriptional flagellar regulation at low and high ATc induction of *flhDC* (**Figure 3**), we wanted to understand the effects of native SoxS concentrations on *flhDC* regulation (**Figure 4**). To achieve physiologically relevant levels of SoxS in the absence of toxic inducers, we utilized a *soxR*^{Con} mutant which results in moderate, constitutive levels of *soxS* expression (**Figure 10**) (Nunoshiba & Demple, 1994). When introduced into the tetracycline-inducible *flhDC* background, *soxR*^{Con} resulted in significantly lower levels of Class III activation across two logs of ATc concentrations, compared to *soxR*^{WT} (Student's *t*-test, all $P \leq 0.0018$) (**Figure 4A**). To test whether translation of *flhDC* mRNA was inhibited by SoxS expression, we generated a 3xFLAG epitope tagged version of FlhC (FlhC-3xFLAG) that was expressed by the tetracycline-inducible *flhDC* construct, similar to Saini and coworkers (Saini et al., 2008). At high ATc concentrations, moderate levels of SoxS were insufficient to reduce levels of FlhC-3xFLAG. On the other hand, low ATc induction resulted in lower FlhC-3xFLAG levels. This is consistent with translation of *flhDC* mRNA being inhibited by a mechanism controlled by SoxS **(Figure 4B).** Similarly, at high ATc the *soxR*^{Con} mutant had a modest effect on motility, while at low ATc it resulted in reduced motility (**Figure 4C**). Taken together, these data indicate that physiological levels of SoxS are capable of

reducing flagellar expression and that this repression appears to occur at the level of *flhDC* translation.

Post-transcriptional repression of *flhDC* expression by SoxS is Hfq-independent.

Since elevated *soxS* expression resulted in reduced *flhDC* translation, we looked to better understand the mechanism behind SoxS-dependent post-transcriptional regulation of *flhDC*. Specifically, we explored the possibility of an Hfq-dependent sRNA mediating repression of *flhDC* when SoxS is expressed (**Figure 5**). The most common mechanism of post-transcriptional regulation of *flhDC* is via action of sRNAs, resulting in both positive and negative effects on *flhDC* expression. Exemplifying this fact is the severe motility defect in an *hfq* mutant in *S*. Typhimurium (Sittka et al., 2007). Hfq is a highly conserved sRNA chaperone required for the function of many sRNAs (Vogel & Luisi, 2011). Should SoxS require an Hfq-dependent sRNA, reductions in flagellar expression and motility caused by *soxR*^{Con} (elevated SoxS levels) should be masked by the effects of a *hfq* mutant. Consistent with observations of Sittka and coworkers, our *hfq* mutant had severely reduced *fliC* transcription and motility (**Figure 5**) (Sittka et al., 2007). The *soxR*^{Con} and *hfq* mutants both caused significant decreases in *fliC* expression compared to wild-type (Student's *t*-test, both $P \leq 2.4 \times 10^{-7}$) (**Figure 5A**). Notably, the *soxR*^{Con} *hfq* double mutant had significantly lower *fliC* levels compared to the *soxR*^{Con} mutant (Student's *t*-test, $P = 8.1 \times 10^{-11}$) and the *hfq* mutant (Student's *t*-test, $P = 1.7 \times 10^{-8}$). These data support a regulatory model

where the negative effects of *soxR*^{Con} (*i.e.* elevated levels of SoxS) on flagellar gene expression are acting independently of Hfq. We attempted to further validate these findings by measuring levels of FliC protein but found quantities were too low to quantify differences by our ELISA method (**Figure 11**).

Measuring the effects of *soxR*^{Con} and *hfq* on motility further demonstrated *soxR*^{Con} acts independently of *hfq* in repressing flagellar expression. Consistent with SoxS inhibiting *flhDC* expression, the *soxR*^{Con} mutant reduced motility compared to wild-type (**Figure 5B**). Similar to *fliC* transcription, combining the *soxR*^{Con} and *hfq* mutations resulted in complete loss of motility compared to the decreases observed in either single mutant. We note an inconsistency between *fliC* expression and motility in the *soxR*^{Con} mutant that is likely due to differences in oxygen concentrations in the medium, required for oxidation of the Fe-S cluster in SoxR (Hidalgo et al., 1995). Regardless, both transcription and motility support a model where SoxS controls expression of an Hfq-independent post-transcriptional pathway to control *flhDC* expression.

Exposure to chemical inducers of *marRAB* and *soxRS* results in reduced flagellar gene expression and motility.

To complement our ectopic expression studies performed in a *marRAB soxRS rob ramRA* mutant background, we explored the effects on flagellar gene expression and motility in a wild-type genetic background of two well-characterized chemical inducers of *marA* and *soxS* expression (**Figure 6**). Specifically, we examined the effects of salicylic acid and paraquat, inducers of

the *marRAB* and *soxRS* systems, respectively. Salicylic acid is also a Rob activator and chemorepellent for many bacteria working through the chemotaxis sensing network and has been shown to inhibit production of flagella in diverse species (Chubiz et al., 2012; Kunin et al., 1995; Tso & Adler, 1974). In contrast, the effects of paraquat, a redox-active electron transfer inhibitor, on bacterial motility are not well characterized.

Consistent with our ectopic expression data, chemical induction of MarA expression and activation of Rob with salicylic acid significantly reduced expression of all classes of flagellar genes (Student's *t*-test, all $P \leq 1.8 \times 10^{-5}$), as did paraquat induction of SoxS (Student's *t*-test, all $P \leq 1.5 \times 10^{-7}$) (**Figure 6A**). Reductions in Class II and Class III expression were larger in the presence of paraquat than salicylic acid (Student's *t*-test, both $P \leq 3.0 \times 10^{-9}$). This is in agreement with the existence of a post-transcriptional pathway activated preferentially by SoxS, characterized above.

Both chemical treatments resulted in reductions in FliC levels and motility. Commensurate with reductions in *fliC* expression, levels of FliC protein are reduced in the presence of salicylic acid or paraquat with paraquat resulting in less FliC than salicylic acid (**Figure 6B**). Conversely, motility was reduced in the presence of salicylic acid or paraquat with salicylic acid having more pronounced effects (**Figure 6C**). This is likely due to salicylic acid functioning as a chemorepellent, in addition to MarA and Rob-dependent transcriptional repression. Collectively, these data demonstrate that canonical chemical

inducers of the *marRAB* and *soxRS* gene systems cause reductions in flagellar gene expression and motility.

MarA and Rob mediate inhibition of motility in the presence of salicylic acid.

Having observed salicylic acid-dependent reductions in motility, we looked to test whether MarA and Rob were involved and found they are principal regulators of this response. Both MarA and Rob are known to respond to salicylic acid, forming a coherent feed forward regulatory loop to control expression of target genes (Chubiz et al., 2012; Jain & Saini, 2016). On systematic deletion of *marRAB* and *rob* in an otherwise wild-type genetic background, we found that salicylate-induced reductions in all classes of flagellar genes were at least partially restored (**Figure 7A**). For *flhDC* (Class I), deletion of *marRAB* or *rob* resulted in an equivalent, modestly-significant increase in Class I expression (Tukey HSD, P=0.037 and P=0.041, respectively) while the *marRAB rob* mutant resulted in a further increase to 79.2±3.7% of untreated levels (Tukey HSD, $P<1x10^{-7}$). Expression of *flhB* (Class II) displayed similar increases, however, the *marRAB rob* mutant had no significant increase compared to either of the single mutants (Tukey HSD, both P=0.99). Finally, *fliC* (Class III) transcription showed the largest recovery from 12.5±1.7% to 81.8±6.0% of untreated levels, comparing wild-type and the *marRAB rob* mutant during salicylic acid treatment (Tukey HSD, $P<1.0x10^{-7}$). Correspondingly, the *marRAB* and *rob* mutants each had nearly equivalent recoveries in *fliC* expression, although differences were

significant (Tukey HSD, P=0.017). Notably, the differences between the *marRAB* or *rob* single mutants and the double mutant, while significant (Tukey HSD, both $P \leq 3.3 \times 10^{-5}$), are not as large as changes from wild-type and may not be physiologically relevant. Although increases of flagellar expression in the presence of salicylic acid were observed in all permutations of *marRAB* and *rob* mutants, expression from all classes of promoters tested remained significantly below wild-type, untreated levels (Student's *t*-test, all $P \leq 0.0023$). These differences may be due to other stress responses to salicylic acid reducing flagellar gene expression.

Increases in flagellar gene expression in *marRAB* and *rob* mutants during salicylic acid exposure resulted in increased production of FliC. We found that in *marRAB*, *rob*, and *marRAB rob* mutants, FliC levels increased in the presence of salicylic acid, with the *marRAB rob* mutant showing slightly higher levels of FliC (**Figure 7B**). However, quantifying these differences by ELISA demonstrated no significant difference in FliC between any of the mutants, but all mutants had significantly elevated levels of FliC compared to wild-type (**Figure 7C**). The modest differences between single and double mutants is likely a result of the interconnected feed forward loop formed by MarA and Rob, wherein loss of either regulator hampers the overall downstream response (Chubiz et al., 2012; Jain & Saini, 2016).

Recovery of flagellar gene expression in *marRAB* and *rob* mutants in the presence of salicylic acid was also reflected in increases in motility (**Figure 7D**). Phenotypically, we observed a larger increase in motility in the *marRAB rob*

mutant compared to wild-type or either single mutant. Notably, the *marRAB* mutant did not result in the increase in motility as did the *rob* mutant, contrasting with gene expression data where *marRAB* and *rob* mutants appeared nearly equivalent (**Figure 7A**). These differences may reflect differences in liquid versus motility agar culturing conditions, where Rob seems to have a stronger repressive effect in the presence of salicylic acid. Notwithstanding, these data indicate that MarA and Rob-dependent repression of flagellar genes likely works in concert with a known chemosensing pathway to enhance negative chemotaxis away from repellents like salicylic acid.

4.3) Discussion

The expression of genes involved in flagellar biosynthesis and chemotaxis in *S.* Typhimurium, and related flagellated *Enterobacteriaceae*, is strongly influenced by many environmental signals through binding of transcription factors to the *flhDC* promoter and sRNAs interacting with *flhDC* mRNA. Here, we add an additional layer to the flagellar regulatory model. We demonstrated the homologous transcription factors MarA, SoxS, Rob and RamA directly inhibit motility by reducing expression of flagellar genes in *Salmonella*. In the case of MarA and Rob, this largely occurs through binding repression of the *flhDC* promoter. SoxS, on the other hand, primarily controls *flhDC* expression through a post-transcriptional pathway resulting in decreased translation of *flhDC*. While RamA regulation of *flhDC* was not explored in detail, here, our data indicates it behaves similarly to SoxS. This provides a new role for these regulators beyond

canonical association with multidrug resistance. Importantly, *S.* Typhimurium's use of these homologous transcription factors to control the flagellar regulon contrasts with regulatory patterns observed in *E. coli*. More broadly, these results demonstrate the flexibility of various cellular processes to be integrated into the global *mar-sox-rob* regulon, a regulon conserved throughout the *Enterobacteriaceae*.

Regulation of flagellar gene expression by MarA, SoxS, Rob, and RamA occurs in *S.* Typhimurium but has not been observed in *E. coli*. MarA, SoxS, and Rob have been well-studied in *E. coli*. In transcriptomic studies by Barbosa and colleagues and Martin and Rosner, no differential expression of the flagellar regulon was observed under ectopic expression conditions similar to those used in aspects of our study (Barbosa & Levy, 2000; Robert G. Martin & Rosner, 2002). Transcriptional profiling under salicylate and paraquat inducing conditions found no significant changes in expression of any flagellar or chemotaxis genes shared between *S.* Typhimurium and *E. coli* (Pomposiello, Bennik, & Demple, 2001). Contrasting these data with our findings suggests transcriptional repression of flagellar genes by MarA homologs may be unique to *S.* Typhimurium. Whether these differences are mediated by differences in MarA homolog promoter discrimination between the two species or divergence in regulatory regions of promoters in the flagellar regulon (namely *flhDC*) is not known. However, given the near complete similarity of MarA, SoxS, and Rob between *S.* Typhimurium and *E. coli* we hypothesize this is due to variation in the *flhDC* promoter between these species.

A number of historical differences in flagellar gene expression have been characterized between *Salmonella* and *E. coli*, reflected in sequence variation in the *flhDC* promoter region between these species (Mouslim & Hughes, 2014; O. A. Soutourina & Bertin, 2003; Wada, Hatamoto, & Kutsukake, 2012). Illustrating these differences, several studies have demonstrated the interactions of various regulators on SPI-1 and SPI-2 (not present in *E. coli*) with the *flhDC* promoter in regions not present in the *E. coli flhDC* promoter (C. D. Ellermeier & Slauch, 2003; Ilyas et al., 2018; Mouslim & Hughes, 2014; Singer et al., 2014). In fact, variation in interaction of these regulators, specifically SsrB from SPI-2, with *flhDC* has recently been shown to be part of the evolutionary transition of non-invasive *S. bongori* into *S. enterica*; suggesting the expression of the flagellar regulon is subject to intense selective pressure during adaptation to different lifestyles or environments such as within macrophages (Ilyas et al., 2018). While we have not yet defined a binding box for MarA and its homologs, it will be interesting to delineate where these interactions occur and if these regions are conserved in *E. coli*. Further examination of how the flagellar regulon is integrated into the broader *mar-sox-rob* stress response regulon may offer deeper insights into the functional role of *mar-sox-rob* in the *Enterobacteriaceae*, surprisingly cryptic despite over 20 years of ongoing research (Duval, 2013).

MarA homolog-dependent post-transcriptional regulation of flagellar gene expression also plays a role in repressing flagellar genes in *Salmonella* in response to chemical stress. Indeed, several sRNA interactions with *flhDC* mRNA have been characterized (de Lay & Gottesman, 2012). The MarA

homologs in *S.* Typhimurium do not have any known regulatory interactions with these sRNA. In ongoing transcriptomics research, we have observed increases in OmrA and OmrB production, two sRNAs involved in regulating porin and flagellar gene expression (de Lay & Gottesman, 2012), during ectopic expression of MarA homologs. However, targeted genetic knockouts of *omrA/B* yielded no changes in motility phenotypes (data not shown). Therefore, if this post-transcriptional mechanism is sRNA-based, and the sRNA is activated by MarA homologs similar to MicF (Chubiz & Rao, 2011; Liu & Ferenci, 1998), the acting sRNA is likely unknown as no small RNA sequencing efforts have been conducted under chemical or ectopic induction of MarA homologs. Adding additional complexity, the post-transcriptional mechanism we have observed is Hfq-independent. While this does not preclude sRNA-mediated repression as a potential mechanism, it excludes correlating Hfq RNA immunoprecipitation data to identify possible candidates (Holmqvist et al., 2016). Finally, we have seen activation of cryptic MarA-dependent post-transcriptional regulatory pathways affect porin expression in *E. coli*, mirroring results seen here (Chubiz & Rao, 2011). Future efforts in small RNA sequencing under conditions explored in this study will shed light on novel sRNA regulator(s) of flagellar gene expression.

Why might *Salmonella* integrate control of flagellar gene expression into the global *mar-sox-rob* regulon? An answer may be that periods of *Salmonella's* lifestyle in the host are benefited by decreasing flagellar synthesis and motility. One such location is within macrophages. Notably, within macrophages *marA*, *soxS*, and *ramA* expression is induced 4 to 25 fold and flagellar gene expression

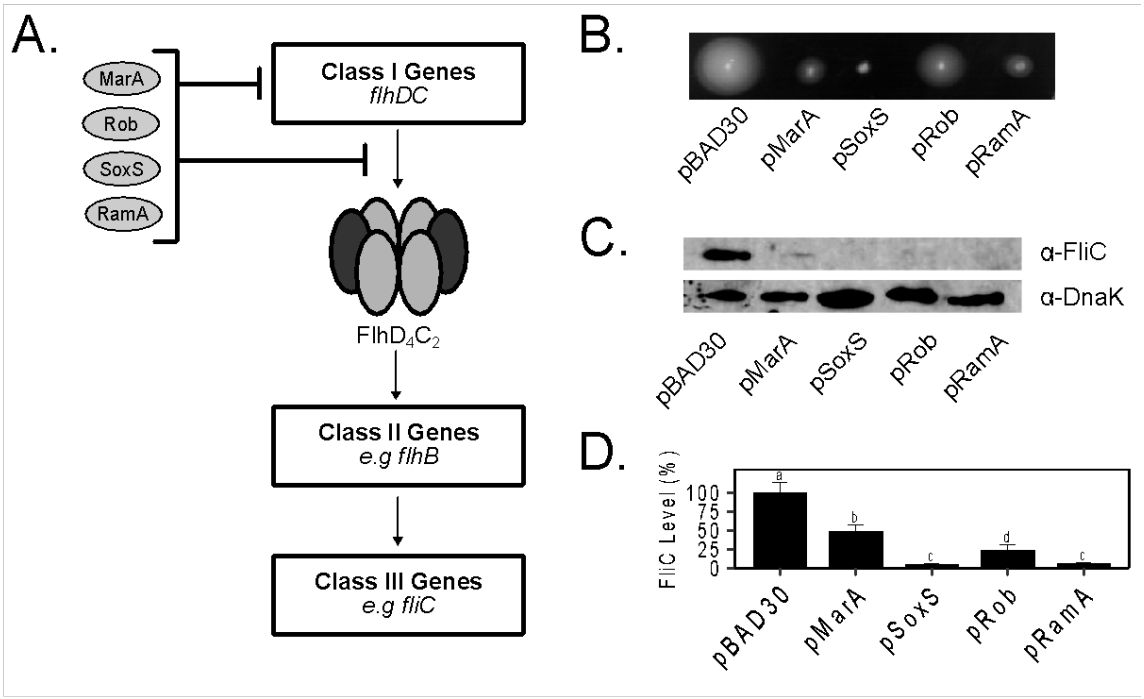
is decreased 20 fold, similar to our *ex vivo* findings (Kröger et al., 2013; Srikumar et al., 2015). The degree to which these events are correlated has not been explored. While it is intriguing to speculate on the importance of these concomitant changes, it is worth noting that Fang and coworkers found that a *soxS* mutant has no apparent effect on virulence in a murine infection model or survival in murine macrophages (Fang, Vazquez-Torres, & Xu, 1997). Contradicting these findings, Bailey and coworkers have more recently demonstrated mutants in *ramA* do attenuate within host survival in a number of infection models including mice (A. M. Bailey et al., 2010). Cast in the light of our current results, it may be overlapping functional redundancy of MarA homologs that masks singular loss of *marA*, *soxS*, *rob*, or *ramA*. Further, each regulator may contribute differently such as RamA having stronger effects on virulence than MarA, SoxS, or Rob alone, much like flagellar regulation (A. M. Bailey et al., 2010; Fang et al., 1997; Prouty et al., 2004; Sulavik et al., 1997). Likewise, MarA homologs may work in conjunction with other regulators, such as SsrB, to enable more robust repression of flagellar expression within macrophages. In this way, *mar-sox-rob* may serve as an additional mechanism of regulatory reinforcement during *S.* Typhimurium's transition to an intracellular lifestyle.

Salmonella uses a transcriptional control to enhance negative chemotaxis. Salicylate is a well-documented chemorepellent in *E. coli* occurring through the chemosensing network via the receptor Tsr as well as alteration of intracellular pH (Repaske & Adler, 1981; Tso & Adler, 1974). Here, we have found that *Salmonella* uses transcriptional and post-transcriptional regulation, in addition to

chemotaxis, to avoid toxic aromatic acids. By adding a transcriptional layer of control to negative chemotaxis, *S.* Typhimurium may be able to more finely tune concentrations of chemoreceptors to enhance sensing of repellent compounds in the midst of attractants. Given salicylate is sensed by Tsr, which also senses a variety of attractants such as serine, lowering levels of Tsr may allow salicylate to outcompete binding of attractants thereby enhancing negative chemotaxis (Kalinin, Neumann, Sourjik, & Wu, 2010; Y. Yang & Sourjik, 2012).

Regulation of flagellar gene expression in *S.* Typhimurium is complex. Here, we add an additional mechanism of control during chemical stress, repression by the global stress response transcription factors MarA, SoxS, Rob, and RamA. Broadly, this result highlights the evolutionary plasticity of global gene regulation within the *Enterobacteriaceae*. More pointedly, our findings add to a growing understanding of the diverse targets of the *mar-sox-rob* regulatory system throughout this bacterial family. Continued exploration of *mar-sox-rob* regulatory targets will undoubtedly shed light on the role of MarA homologs in regulating the diverse physiology and metabolism in *E. coli's* many relatives.

Figure 1.



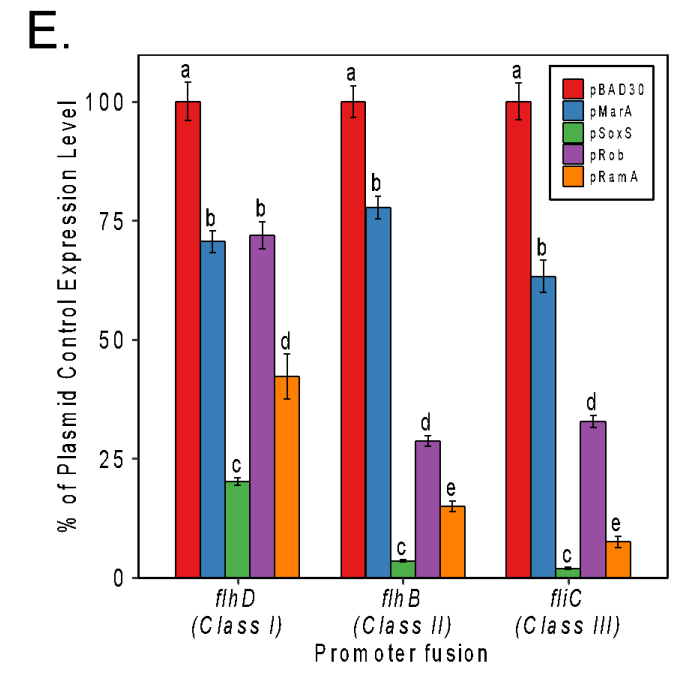
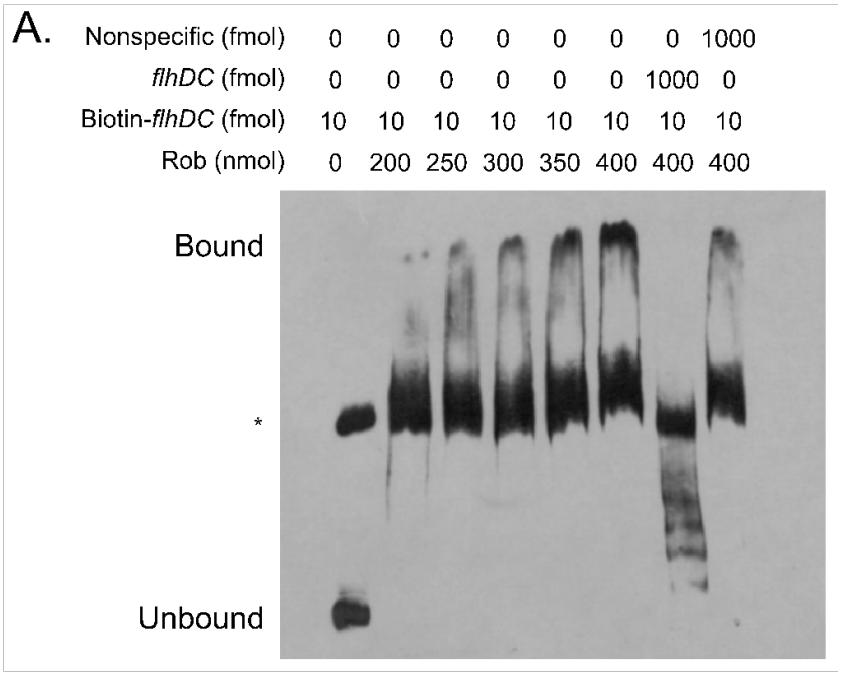


Figure 1. Repression of motility, flagellin levels and flagellar gene expression by MarA, SoxS, Rob, and RamA. Each transcription factor was expressed from

pBAD30 (pMarA, pSoxS, pRob, or pRamA) in a *marRAB soxRS rob ramRA* quadruple mutant genetic background (strain LCM2380) unless otherwise noted. **A)** A proposed model of MarA, SoxS, Rob, and RamA repression of flagellar gene expression. **B)** The effects of MarA, SoxS, Rob, and RamA production on motility compared to a pBAD30 plasmid control. Motility assays were conducted at room temperature in soft tryptone agar supplemented with 0.2% arabinose to induce expression of *marA*, *soxS*, *rob*, or *ramA*. **C)** The effect of MarA, SoxS, Rob, and RamA production on levels of flagellin (FliC) compared to a pBAD30 plasmid control as determined by Western blot. Cell extracts from cultures grown in tryptone broth supplemented with 0.2% arabinose were displayed (100 µg total protein) on 12% acrylamide SDS-PAGE prior to transfer to a PVDF membrane and immunoblotting for FliC and DnaK. DnaK levels were used as an internal loading control for each sample. **D)** The effect of MarA, SoxS, Rob, and RamA production on levels of flagellin (FliC) compared to a pBAD30 plasmid control quantified by an indirect enzyme-linked immunosorbent assay (ELISA) using FliC primary and HRP-conjugated secondary antibodies. Cell extracts (25 µg total protein) used for ELISA were obtained under the same conditions for Western blots, above. Light emission from each sample was normalized to the pBAD30 plasmid control and presented as a percentage of the FliC level in the pBAD30 plasmid control. Six replicate measurements were made for each plasmid bearing strain. Letter labels (a-d) represent statistically significant groups (Tukey HSD, $P<0.05$). **E)** The effects of MarA, SoxS, Rob, or RamA production on *flhD*, *flhB*, and *fliC* transcription compared to a pBAD30 plasmid control. *marA*, *soxS*,

rob, or *ramA* were expressed from pBAD30 in strains where f*lhD*, *flhB*, and *fliC* promoters were transcriptionally fused to *yfp* in the quadruple mutant genetic background (strains LCM2416, LCM2431, and LCM2446). Fluorescence measurements were made with mid-logarithmic cultures grown in tryptone broth supplemented with 0.2% arabinose and normalized to culture density. Levels of *flhD*, *flhB*, and *fliC* promoter activity in each plasmid bearing background are presented as a percentage of the expression from each promoter in the pBAD30 plasmid control. Six replicate measurements were made for each plasmid bearing strain. Letter labels (a-e) represent statistically significant groups (Tukey HSD, $P<0.05$) for each transcriptional fusion.

Figure 2.


A.
Nonspecific (fmol) 0 0 0 0 0 0 0 1000
flhDC (fmol) 0 0 0 0 0 0 1000 0
Biotin-flhDC (fmol) 10 10 10 10 10 10 10 10
Rob (nmol) 0 200 250 300 350 400 400 400
Bound
*
Unbound

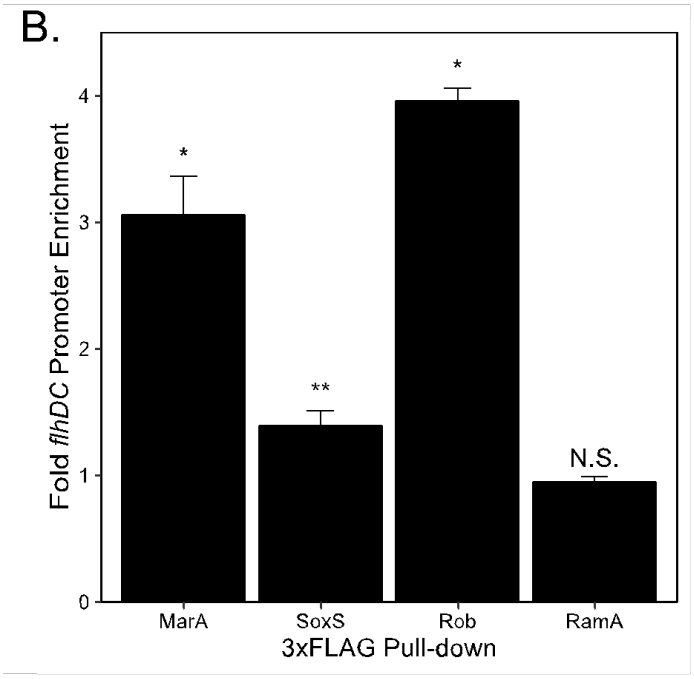

B.
Fold flhDC Promoter Enrichment
0
1
2
3
4
*
**
*
N.S.
MarA
SoxS
Rob
RamA
3xFLAG Pull-down

Figure 2. Binding of MarA homologs to the *flhDC* promoter region. **A)** Electromobility shift assay (EMSA) of a 559 bp *flhDC* promoter fragment in the presence of indicated concentrations of purified Rob protein, biotinylated *flhDC* promoter DNA, and competitor DNA (unlabeled 172bp region of *gyrA* was used as non-specific competitor). The asterisk (*) indicates a non-specific biotinylated DNA band. **B)** Fold enrichment of a *flhDC* promoter fragment in DNA co-immunoprecipitated by capture of 3xFLAG tagged MarA, SoxS, Rob and RamA proteins expressed from pBAD30 in a wild-type background (LCM1930). Fold enrichment was determined by qPCR using *gyrA* as an internal control. Asterisks (*) and (**) indicate Student's *t*-test $P<0.005$ and $P<0.05$, respectively.

Figure 3.

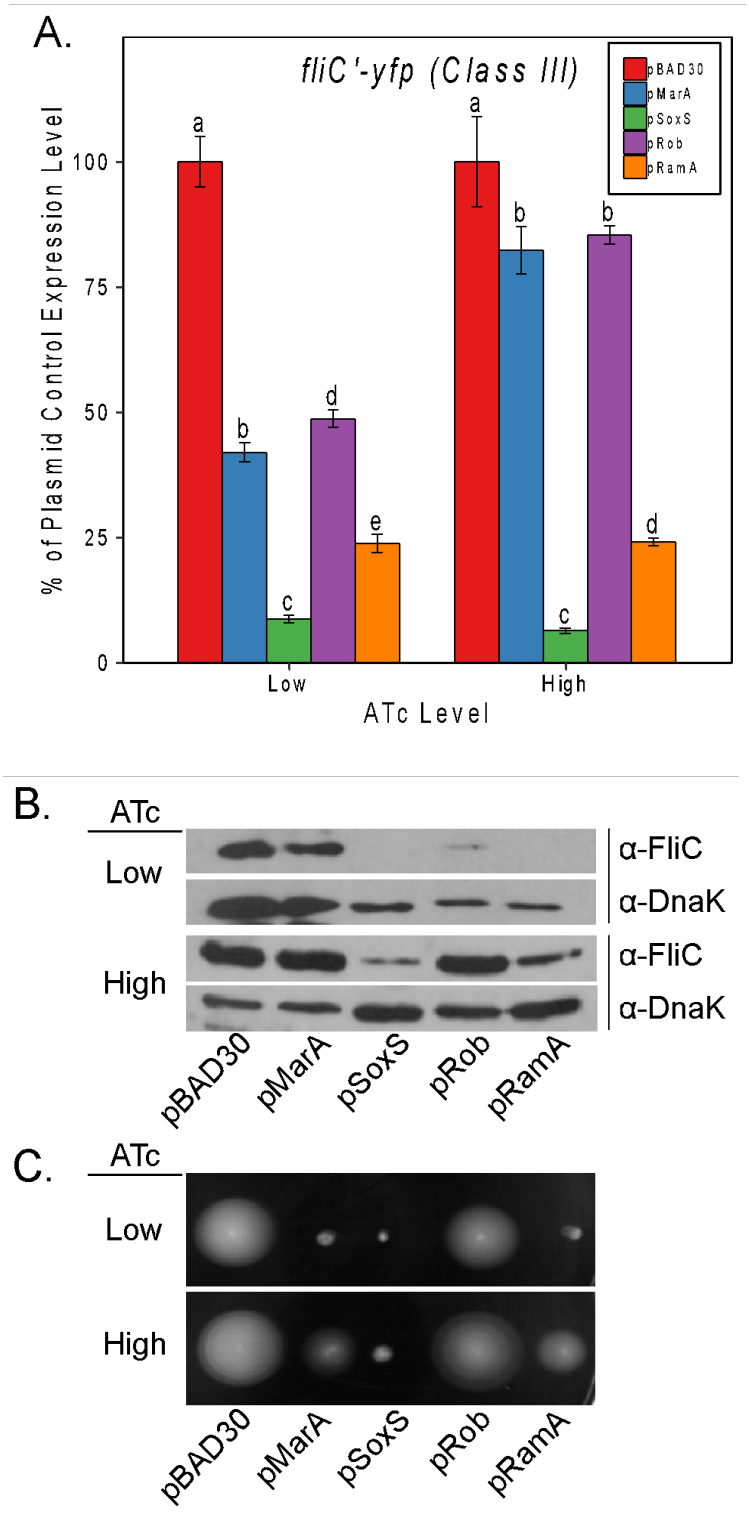

A.
fliC'-yfp (Class III)
pBAD30
pMarA
pSoxS
pRob
pRamA
% of Plasmid Control Expression Level
0
25
50
75
100
a
b
c
d
e
a
b
c
b
d
Low
High
ATc Level
B.
ATc
Low
High
α-FliC
α-DnaK
α-FliC
α-DnaK
pBAD30
pMarA
pSoxS
pRob
pRamA
C.
ATc
Low
High
pBAD30
pMarA
pSoxS
pRob
pRamA

Figure 3. Production of MarA, SoxS, Rob, and RamA results in post-transcriptional repression of *flhDC*. Expression of *flhDC* was driven by a tetracycline-inducible promoter at ATc concentrations of 1 ng/ml (Low) and 100 ng/ml (High) and *marA*, *soxS*, *rob*, and *ramA* were ectopically expressed from pBAD30. **A)** The effects of MarA, SoxS, Rob, and RamA on *fliC* transcription in a tetracycline-inducible *flhDC* genetic background (strain LCM2701) where native *flhDC* regulation has been removed. Fluorescence measurements were made with mid-logarithmic cultures grown in tryptone broth supplemented with 0.2% arabinose and indicated ATc levels, followed by normalization to culture density. Data for each transcriptional fusion are presented as a percentage of *fliC* expression observed in the pBAD30 plasmid control. Six replicate measurements were made for each plasmid bearing strain. Letter labels (a-e) indicate statistically significant groups (Tukey HSD, $P<0.05$) for each ATc treatment. **B)** MarA, SoxS, Rob, and RamA effects on FliC levels in low and high ATc treatments as measured by Western blot. Cell extracts from cultures grown in tryptone broth supplemented with 0.2% arabinose were displayed (100 µg total protein) on 12% acrylamide SDS-PAGE prior to transfer to a PVDF membrane and immunoblotting for FliC and DnaK. DnaK levels were used as an internal loading control for each sample. **C)** Effects of MarA, SoxS, Rob, and RamA on motility in low and high ATc treatments. Motility assays were conducted at room temperature in soft tryptone agar supplemented with 0.2% arabinose. Both western blot and motility assays were performed using strain LCM2678.

Figure 4.

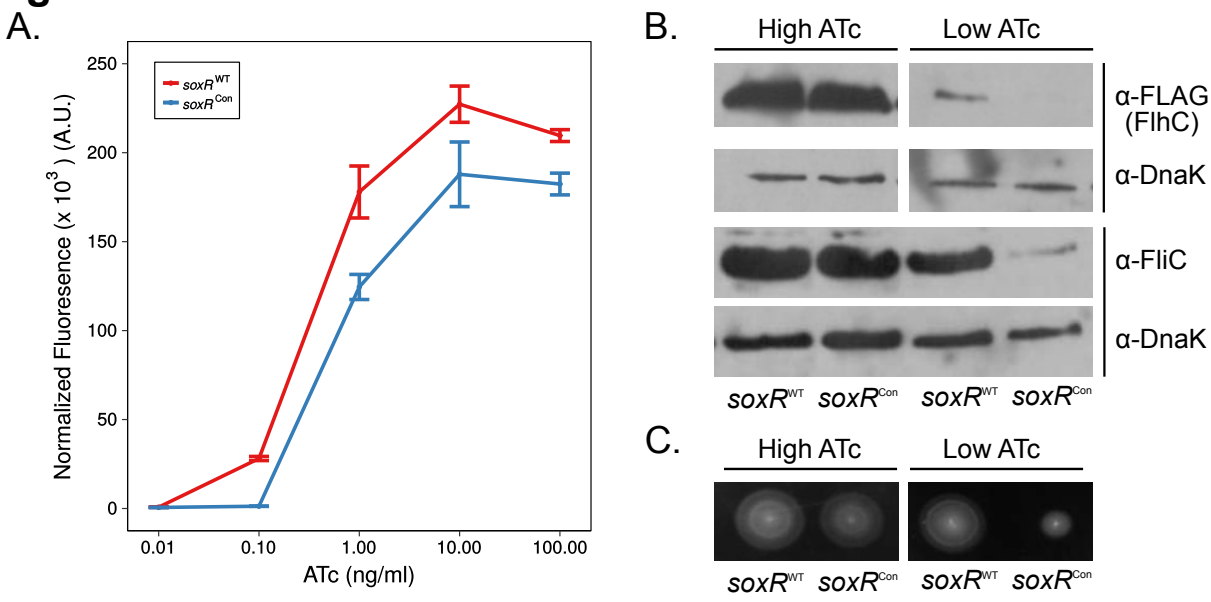


Figure 4. Moderate, constitutive levels of SoxS result in post-trancriptional repression of *flhDC*. **A)** Levels of *fliC* transcription in a tetracycline-inducible *flhDC* genetic background with wild-type (*soxR*^{WT}) and mutant with constitutive *soxS* expression (*soxR*^{Con}) (strains LCM2701 and LCM2716). Fluorescence measurements were made with mid-logarithmic cultures grown in tryptone broth supplemented with indicated ATc concentrations and normalized to culture density. Differences between *soxR*^{WT} and *soxR*^{Con} at all ATc concentrations are significant (Student's *t*-test, $P \leq 0.0018$). **B)** The effects of *soxR*^{WT} and *soxR*^{Con} on FlhC-3xFLAG (strains LCM2712 and LCM2713) and FliC (strains LCM2678 and LCM2687) levels in a tetracycline-inducible genetic *flhDC* genetic background as measured by Western blot. Expression of *flhDC* was induced with low (1 ng/ml) and high (100 ng/ml) ATc treatments. Cell extracts from cultures grown in tryptone broth supplemented with indicated ATc concentrations were displayed

(100 µg total protein) on 12% acrylamide SDS-PAGE prior to transfer to a PVDF membrane and immunoblotting for 3xFLAG (FlhC), FliC, and DnaK. DnaK levels were used as an internal loading control for each sample. **C)** Effects of *soxR*^{WT} (LCM2678) and *soxR*^{Con} (LCM2687) on motility in the tetracycline-inducible *flhDC* background. Motility assays were conducted at room temperature in soft tryptone agar supplemented with low or high ATc concentrations, mentioned above.

Figure 5.

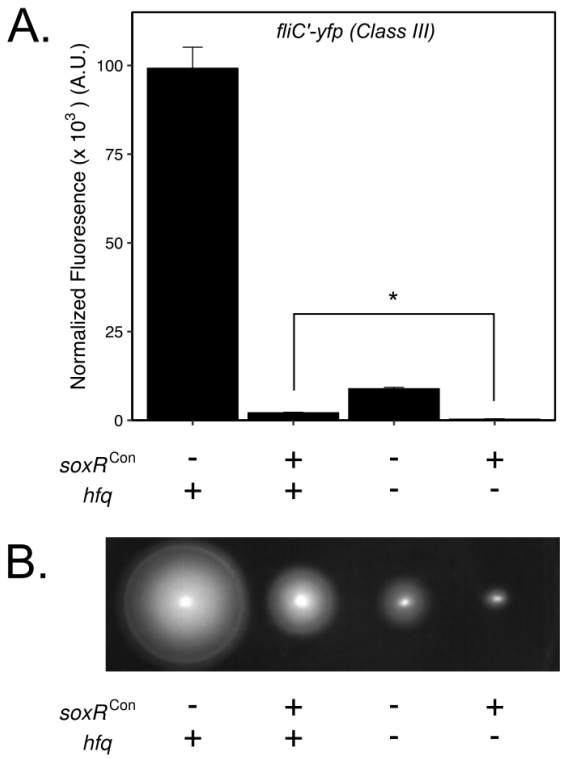
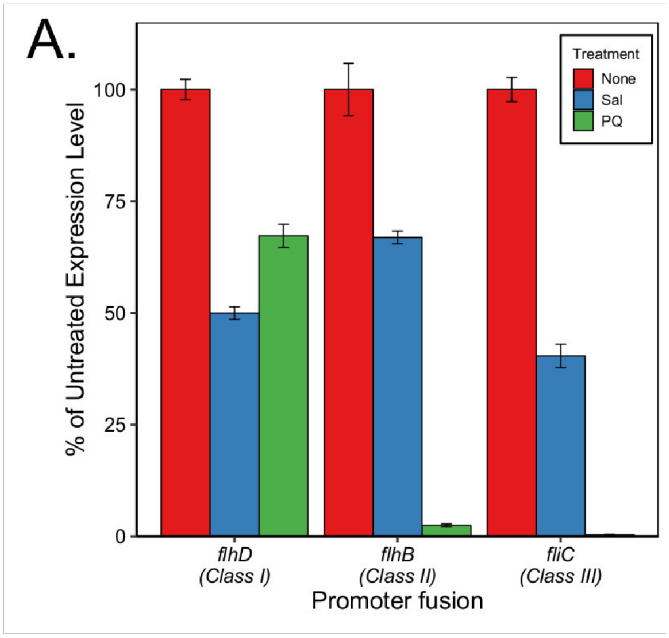
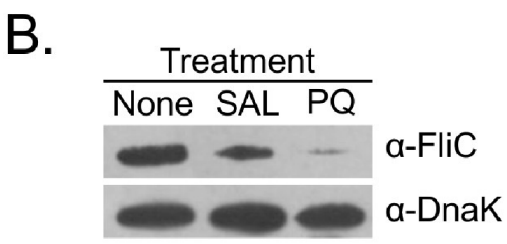


Figure 5. Post-transcriptional regulation of *flhDC* is independent of Hfq. **A)** Levels of *fliC* transcription in wild-type, *soxR*^{Con}, *hfq*, and *soxR*^{Con} *hfq* genetic backgrounds (strains LCM2326, LCM2473, LCM2714, and LCM2715). Fluorescence measurements were made with mid-logarithmic cultures grown in tryptone broth. All mutants are significantly lower than wild-type (Student's *t*-test, $P \leq 2.4 \times 10^{-7}$). The asterisk (*) indicates a significant difference between *soxR*^{Con} and *soxR*^{Con} *hfq* mutants (Student's *t*-test, $P = 8.1 \times 10^{-11}$) **B)** Motility in wild-type, *soxR*^{Con}, *hfq*, and *soxR*^{Con} *hfq* backgrounds (strains LCM1930, LCM2449, LCM2597, and LCM2598). Motility assays were conducted at room temperature in soft tryptone agar.

Figure 6.


A.
Treatment
None
Sal
PQ
% of Untreated Expression Level
100
75
50
25
0
flhD
(Class I)
flhB
(Class II)
fliC
(Class III)
Promoter fusion


B.
Treatment
None
SAL
PQ
α-FliC
α-DnaK

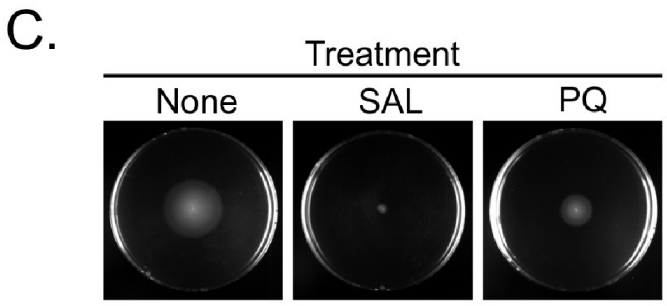
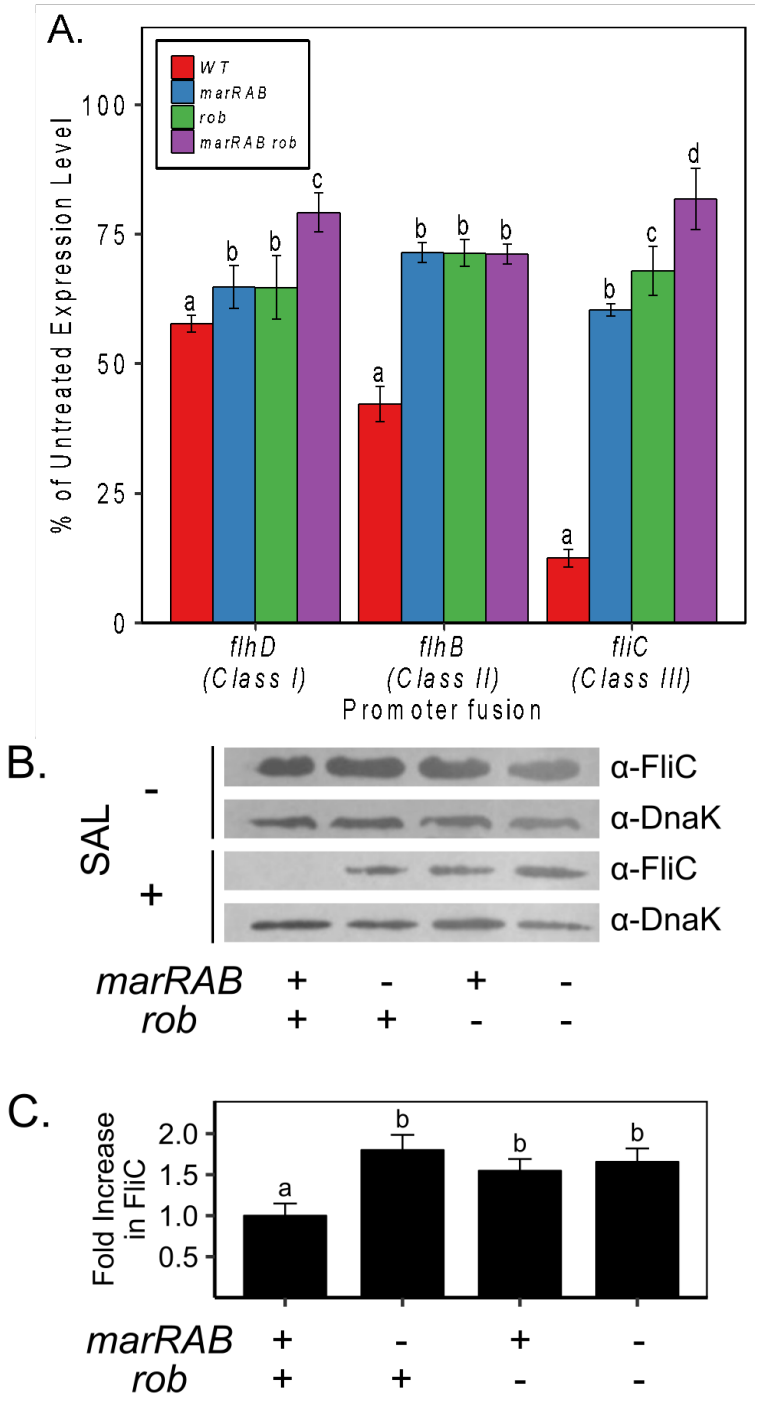

C.
Treatment
None
SAL
PQ

Figure 6. Repression of flagellar genes, motility, and flagellin production by salicylic acid and paraquat. **A)** Effects of salicylic acid and paraquat on transcription of *flhD*, *flhB*, and *fliC* promoters (strains LCM2324, LCM2325, and LCM2326). Fluorescence measurements were made with mid-logarithmic cultures grown in tryptone broth supplemented with sodium salicylate (3 mM) or paraquat (50 μM). Fluorescence measurements were normalized to culture density and presented as a percentage of the untreated expression level for each promoter fusion. All decreases in expression were significant (Student's *t*-test, $P \leq 1.8 \times 10^{-5}$). **B)** Levels of FliC protein as determined by Western blot in the presence of salicylic acid or paraquat in wild-type (LCM1930). Cell extracts from cultures grown in tryptone broth supplemented with indicated concentrations of sodium salicylate or paraquat were displayed (100 μg total protein) on 12% acrylamide SDS-PAGE prior to transfer to a PVDF membrane and immunoblotting for FliC and DnaK. DnaK levels were used as an internal loading control for each sample. **C)** Effects of salicylic acid and paraquat on motility in wild-type (LCM1930). Motility assays were conducted at room temperature in soft tryptone agar supplemented with sodium salicylate or paraquat at concentrations described above.

Figure 7.


A.
WT
marRAB
rob
marRAB rob
% of Untreated Expression Level
100
75
50
25
0
a
b
b
c
a
b
b
b
a
b
c
d
flhD
(Class I)
flhB
(Class II)
fliC
(Class III)
Promoter fusion
B.
SAL
-
+
α-FliC
α-DnaK
α-FliC
α-DnaK
marRAB + - + -
rob + + - -
C.
Fold Increase in FliC
2.0
1.5
1.0
0.5
a
b
b
b
marRAB + - + -
rob + + - -

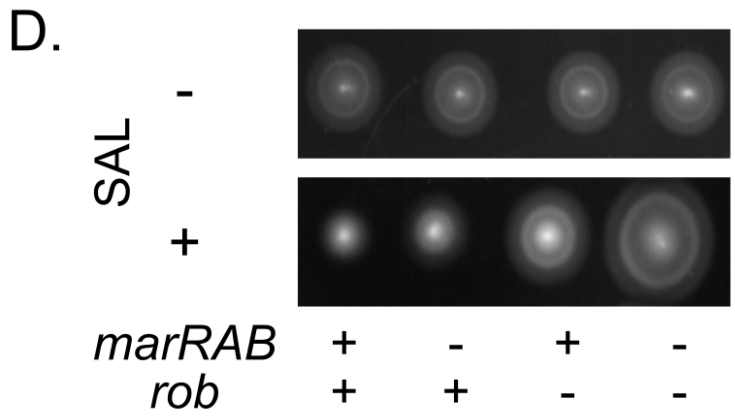


Figure 7. Recovery of flagellar gene expression and motility in the presence of salicylic acid when MarA- and Rob-dependent repression is alleviated. **A)** Transcription levels of *flhD*, *flhB*, and *fliC* promoter transcriptional fusions to *yfp* in wild-type (strains LCM2324, LCM2325, and LCM2326), *marRAB* (strains LCM2399, LCM2417, LCM2432), *rob* (strains LCM2401, LCM2419, and LCM2434), and *marRAB rob* (strains LCM2407, LCM2422, and LCM2437) genetic backgrounds. Fluorescence measurements were made with mid-logarithmic cultures grown in tryptone broth supplemented with sodium salicylate (3 mM). Fluorescence measurements were normalized to culture density and presented as a percentage of the untreated expression level for each promoter fusion. Letter labels (a-d) represent statistically significant groups (Tukey HSD, $P<0.05$) for each transcriptional fusion. **B)** Levels of FliC protein in wild-type (LCM1930), *marRAB* (LCM2366), *rob* (LCM2368) and *marRAB rob* (LCM2371) as measured by Western blot. Cell extracts from cultures grown in tryptone broth supplemented with or without sodium salicylate (3 mM) were displayed (100 µg total protein) on 12% acrylamide SDS-PAGE prior to transfer to a PVDF membrane and immunoblotting for FliC and DnaK. DnaK levels were used as an

internal loading control for each sample. **C)** The effect of *marRAB*, *rob*, and *marRAB rob* mutations on FliC levels in the presence of salicylic acid quantified by ELISA. Cell extracts (25 µg total protein) used for ELISA were obtained under the same conditions for Western blots, above. Light emission from each sample was normalized to wild-type FliC levels and presented as a fold increase in FliC abundance. Six replicate measurements were made for each plasmid bearing strain. Letter labels (a, b) represent statistically significant groups (Tukey HSD, $P<0.05$) between each genetic background. **D)** The effects of *marRAB* (LCM2366), *rob* (LCM2368), and *marRAB rob* (LCM2371) deletions on motility in the presence and absence of salicylic acid compared with wild-type (LCM1930). Motility assays were conducted at room temperature in soft tryptone agar supplemented with or without sodium salicylate (3 mM).

Figure 8.

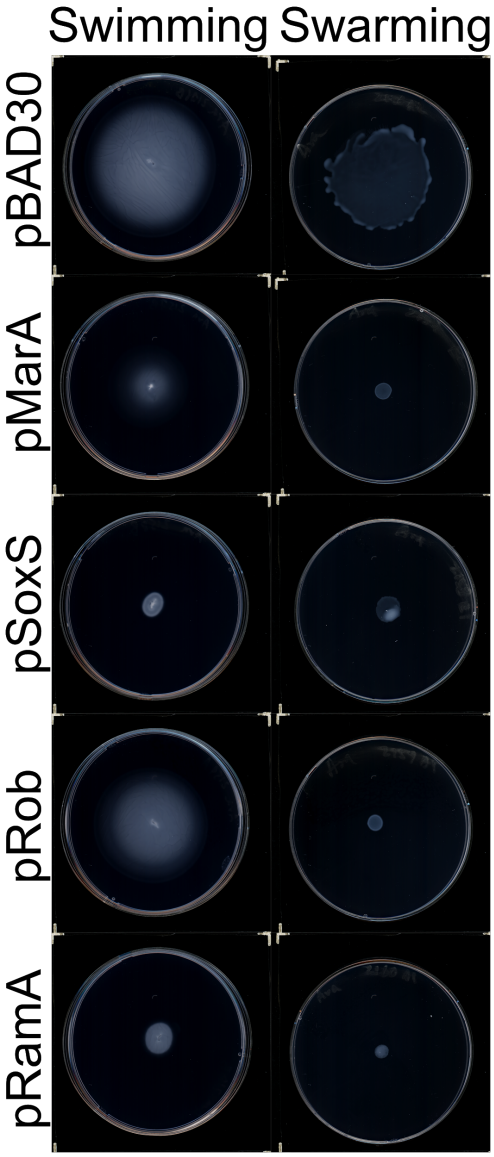


Figure 8. Swimming (see Methods and Materials) and swarming motility wild-type *Salmonella enterica* serovar Typhimurium LT2 (LCM1930) with *marA*, *soxS*, *rob*, or *ramA* ectopically expressed from pBAD30 compared to a plasmid control. Swimming and swarming assays were conducted at room temperature using tryptone agar plates with 0.3% agar for swimming and 0.6% agar and 0.02% Tween-80 for swarming.

Figure 9.

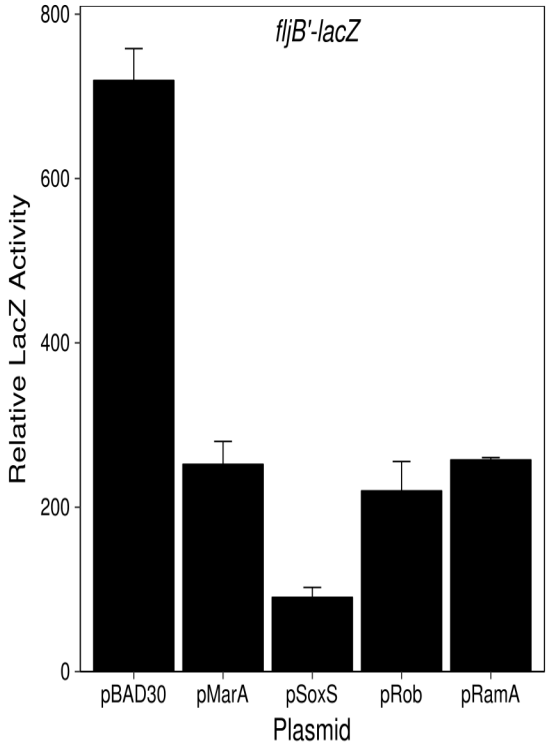


Figure 9. Expression of a *fljB'-lacZ* transcriptional fusion (LCM2700) when *marA*, *soxS*, *rob*, or *ramA* are ectopically expressed from pBAD30. LacZ activities are presented as $(A_{420}*min^{-1})/(ml*A_{600})$. All reductions in *fljB* expression were significant compared to the pBAD30 plasmid control (Student's *t*-test, $P<1x10^{-7}$).

Figure 10.

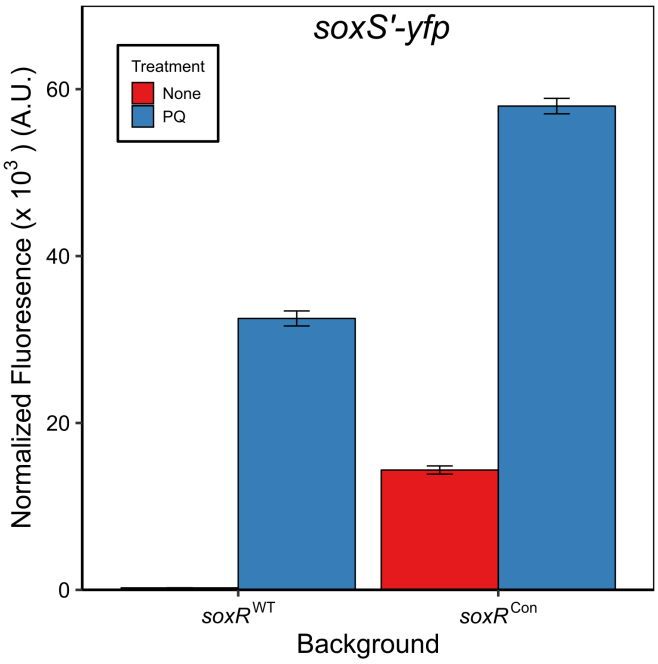


Figure 10. Expression of *soxS* measured via a *soxS* transcriptional fusion to *yfp* in wild-type (*soxR*^{WT}, LCM2669) and consitutively active SoxR (*soxR*^{Con}, LCM2672) genetic backgrounds, with or without paraquat (PQ). Fluorescence measurements were made with mid-logarithmic cultures grown in tryptone broth with or without paraquat (50 μM) and normalized to optical density (OD_{600}).

Figure 11.

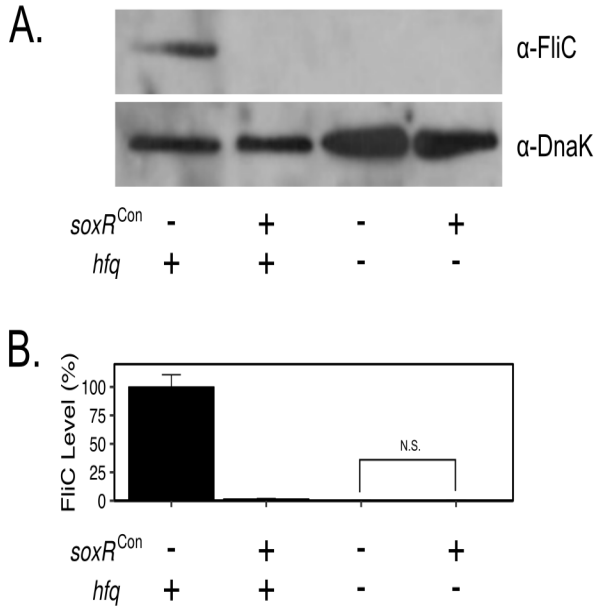


Figure 11. FliC is repressed in *soxR*^{Con} and *hfq* genetic backgrounds. **A)** Levels of FliC protein in wild-type (LCM1930), *soxR*^{Con}(LCM2449), *hfq* (LCM2597), and *soxR*^{Con} *hfq* (LCM2598) mutant backgrounds measured by Western blot. Cell extracts from cultures grown in tryptone broth supplemented with 0.2% arabinose were displayed (100 µg total protein) on 12% acrylamide SDS-PAGE prior to transfer to a PVDF membrane and immunoblotting for FliC and DnaK. DnaK levels were used as an internal loading control for each sample. **B)** Levels of FliC protein in wild-type (LCM1930), *soxR*^{Con}(LCM2449), *hfq* (LCM2597), and *soxR*^{Con} *hfq* (LCM2598) mutant backgrounds quantified by an indirect enzyme-linked immunosorbent assay (ELISA) using FliC primary and HRP-conjugated secondary antibodies. Cell extracts (25 µg total protein) used for ELISA were obtained under the same conditions for Western blots, above. Light emission from each sample was normalized to wild-type and presented as a percentage of

the FliC level in wild-type. Six replicate measurements were made for each plasmid bearing strain. All reductions in FliC levels measured by ELISA are significant (Student's t-test, $P<1x10^{-7}$), unless indicated as non-significant (N.S.).

Chapter 5: Regulation of SPI-1 genes by MarA homologs in *Salmonella enterica* serovar Typhimurium

5.1) Introduction

In *S.* Typhimurium two type 3 secretion systems (T3SSs) are regulated temporally and spatially for successful infection of host cells (Hapfelmeier et al., 2004). T3SS-1 is coded in a gene cluster on the chromosome termed as *Salmonella* pathogenic island (SPI-1). SPI-1 comprises all genes that code for proteins that make up the needle-like complex, effector proteins, and translocases. *S.* Typhimurium uses the T3SS-1 needle complex to inject effector proteins into epithelial cells and macrophages (Zhou & Galán, 2001). The secretion of effector proteins into epithelial cells is important for *S.* Typhimurium entry and activation of host pathways that signal the recruitment of neutrophils into the intestine. The effector proteins of *S.* Typhimurium also facilitate its replication and survival in *Salmonella* containing vacuole (SCV) inside epithelial cells and macrophages (Brawn, Hayward, & Koronakis, 2007). T3SS-1 effector proteins suppress expression of pro-inflammatory cytokines in macrophages to stall a premature inflammatory response. *S.* Typhimurium uses T3SS-1 primarily for invasion and T3SS-2 coded by a separated pathogenicity island (SPI-2) for intracellular survival. SPI-1 and SPI-2 genes are inversely regulated (Waterman & Holden, 2003; Zhou & Galán, 2001). This requires integration of numerous signals and regulatory networks. Elucidating these regulatory networks further is an important step in understanding *S.* Typhimurium pathogenesis.

The SPI-1 locus is an approximately 40 kb long region, which codes for at least 39 different proteins that make up the T3SS-1 needle complex, regulatory proteins, effector proteins and translocases. Also, some of the genes that code for T3SS-1 proteins are located outside the SPI-1 locus (Lou et al., 2019a). The T3SS needle, which is evolutionarily related to bacterial flagella spans both the membranes of bacteria and extends outside of the cell. The needle complex consists of a multi-ring cylindrical base formed by InvG, PrgH and PrgK proteins; an internal rod embedded in the base formed by PrgJ, which is connected to the needle that starts outside outer membrane formed by PrgI. SipD, SipC and SipB form a translocase complex at the tip that assists effectors to reach the host cells. SipB and SipC form a channel inside the host membrane that connects to the needle channel via SipD (Kubori, Sukhan, Aizawa, & Galán, 2000; Lara-Tejero & Galán, 2009).

Following invasion, SPI-1 effectors (importantly SipA, SopA and SptP) serve as proinflammatory signals in epithelial cells. The epithelial cells subsequently secrete cytokines that recruit neutrophils and other immune cells that promote clearing of many commensal microorganisms. This ultimately reduces competitive pressure on *S.* Typhimurium in the lumen (Hapfelmeier et al., 2004; Malik-Kale et al., 2011; Rivera-Chávez & Bäumler, 2015).

The virulence-associated gene product AvrA, *Salmonella* invasion proteins (Sips), *Salmonella* protein tyrosine phosphatase (SptP), and *Salmonella* outer proteins (Sops) are important among effector proteins that play a role in invasion, replication of *S.* Typhimurium inside the host cell and immune modulation

(Johnson et al., 2017; Wallis et al., 2000; H. Wu, Jones, & Neish, 2012). SipA polymerizes actin filaments of cytoskeleton leading to membrane ruffling of host cells. This enables *S.* Typhimurium to enter non-phagocytic epithelial cells. SipA also enables *S.* Typhimurium replication in the SCV. SptP on the other hand dissociates actin filaments and reduces membrane ruffling after invasion. AvrA down-regulates the expression of the NF-kB transcription factor, an activator of proinflammatory cytokines delaying early immune response. SopA induces recruitment of neutrophils into the intestinal lumen that will eventually cause inflammation and fluid secretion into the lumen that causes diarrhea. Other Sop proteins like SopD, $SopD_2$, and SopE modulate host mechanisms for *Salmonella* intracellular replication (Johnson et al., 2017; Wallis et al., 2000; H. Wu et al., 2012; Zhou & Galán, 2001).

A complex regulatory network that senses numerous environmental signals control the expression of SPI-1 genes (**Figure 1**). HilA is the master regulator of SPI-1 genes. It activates the expression of InvF, which activates several SPI-1 genes. *hilA* is activated by HilD, HilC and RtsA of which HilD is the primary activator. HilD, HilC and RtsA activate each other and auto-activate themselves (Boddicker, Knosp, & Jones, 2003; C. D. Ellermeier & Slauch, 2003; Lucas & Lee, 2001). HilE negatively regulates expression of SPI-1 genes by destabilizing HilD (Baxter, Fahlen, Wilson, & Jones, 2003). A flagellar gene regulator FliZ activates *hilD* post-transcriptionally and represses *fimZ*, an activator of *hilE*. A carbohydrate metabolism regulator Mlc represses transcription of *hilE* resulting in activation of SPI-1 genes. CsrA acts as a

negative regulator of SPI-1 genes. It binds to *hilD* RNA and stalls its translation. BarA/SirA – a two component system activates SPI-1 genes by activating the expression of *csrB/C* sRNAs that sequester CsrA. This relieves *hilD* from CsrA repression (Altier, Suyemoto, & Lawhon, 2000; Cott Chubiz, Golubeva, Lin, Miller, & Slauch, 2010; Lim et al., 2007; Lucas & Lee, 2001).

S. Typhimurium turns on SPI-1 virulence genes specifically in the ileum of human hosts to invade epithelial cells. After entry into the host cell, SPI-1 genes are turned off followed by subsequent activation of the SPI-2 T3SS-2 that is essential for survival of *S.* Typhimurium inside the host cell. Expression of virulence traits in unwanted regions could be deleterious for *S.* Typhimurium due to the high energy burden and premature activation of the immune response. To temporally and spatially regulate virulence genes, *Salmonella* successfully senses the anatomical location in the intestine. It integrates the environmental signals specific to an intestinal location with its regulatory networks. Bile secreted by the gall bladder into the intestine is a known repressor of SPI-1 genes in *Salmonella enterica*. Indole, a biproduct of bacterial metabolism of intestinal microbiota also represses SPI-1 genes (Gart et al., 2016; Hung et al., 2016). SPI-1 genes are also repressed when *S.* Typhimurium is inside macrophages and epithelial cells. Multidrug resistance genes regulator Rob is post-translationally activated by bile and RamA, a Rob homolog is activated by indole (Griffith et al., 2009; E. Nikaido et al., 2008). Recent transcriptomic studies of *S.* Typhimurium within SCVs have shown that expression of all four MarA homologs is upregulated inside macrophages concomitant with downregulation of SPI-1

genes (Avital et al., 2017). My transcriptomic data (**Figure 2, Chapter 3**) also indicated that over-expression of MarA homologs downregulated SPI-1 genes. Data presented in Chapter 4 demonstrated that all flagellar genes are down regulated by MarA homologs. MarA homologs directly repress the expression of *flhDC*, the master regulator of flagellar genes (Thota & Chubiz, 2019). FliZ, a class II flagellar gene, activates *hilD* (Cott Chubiz et al., 2010). So, MarA homologs may regulate SPI-1 genes via FliZ or through other direct pathways. In this chapter, I looked at regulation of *hilA* and its regulators *hilD* and *hilC* by MarA homologs. I also checked 3,4-dimethylbenzoic acid, a bile, which represses *hilA* (Peixoto et al., 2017) has any effect on MarA homologs.

5.2) Results

MarA homologs repress *hilA*

The transcriptomic data (**Figure 2, Chapter 3**) suggested that all the SPI-1 genes are down-regulated by MarA homologs. I wanted to test if MarA homologs work at HilA level, since it is the master regulator of SPI-1 genes. I used a transcriptional fusion of the *hilA* promoter and *lacZ* in *S.* Typhimurium strain 14028. I performed a β-galactosidase assay to measure expression of the *hilA* promoter in the control strain with pBAD30 and strains harboring vectors expressing MarA homologs. The data indicated substantial decrease in *hilA* expression in strains over-expressing MarA to 0.65±1.4% (Student's *t*-test, $P=4 \times 10^{-4}$), SoxS to 6.3±3.3% (Student's *t*-test, $P=5 \times 10^{-4}$), Rob to 5.9±1.33%

(Student's *t*-test, $P=5 \times 10^{-4}$) and RamA to 1.09±0.63% (Student's *t*-test, $P=4 \times 10^{-4}$) of the control strain, respectively (**Figure 2)**.

RamA represses transcription of *hilD*

HilD is the primary activator of *hilA* expression. Given the effects of MarA homologs on *hilA* expression, I examined whether this occurs through decreases in *hilD* transcription. I tested the role of MarA homologs in regulating *hilD* transcription by constructing a strain with the *hilD* promoter fused to *lacZ*. I observed that none of the MarA homologs repressed the transcription of *hilD* except RamA (**Figure 3**). The expression of *hilD* in the presence of RamA is 48.3±13.7% (Student's *t*-test, $P=3 \times 10^{-5}$) of the control strain harboring the empty vector pBAD30. It needed to be further tested if the other MarA homologs regulate *hilD* at post-transcriptional level.

SoxS and RamA repress *hilC*

In conjunction with HilD, HilC is another regulator that activates *hilA*. I sought to understand whether effects on *hilA* may be working through *hilC* expression. I constructed a strain with the *hilC* promoter fused to *lacZ* to test this possibility. I measured *hilC* expression by over-expressing MarA homologs. I noticed the levels of *hilC* diminished substantially in the strains that over-expressed SoxS and RamA to 14.4±0.56% (Student's *t*-test, $P=4 \times 10^{-4}$) and 8.1±4.7% compared (Student's *t*-test, $P=2 \times 10^{-7}$) to the control strain harboring the empty vector pBAD30, respectively (**Figure 4**). *hilC* was only slightly repressed in strains over-expressing MarA and Rob to 84.9±6.19% (Student's *t*-

test, P=2 x 10^{-9}) and 78±2.1% (Student's t-test, P=1.6 x 10^{-9}) of the control strain, respectively. This indicates *hilC* transcription was reduced when SoxS and RamA are over-expressed. Given *hilC* expression is activated by HilD, these data may also be reflective of decreased levels of HilD protein despite limited effects of MarA homologs on *hilD* transcription.

Activation of MarA by a bile salt

There is ample evidence for regulation of SPI-genes and MarA homologs by same intestinal compounds. It was shown by Peixoto and co-workers that a bile salt 3,4-dimethylbenzoic acid represses *hilA* (Peixoto et al., 2017). I constructed a strain with *marRAB* promoter fused to *yfp* to test its expression in the presence of 3,4-dimethylbenzoic acid. I observed that the *marRAB* expression in the presence of the bile salt is significantly higher than in the strain grown in its absence (Student's t-test, P=1.08 x 10^{-8}). (**Figure 5)**. I found out that *hilA* repression by 3,4-dimethylbenzoic acid is independent of MarA (data not shown).

5.3) Discussion

The expression of MarA homologs and virulence traits of *S.* Typhimurium are controlled by similar intestinal compounds (Hung et al., 2016; Rosenberg et al., 2003). It is already known from my transcriptomic studies that MarA homologs repress SPI-1 genes (**Figure 2, Chapter 3**). My genetic studies discussed chapter 4 showed that MarA homologs repress the flagellar master regulator

flhDC at both transcriptional and post-transcriptional levels. Flagellar genes are hierarchically divided into three classes. FliZ, a class II flagellar gene activates *hilD,* the master regulator of SPI-1 genes (Cott Chubiz et al., 2010). MarA homologs repress all three classes of flagellar genes. All of the above evidences motivated me to verify the hierarchical level of the SPI-1 regulatory chain do the MarA homologs work. My results have shown *hilD* transcription is reduced only when RamA is over-expressed. I have also shown that *hilA* and *hilC* expression was reduced when MarA homologs were over-expressed. It remains to be verified if the other three MarA homologs – MarA, SoxS and Rob – regulate *hilD* at post-transcriptional or post-translational stages. I still need to verify how MarA homologs regulate HilE the negative regulator of HilD and *rtsA* the activator of *hilD* and *hilA*. I have shown that a bile salt 3,4-dimethylbenzoic acid that represses *hilA* activate the expression of *marA*. However, the preliminary data indicates that *hilA* is repressed by 3,4 dimethylbenzoic acid in a manner independent of MarA (data not shown). After conducting genetic assays mentioned above, I will perform in vitro infection assays in human epithelial cells, with WT strains over-expressing MarA homologs, to support genetic assays.

Figure 1.

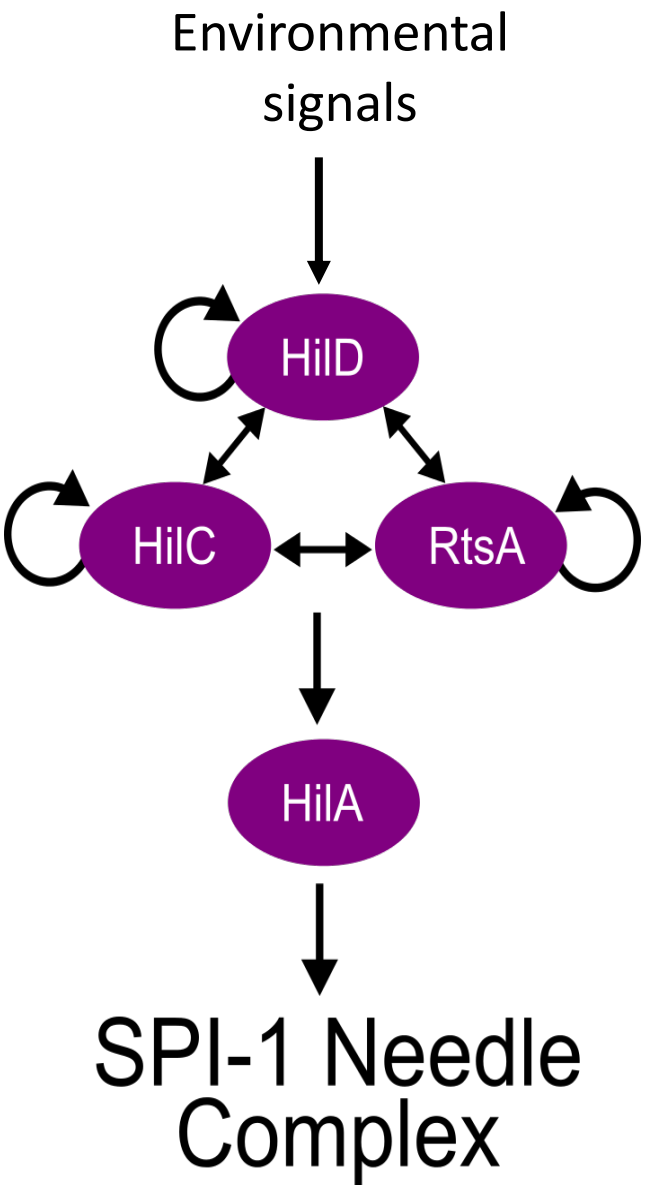


Figure 1. Model for regulation of HilA, the master regulator of SPI-1 genes (C. D. Ellermeier, Ellermeier, & Slauch, 2005).

.**Figure 2.**

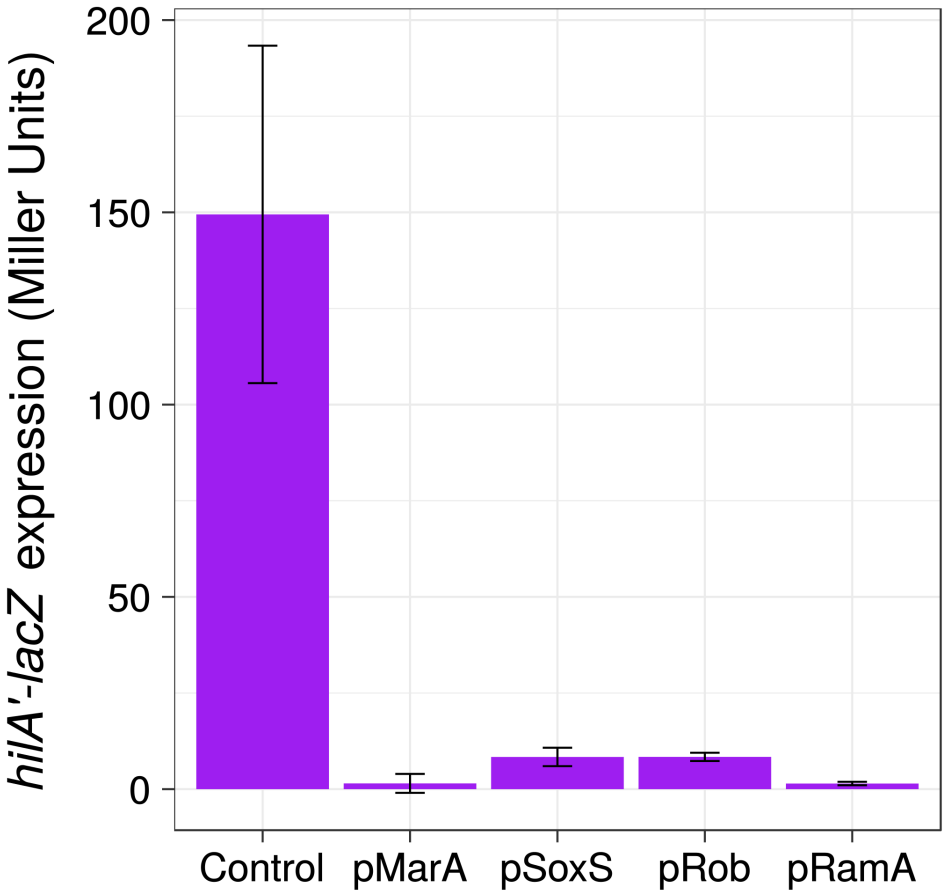


Figure 2. Exression of *hilA'-lacZ* transcriptional fusion (JS575) in strains with pBAD30, pMarA, pSoxS, pRob and pRamA plasmids. *hilA* expression is repressed to 0.65%±1.4 (Student's *t*-test, $P=4 \times 10^{-4}$) by MarA, to 6.3%±3.3 (Student's *t*-test, $P=5 \times 10^{-4}$) by SoxS, to 5.9%±1.33 (Student's *t*-test, $P=5 \times 10^{-4}$) by Rob and to 1.09%±0.63 (Student's *t*-test, $P=4 \times 10^{-4}$) by RamA compared to the control strain, respectively. Expression of MarA homologs from the pBAD30 is induced by adding 0.2% of arabinose to the cultures.

Figure 3.

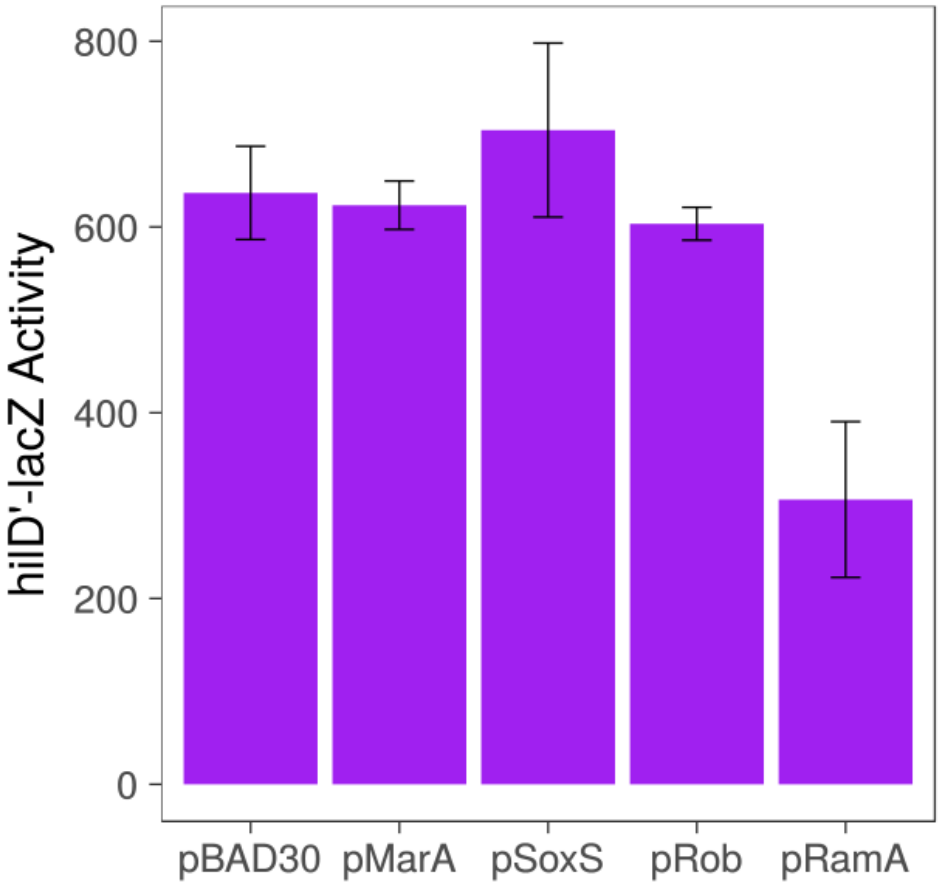


Figure 3. Expression of *hilD'-lacZ* transcriptional fusion (LCM2250) in strains with pBAD30, pMarA, pSoxS, pRob and pRamA plasmids. *hilD* expression is repressed only by RamA.to 48.3%±13.7 (Student's *t*-test, $P=3 \times 10^{-5}$) of the control strain. Expression of MarA homologs from the pBAD30 is induced by adding 0.2% of arabinose to the cultures.

Figure 4.

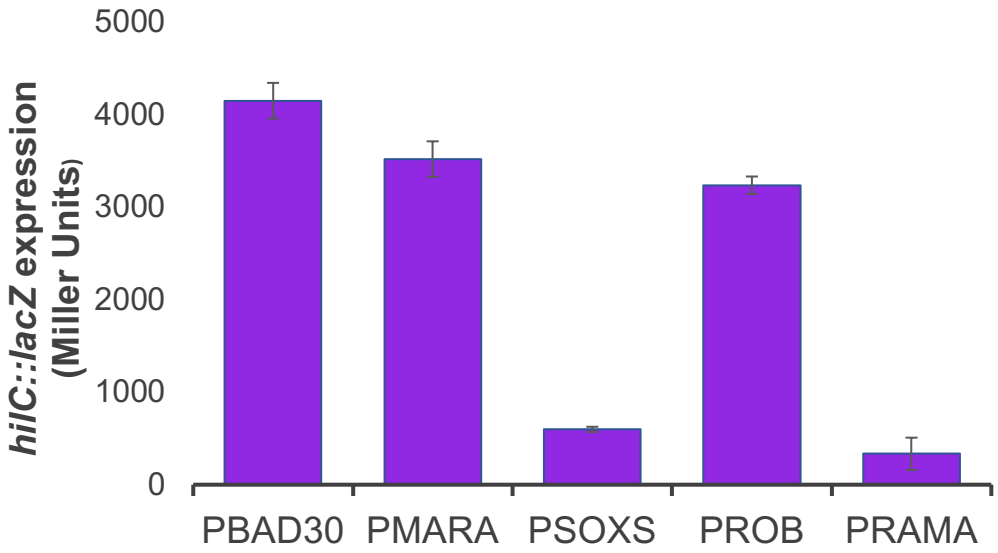


Figure 4. Exression of *hilC'-lacZ* transcriptional fusion (LCM2249) in strains with pBAD30, pMarA, pSoxS, pRob and pRamA plasmids. *hilc* expression is repressed significantly by SoxS and RamA to 14.4±0.56% (Student's *t*-test, $P=4 \times 10^{-4}$) and 8.1±4.7% (Student's *t*-test, $P=2 \times 10^{-7}$) compared to the control strain harboring the empty vector pBAD30, respectively. MarA and Rob repressed *hilC* to 84.9±6.19% (Student's *t*-test, $P=2 \times 10^{-9}$) and 78±2.1% (Student's *t*-test, $P=1.6 \times 10^{-9}$) of the control strain, respectively. Expression of MarA homologs from the pBAD30 is induced by adding 0.2% of arabinose to the cultures.

Figure 5.

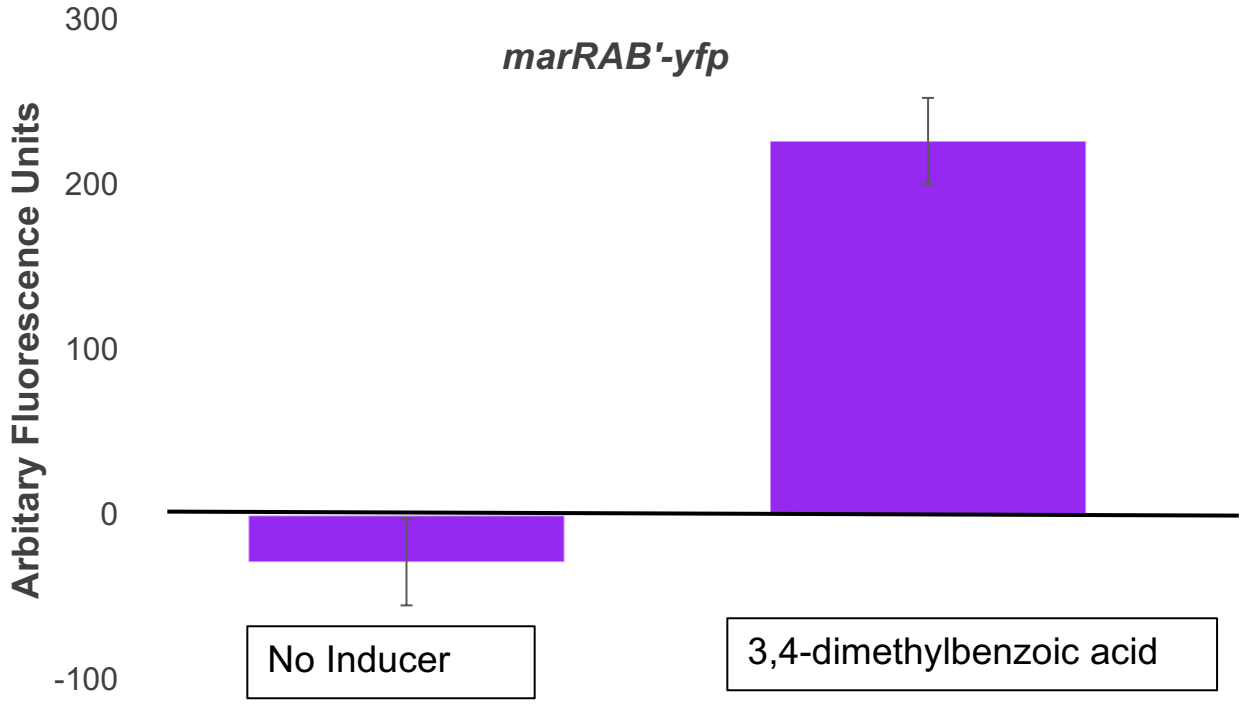


Figure 5. Exression of *marRAB'-yfp* transcriptional fusion (LCM2720) in the presence and absence of 3,4-dimethylbenzoic acid. *marRAB* expression was observed only in the presence of the bile salt (Student's *t*-test, $P=1.08 \times 10^{-8}$). Fluorescence measurements were made in overnight stationary cultures grown in LB at 37°C with or without the inducer.

Chapter 6: Identification of direct targets of MarA homologs in *Salmonella enterica* serovar Typhimurium

6.1) Introduction

As discussed in Chapter 3, transcriptomic analysis does not give information about direct targets of transcription factors. Identifying the direct targets of transcription factors complements the transcriptomic data to better understand regulatory networks of a cell. Information about all the promoters a transcription factor binds in a genome will enable development of a better consensus DNA sequence to which the transcription factor of interest binds. This facilitates the search for targets of the transcription factor in other closely related species of *S*. Typhimurium. Similar to the transcriptomics experiments, strains that over-express MarA homologs from inducible plasmids must be used in order to avoid under representation of weaker targets. Over-expression will also ensure long half-life of MarA homologs in the cell which are otherwise degraded by proteases.

Chromatin immunoprecipitation sequencing (ChIP-Seq) experiments were performed to identify all the DNA regions in the genome bound by transcription factors. Cells were cross-linked with formaldehyde which makes transcription factors stay bound to their target DNA regions. Cells were later lysed, the transcription factor of interest is pulled down using antibody and any DNA bound to the transcription factor is sequenced (Petrone et al., 2014). In this case, MarA homologs were tagged with a FLAG peptide at the C-terminal end. The reason to choose C-terminal end for FLAG tagging is to avoid masking of DNA binding

domain present at the N-terminal end of MarA homologs. Anti-FLAG peptide antibody was used to pull down MarA homologs.

S. Typhimurium harbors a large plasmid, pSLT, approximately 94 kb in size. pSLT is essential for virulence in animal hosts and contains a large number of virulence-associated genes that include those that code for attachment (Lobato-Márquez, Molina-García, Moreno-Córdoba, García-del Portillo, & Díaz-Orejas, 2016). Though the plasmid has been sequenced most of the genes are not characterized. There is a possibility for these genes on pSLT to be regulated by global regulators present on the chromosome.

In this chapter, I present the results from a pilot ChiP-Seq experiment using strains expressing FLAG tagged Rob from its chromosomal locus. I identified several direct targets that are known to be regulated by Rob from previous transcriptomic data. Of all the targets, we focused on an uncharacterized gene annotated as *pSLT026* due its location in the pathogenic plasmid pSLT. We conducted genetic and biochemical assays to identify how the *pSLT026* is regulated by Rob and identified its localization.

6.2) Results

Chromatin immunoprecipitation sequencing of Rob

In order to identify the direct targets of Rob in *S.* Typhimurium, I tagged chromosomally expressed Rob with a C-terminal 3XFLAG peptide (Rob3XFLAG) to conveniently use anti-FLAG antibody for immunoprecipitation. Cross-linked cells were lysed followed by incubation with protein A/G beads attached with anti-

FLAG antibodies to specifically bind Rob3XFLAG-DNA complexes. Rob3XFLAG-DNA complexes are eluted after washing off non-specific protein-DNA complexes. Rob3XFLAG protein is degraded using proteinase K and DNA bound to it is sequenced to identify the Rob targets (**Materials and methods, Chapter 2**). I identified a number of Rob binding targets across the *S*. Typhimurium genome. For example, the promoter regions of *lpxC* (lipid biosynthesis), *modA*-(Metal binding protein in periplasm), *micF* (sRNA that represses OmpF), and *deoB* (a phosphopentomutase) were enriched by Rob3XFLAG ChIP (**Figure 1**). I also identified an uncharacterized gene *pSLT026* to be the only target of Rob on the pathogenic plasmid pSLT. This appeared to be strongly bound by Rob as this region has the highest read depth compared to other regions sequenced (**Figure 1).**

Rob represses *pSLT026*

I next tested how Rob regulated *pSLT026.* We constructed a *pSLT026* promoter fusion of *lacZ* strain to measure the transcription of *pSLT026* in the presence and absence of Rob. I observed that the expression of *pSLT026* is 33.7±0.9 fold (Student's *t*-test $P = 3.2 \times 10^{-5}$) more in the *Δrob* strain compared to the WT (**Figure 2**). This indicates that Rob is strong repressor of *pSLT026*.

Rob interacts *pSLT026* promoter

The ChIP-Seq data clearly indicated that Rob binds *pSLT026* promoter with strong affinity. I further tested it by performing an electrophoretic mobility

shift assay. Purified Rob protein has retarded the mobility of a biotin labeled *pSLT026* promoter DNA during electrophoresis (**Figure 3**). The shift disappeared when a specific competitor (unlabeled *pSLT026* promoter region) was added to the reaction but not when a non-specific competitor (*gyrA* gene region) was added. This clearly indicated that Rob binds to the *pSLT026* promoter region.

PSLTO26 is a cytoplasmic protein

The function of *pSLT026* is still unknown. As a part of characterizing this gene, I wanted to first test its location in the cell. Few virulent proteins are translocated into the periplasm or secreted into the outside environment. Since the translocation signal peptides for these proteins are located on the N-terminal end, we tagged PSLT026 with 3XFLAG peptide on its C-terminal end. Cytoplasmic and periplasmic components were fractionated and were used to conduct an immunoblot. Anti-3XFLAG was used to detect PSLT026-3xFLAG with anti-DnaK (a marker for cytoplasmic proteins) and anti-DsbA (a marker for periplasmic proteins) antibodies used to verify cytoplasmic and periplasmic fractionation. I observed that the PSLT026 protein is localized in the cytoplasm. I found traces of it present in the periplasmic fraction but that could be due to cell lysis (**Figure 4**).

MEME analysis of promoters bound by Rob

Binding of Rob specifically to the *pSLT026* promoter region in the EMSA experiment validated Rob3XFLAG - ChIP-seq data. I used the MEME-ChIP

bioinformatic tool to define the consensus sequence of Rob binding site (Machanick & Bailey, 2011). Maximum number of motifs allowed to retain by the tool was set to 1 and the length of the motif sequence to be searched was set between 15-20 bp. This resulted in identification of a 19 bp motif which is shown below in the **Figure 5.**

The consensus sequence where the MarA homologs bind specifically is called marbox and is well characterized in *E. coli* by (Robert G. Martin et al., 1999). The marbox is a 20 bp long sequence that is defined as AYnGCACnnWnnRYYAAAYn (R, A or G; Y, C or T; W, A or T; n, any nucleotide). The motif obtained here by MEME-ChIP analysis of Rob target promoters in *S.* Typhimurium have some similarities with the known marbox of *E.coli* in length and the fact that both the motifs are flanked by A's on both 5' and 3' ends.

Interestingly, the core GCAC motif is not conserved in the Rob binding consensus but is instead RCnG. From these data, we can conclude that Rob may have subtle differences in binding recognition compared to MarA and SoxS whose binding has been better characterized (Seo et al., 2015; Sharma et al., 2017). Importantly, this provides an additional structural basis for differences in MarA homolog promoter binding preferences.

Since the Rob3XFLAG is expressed from the chromosomal locus (low level expression) and absence of post-translational activator of Rob in the current experimental setting, there might be a limitation to identify weaker Rob targets.

By identifying both stronger and weaker Rob targets, the consensus sequence of Rob binding site can be further resolved.

6.3) Discussion

I am still in the initial stages of standardizing the ChIP-Seq protocol. This experiment was performed in a strain expressing Rob from the chromosomal locus. Since Rob is post-translationally activated only in the presence of inducers like decanoate or dipyridyl and absence of these compounds in the media used to grow this strain, there might be very low levels of activated Rob available in the strain. I therefore might have not retrieved all the targets of Rob. The genes we identified here must be strong targets of Rob. Future experiments will be performed in strains over-expressing MarA homologs from an inducible plasmid (pBAD30) followed by MEME-ChIP analysis to further resolve the Rob binding consensus sequence. From my current data, of all the Rob targets, *pSLT026* is being considered as the most important because of its location on the pSLT plasmid and its multi-fold repression by Rob. I also observed that PSLT026 is localized in the cytoplasm. Since most of the genes present on the plasmid pSLT are pathogenic, I speculate that this gene might have a role in virulence. This will be confirmed by performing in vitro infection assays with WT and *pSLT026* deletion mutants on human epithelial cells.

Figure 1.

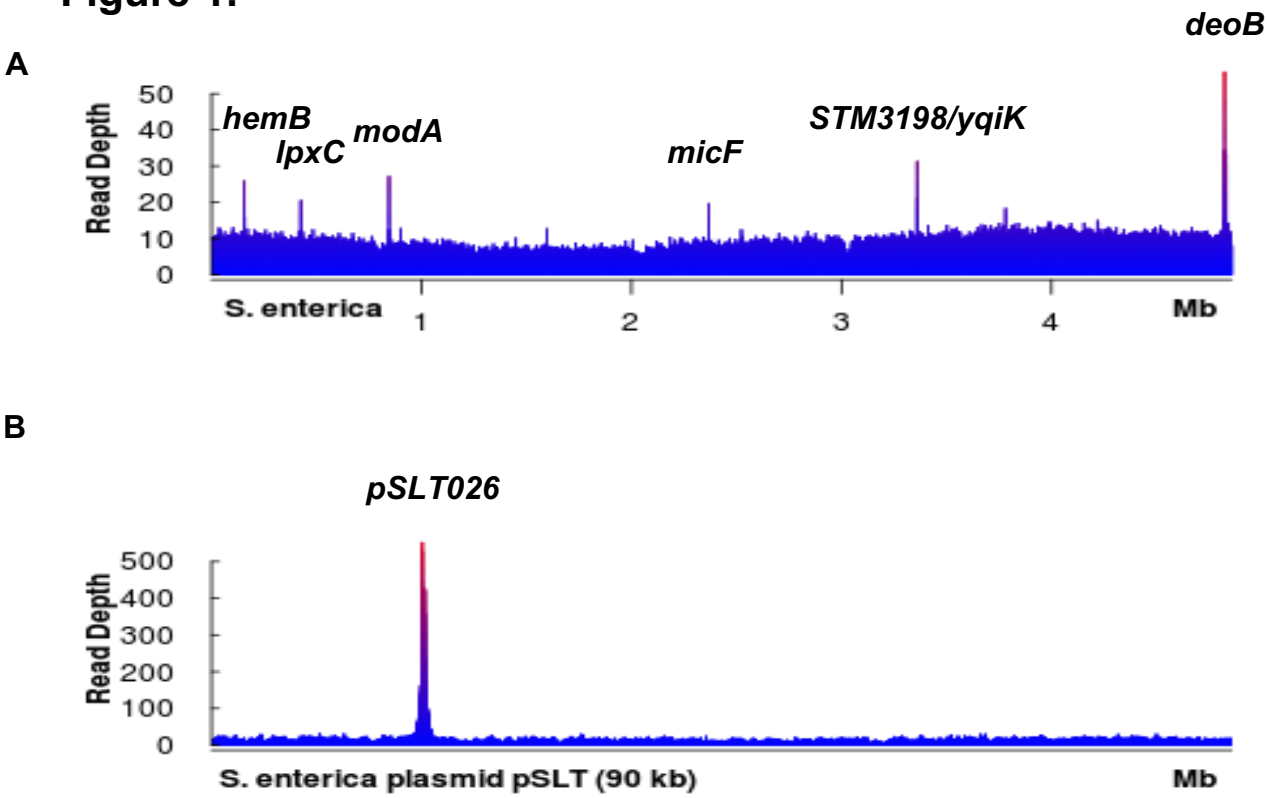


Figure 1. Bedgraph images of Rob targets across chromosome (**A**) and pSLT plasmid (**B**) in *Salmonella enterica* LT2 expressing 3XFLAG tagged Rob (LCM1972).

Figure 2.

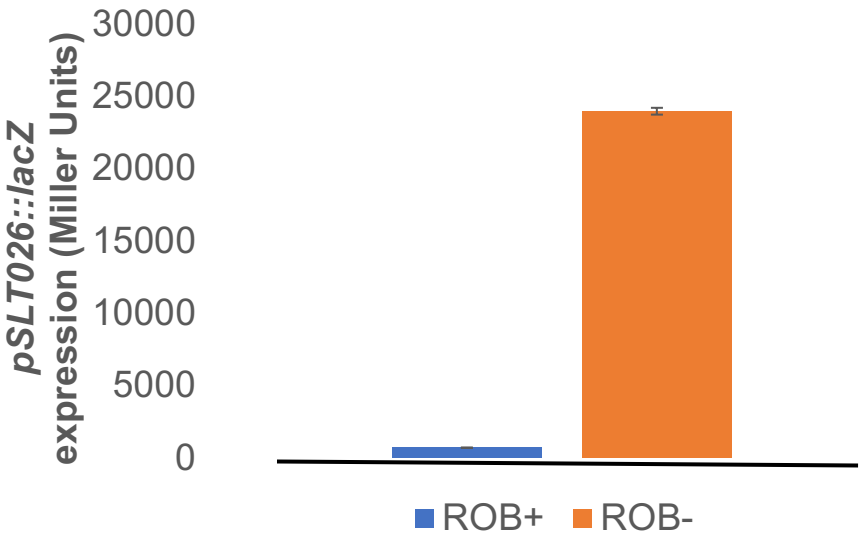


Figure 2. Rob represses the transcription of *pSLT026*. We performed β-galactosidase assay on strains with *pSLT026* promoter and *lacZ* fusion in the presence and absence of Rob (LCM2087, LCM2088). *pSLT026* is expressed 33.7±0.9 fold (Student's *t*-test $P = 3.2 \times 10^{-5}$) more in *Δrob* strain compared to WT.

Figure 3.

Nonspecific (fmol)	0	0	0	0	0	0	0	100
pSLT026 (fmol)	0	0	0	0	0	0	100	0
Biotin-*pSLT026* (fmol)	10	10	10	10	10	10	10	10
Rob (nmol)	0	20	40	60	80	100	100	100

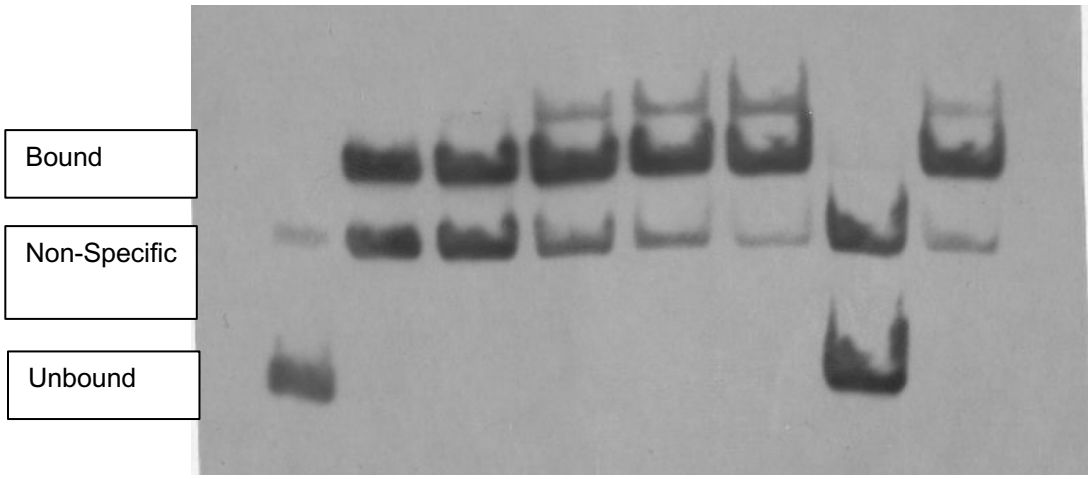


Figure 3. Binding of Rob to the *pSLT026* promoter region. Electromobility shift assay (EMSA) of a 135 bp *pSLT026* promoter fragment in the presence of indicated concentrations of purified Rob protein, biotinylated *pSLT026* promoter DNA, unlabeled *pSLT026* promoter DNA (specific competitor) and unlabeled 172bp region of *gyrA* (non-specific competitor).

Figure 4.

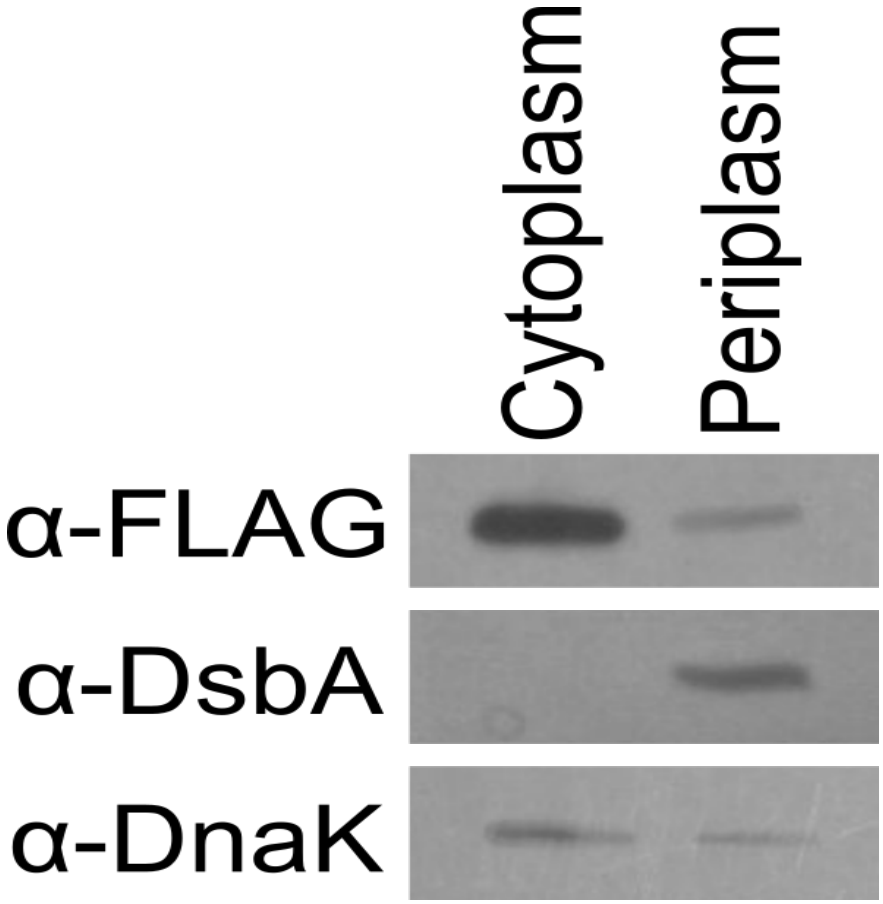


Figure4. Immunoblot to detect Pslt0263XFLAG in cytoplasmic and periplasmic fractions. Cytoplasmic and periplasmic components of the strain expressing Pslt0263XFLAG protein in a r*ob* deletion background (LCM2027) is used. DnaK and DsbA serve as cytoplasmic and periplasmic localization controls.

Figure 5.

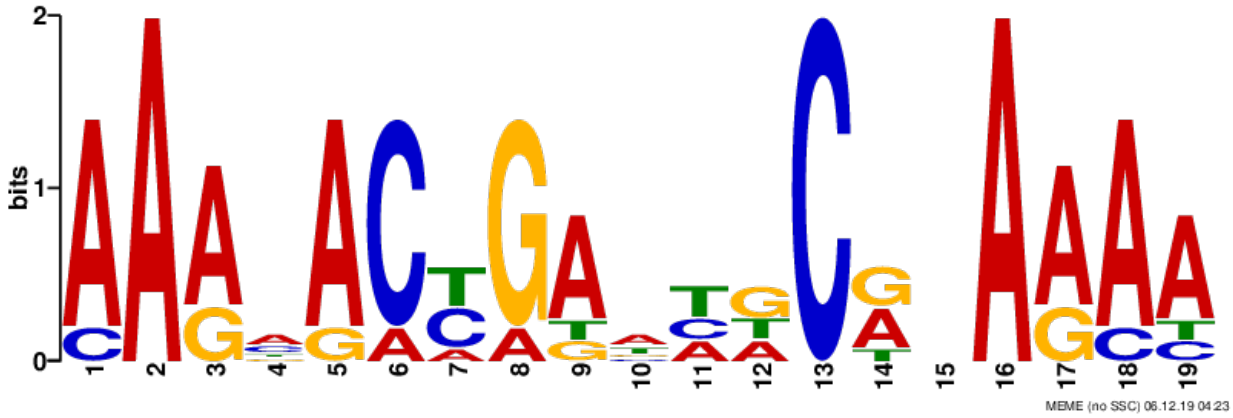


Figure 5. MEME-ChIP motif of Rob binding region. This motif is obtained by uploading all the promoter regions of Rob targets obtained from ChIP-seq to the MEME-ChIP software tool.

Chapter 7: General conclusions and future directions

7.1) General conclusions and significance

MarA homologs in *Enterobacteriaceae* species were primarily considered to be regulators of multidrug resistance genes because the early isolated *E. coli* mutants of these transcription factors were susceptible to antibiotics (Cohen, Hachler, et al., 1993; Cohen, Levy, Foulds, & Rosner, 1993; Cohen et al., 1988). There are a few examples in the literature that suggest MarA homologs may have roles more than rendering antibiotic resistance to various *Enterobacteriaceae* species. For example, Δ*marA* Δ*soxS* Δ*rob* triple knockout of uropathogenic *E.coli* (UPEC) has attenuated virulence in a murine infection model (Casaz et al., 2006). This suggest that MarA homologs may actually up-regulate virulence genes in *E. coli*. It is logical to expect a similar trend in *S*. Typhimurium, a closely related species of *E. coli*. I performed RNA sequencing in *S*. Typhimurium to study genes regulated by MarA homologs. Surprisingly, I observed that the expression of flagellar and SPI-1 genes, which code for virulence traits are reduced in strains where MarA homologs are over-expressed (**Chapter 3**). This is contrary to what is known in *E. coli*.

Presently, most studies in *S.* Typhimurium suggest no involvement of MarA homologs in virulence. Fang et. al. could not find any change in survival rates of *ΔsoxS Salmonella* inside macrophages (Fang et al., 1997). Similarly, Sulavik and Miller concluded that the *marRAB* locus of *S.* Typhimurium has no role in virulence in mice (Sulavik et al., 1997). My findings suggest MarA

homologs repress key virulence traits required for *S*. Typhimurium invasion of host epithelial cells. Supporting these findings, Bailey and co-workers have observed reduced virulence phenotypes in a *C. elegans* infection model when *ramA* is highly expressed (A. M. Bailey et al., 2010).

So, why might single deletions not confer any virulence phenotypes in *S*. Typhimurium? Redundancy of MarA homologs might be the reason why no role for MarA homologs in virulence was identified in the above studies. MarA homologs are duplicated genes. Duplicated genes often have novel functions or a new target in the case of a duplicated transcription factor gene (Bratlie et al., 2010). Due to similarity in DNA binding domains of MarA homologs, they have numerous overlapping targets. In this way, a single deletion may simply be masked by the overlapping function of other MarA homologs. This hypothesized mechanism is particularly intriguing because *marA, soxS,* and *ramA* expression are all significantly upregulated during growth in macrophages (Srikumar et al., 2015). Thus, a true virulence phenotype may only be observable in higher order MarA homolog mutants.

Flagellar proteins and SPI-1 needle complex proteins are the key virulence structures of *Salmonella* that elicit host immune response (Lou, Zhang, Piao, & Wang, 2019b; Spöring et al., 2018). Premature expression of these structures would be detrimental for the pathogen since they will be recognized by the host immune system even before adapting to the new niche (Rossez, Wolfson, Holmes, Gally, & Holden, 2015). Above all, these structures have energy costs to maintain. Expressing them prematurely will have huge energy

costs to *Salmonella* in the nutrient deprived conditions during early stages of inhabitation in the human intestine (Sturm et al., 2011). *Salmonella* uses complex regulatory networks to temporally and spatially express flagellar and SPI-1 genes. These structures are turned on when *Salmonella* reaches the ileum. There are several activators and repressors of these virulence genes. The coordinated work of activators and repressors results in fine tuning of expression of virulence genes (Gart et al., 2016). Since the virulence phenotypes and expression of MarA homologs are regulated by similar intestinal compounds, there might be a possibility for a role of MarA homologs in regulating virulence genes in *Salmonella*(Hung et al., 2016; Peixoto et al., 2017) .

There are known chemical inducers for each of the MarA homologs. Salicylic acid, paraquat, decanoic acid and indole activate *marRAB*, *soxS*, Rob (post-translationally) and *ramA*, respectively (Cohen, Levy, et al., 1993; E. Nikaido et al., 2008; Rosenberg et al., 2003; J. Wu & Weiss, 1992). Recently we have found that in the presence of salicylic acid, *S.* Typhimurium exhibited decreased killing of *C. elegans* (data not shown). There is a high possibility that salicylic acid represses this virulence in *C. elegans* in a MarA dependent manner. Screening for more compounds that activate MarA homologs might be a good future direction for drug discovery to treat diseases caused by *S.* Typhimurium and other related species that have MarA homologs.

In this thesis I present the research I have conducted that supports the above hypotheses. My initial transcriptomic data (**Figure 1, Chapter 3**) indicated down regulation of genes that code for these virulence traits (flagella, fimbria and

SPI-1 needle complex) by all four MarA homologs. My genetic assays conclusively indicated repression of flagellar genes by MarA homologs. I have shown that MarA and Rob bind to the *flhDC* promoter region and repress its transcription and SoxS represses *flhDC* via a post-transcriptional method. These MarA homologs also repress motility of *S.* Typhimurium. Salicylate represses motility of *Salmonella* through MarA and Rob homologs (Thota & Chubiz, 2019). The genetic studies have also suggested that the expression of *hilA* and *hilC* genes is reduced when MarA homologs are over-expressed. In an effort to identify direct targets of MarA homologs, I have identified numerous targets of Rob in the chromosome and pSLT of *S.* Typhimurium from initial ChIP-seq experiments. Of all the targets, my attention was drawn more towards an uncharacterized gene *pSLT026* on the pSLT plasmid that harbors several pathogenic genes. I found *pSLT026* is strongly repressed by Rob from genetic assays. I also identified that PSLTO26 is a cytoplasmic protein contrary to its genomic annotation.

Overall, my research further expanded the *mar-sox-rob* regulon and identified novel roles of MarA homologs in *S*. Typhimurium. I worked out the details of how MarA homologs regulated flagellar genes. How MarA homologs regulate SPI-1 genes, characterization of PSLT026, and quantifying the role of MarA homologs in infection of human epithelial cells remain to be studied.

7.2) Future directions

My study shows strong evidence for post-transcriptional repression of *flhDC* by SoxS. Currently, it is unknown if repression occurs at the mRNA or at the FlhD4C2 protein level. The transcriptomic data suggests expression of *ydiV* is increased when SoxS is expressed. The *ydiV* gene codes for a protein that directs the FlhD4C2 complex to the ClpXP protease, thus post-translationally repressing flagella (Takaya et al., 2012). My initial experiments (data not shown here) has suggested that SoxS regulates FlhD4C2 through YdiV. It should also be verified if SoxS activates any small RNA that might destabilize *flhDC* mRNA or stall its translation. The $soxR^{con}$ mutant that expresses SoxS at endogenous levels can repress *fliC* to extremely low levels. I am currently performing transposon mutagenesis in the $soxR^{con}$ strain to screen for mutants that will express normal FliC levels. This will reveal new pathways that SoxS may use to repress flagellar genes. It should also be further checked if MarA homologs directly regulate *fliC* independent of FlhD4C2.

Only RamA of all the MarA homologs represses *hilD* transcription. It remains to be seen if the other three MarA homologs regulate a pathway that represses *hilD* at the post-transcriptional level. Direct regulation of *hilA* and *hilE*, the negative regulator of HilD, by MarA homologs must be checked. These genetic assays should be followed by in vitro invasion assays of human epithelial cells. *pSLT026* must be verified for its role in virulence and its specific function must be characterized.

My research has shown that the multidrug resistance regulators – MarA homologs – have an important role in repressing virulent traits in *S.* Typhimurium.

Compounds that activate MarA homologs may have the potential to suppress virulence of *S.* Typhimurium through MarA homologs. Screening commercially available chemical libraries that activate MarA homologs will be a good direction to identify drugs that treat *S.* Typhimurium infections. Compounds with similar structures as Salicylic acid or those with redox properties like paraquat will be used in these screens.

My research has shed some light on multiple roles of MarA homologs in *S.*Typhimurium. These genes were primarily known to render antibiotic resistance in different species of *Enterobacteriaceae* and up-regulation of virulence genes in *E. coli*. In *S*. Typhimurium, previous researchers overlooked the redundancy of MarA homologs and concluded that they may have no role in regulation of virulence genes. My transcriptomics data in strains that specifically over-express a single MarA homolog from an inducible vector, pBAD30, identified all the genes up-regulated and down regulated by each of the MarA homologs. As expected, overlap of gene targets of MarA homologs has been noticed. Interestingly, genes coded for virulence traits (flagellar, fimbrial and SPI-1) were down-regulated by all MarA homologs (**Chapter 3**), contrary to other studies in *S.*Typhimurium. I elucidated how MarA homologs regulated flagellar genes in a non-redundant quadruple MarA homolog knockout background (**Chapter 4**). Reduction of expression of regulators of SPI-1 genes (*hilA* and *hilC*) was also observed in strains over-expressing MarA homologs (**Chapter 5**). This regulation must be further elucidated. MarA homologs are well conserved across many species of the family *Enterobacteriaceae* that dwell in different habitats

with various nutritional and survival challenges. Few of these species lack some of the MarA homologs. Identifying targets of MarA homologs across different species of *Enterobacteriaceae* will shed light on how duplicated MarA homologs evolved to regulate novel functions specific to a species.

This chapter contains the research I conducted in Dr. Schechter's lab until December 2014.

Chapter 8: Role of HrpRS in regulation of type III secretion system in *Pseudomonas syringae* pathovar tomato strain DC3000

8.1) Abstract

Pseudomonas syringae is a plant pathogen that causes economically significant disease in a wide variety of crop plants. This bacterium uses the type III secretion system (T3SS), to secrete effectors proteins directly into host plant cells. The success of *Pseudomonas* as a pathogen in the host cell depends on proper activation of T3SS, since the effector proteins repress the host defense mechanisms. T3SS genes are tightly regulated by multiple transcription factors. HrpL is an alternate sigma factor that directly regulates expression of genes encoding secreted effector proteins and structural components of the secretion apparatus. In turn, *hrpL* is activated by two members of the bacterial enhancer binding protein (bEBP) family, HrpR and HrpS. Although the consensus binding sequence for HrpL is known, the exact sites that are bound by HrpR and HrpS have not yet been determined. To narrow down the sequences bound by HrpR/S, I constructed *lacZ* reporter plasmids containing various lengths of the *hrpL* promoter region from *P. syringae* pathovar tomato DC3000. I then examined which reporters are activated by HrpR and/or HrpS in *P. syringae*. My results are in agreement with previous studies showing that both HrpR and HrpS are required to activate *hrpL*. I also purified HrpR and HrpS and found that HrpS

and HrpRS complex but not HrpR can bind to the full-length *hrpL* promoter region. These results contradict previously published data that showed both HrpR and HrpS independently binding the *hrpL* promoter. We also constructed a HrpR variant whose HTH domain has been swapped with that of HrpS. This modified protein retarded *hrpL* DNA in a mobility shift assay. We are currently performing DNA footprinting assays to identify the exact HrpS/RS binding site in the *hrpL* promoter region. Identifying the HrpRS binding site will help to search for other genes regulated by HrpRS. We also tested how CorR influenced the activity of *hrpL* in the presence and absence of HrpRS. Overall, these studies will contribute to a better understanding of global gene regulation of in *P. syringae* as well as the molecular mechanisms responsible for activation of the T3SS.

8.2) Introduction

Pseudomonas syringae is a Gram-negative bacterial plant pathogen. The species is divided into 50 pathovars primarily based on host range (Xin & He, 2013). For instance, all isolates characterized as *P. syringae* (pv.) tomato infect tomato plants. Isolates in a particular pathovar may additionally cause disease on other plants, exemplified by the well-studied strain *Pseudomonas syringae* (pv.) tomato (*Pst*) DC3000, which infects tomato and the model plant Arabidopsis. It causes bacterial speck on tomato fruit and its leaves, and chlorosis of leaves in Arabidopsis (Cornelis, 2006; Ishiga, Ishiga, Uppalapati, & Mysore, 2011; Xin & He, 2013).

Pseudomonas syringae lives both as an epiphyte on the leaf surface and an endophyte within the leaf tissue (Xin & He, 2013). After colonizing the leaf surface, bacteria enters the leaf apoplast intercellular space through stomata or wounds, obtain nutrients from plant tissue, and replicate aggressively. Eventually the apoplast cells surrounding *P.syringae* colonies die, leaving a necrotic lesion in the tissue (Xin & He, 2013).

In order to multiply in plant tissue, *P. syringae* must turn off plant defense mechanisms during the endophytic phase of infection. One plant defense that *P.syringae* must overcome is termed PAMP-triggered immunity or PTI. During PTI, plants recognize conserved bacterial features called pathogen associated molecular patterns (PAMPs) via pattern recognition receptors (PRRs) and induce basal defenses like callose production, release of reactive oxygen species (ROS) and phytoalexins, and reduction of pH in the apoplast (Stuart, Paquette, & Boyer, 2013; Xin & He, 2013). Callose accumulates between the cell wall and plasma membrane of plant cell to prevent bacterial entry, whereas ROS, phytoalexins, and acidic pH inhibit bacterial growth. *P. syringae* is able to block these defenses by delivering effector proteins into plant cells that collectively suppress PTI. Currently it is hypothesized that resistance (R) proteins in non-host plants recognize effector proteins by various direct and indirect mechanisms and induce a second more powerful defense response called effector-triggered immunity (ETI). The end result of ETI is hypersensitive response (HR), a rapid and localized death of infected plant cells (Nicaise, Roux, & Zipfel, 2009; Stuart et al., 2013; Xin & He, 2013; J. Zhang et al., 2010). In non-host plants, *P. syringae* is

cleared by the HR, whereas plants that do not possess the appropriate R proteins succumb to infection (Stuart et al., 2013; C. F. Wei et al., 2007; Xin & He, 2013; J. Zhang et al., 2010).

P. syringae injects effector proteins into plant cells through a needle like structure called an injectisome (Cornelis, 2006). This injectisome is made up of many proteins that span the bacterial inner membrane, periplasm, and outer membrane (Cornelis, 2006). The Genes that encode the injectisome proteins, effector proteins and their regulators make up the type III secretion system (T3SS) (Alfano et al., 2000; Stauber, Loginicheva, & Schechter, 2012). T3SS is found in 25 different gram-negative bacterial species that infect plants and animals (Cornelis, 2006). T3SS injectisome genes and few effector genes are clustered in the chromosome in a region called as pathogenic island (PAI) (Blum et al., 1994), and other effector proteins are scattered throughout the chromosome (Alfano et al., 2000). In *P. syringae*, the PAI is called the hypersensitive response and pathogenicity gene cluster (*hrp*). Injectisome proteins are encoded by the *hrp* and *hrc* genes, whereas genes that encode secreted proteins have been named either *avr* or *hop* (Alfano et al., 2000).

T3SS genes appear to be optimally expressed under the specific environmental conditions that bacteria encounter in plants (Rico & Preston, 2008). Gene expression is controlled by a cascade of transcription factors encoded within the *hrp* PAI. Most *hrp/hrc* and *avr/hop* genes are activated by an extracytoplasmic sigma factor called HrpL (Fouts et al., 2002; Stauber et al., 2012). HrpL recruits RNA polymerase to T3SS gene promoters by binding to a

conserved element called the Hrp box (5'-GGAACCNA-N13–14CCACNNA-3') (Fouts et al., 2002). *corR* is one such gene that has a Hrp box in its upstream regions and was shown to be activated by HrpL. CorR also binds to upstream region of *hrpL. hrpL* expression was lowered and delayed by 2 hours in ∆*corR* DC3000 (Sreedharan, Penaloza-Vazquez, Kunkel, & Bender, 2006).

hrpL gene expression is activated by two enhancer binding proteins, termed HrpR and HrpS, which both belong to the AAA^+ family of ATPases (Hutcheson, Bretz, Sussan, Jin, & Pak, 2001; Jovanovic et al., 2011) (**Figure 1**). HrpR and HrpS are 55-65% and 70-79% similar at the amino acid level. Both have a C- terminal DNA binding helix-turn-helix (HTH) domain and an AAA^+ domain, which contains ATP binding, σ^{54} binding, nucleotide hydrolysis, and oligomerization regions (Jovanovic et al., 2011).

Enhancer binding proteins bind upstream activating sequences (UAS) that are located 80-150bp upstream of σ^{54}-RNA polymerase holoenzyme binding site . In conjunction with HrpR/S binding, intergenic host factor (IHF) binds between UAS and promoter regions. IHF bends DNA and brings enhancer binding proteins to the close proximity of σ^{54}. Upon binding ATP, EBPs carry on ATP hydrolysis and provide energy to the holoenzyme. This leads to the formation of open complex at the promoter and lead to transcription (Bush & Dixon, 2012). Usually, NtrC family enhancer binding proteins are active in hexameric or heptameric state. HrpR and HrpS are also active in a hetero-hexameric state (Joly & Buck, 2011; Jovanovic et al., 2011).

Most regulators in the AAA$^+$ enhancer binding protein family are part of two component systems. In addition to the DNA binding and AAA$^+$ domains, they each have a response regulator domain that is phosphorylated by sensor kinases in response to environmental stimuli. HrpR and HrpS are unusual in that they don't have a response regulator domain. The *hrpR* and *hrpS* are transcribed as a single operon and like the PspF regulator; they are constitutively expressed and negatively regulated post-translationally (Hutcheson et al., 2001; Jovanovic et al., 2011). HrpR is degraded by Lon protease when grown in rich media and HrpV binds HrpS and inactivates it. The *hrpV* is encoded downstream of *hrpL* and hence works as a feedback loop (**Figure 1**). In conditions favorable for T3SS expression, the HrpG chaperone releases repression of HrpS by HrpV (Bretz, Losada, Lisboa, & Hutcheson, 2002; Preston, Deng, Huang, & Collmer, 1998; C. F. Wei, Deng, & Huang, 2005).

A microarray study that compared gene expression profiles of Δ*hrpRS* and Δ*hrpL* of *Pst* DC3000 identified genes that are regulated directly by HrpRS independent of HrpL(Lan, Deng, Zhou, & Tang, 2006). These included 60 up regulated genes of which few are transcription factors and some are hypothetical genes and 63 repressed genes that included few flagellar and ribosomal genes. Since flagellin is a PAMP and is recognized by host plants to trigger PTI, down regulating it would be beneficial for pathogenesis. All the up regulated genes might not be activated directly by HrpRS. Few could be induced indirectly by other transcription factors. HrpRS could activate negative regulators that can repress other genes. So, identifying direct targets of HrpRS could give an insight

into the complex gene regulation involved in the pathogenesis of *Pst* DC3000.The overall goal of my work is to determine the location(s) of the HrpR/HrpS DNA binding site(s) upstream of the *hrpL* promoter in *Pst* DC3000 and utilize this information to identify other genes directly regulated by HrpR/HrpS. As a first step, I created epitope-tagged versions of HrpR and HrpS and measured their activity in wild-type and mutant derivatives of *Pst* DC3000. I then carried out genetic and bioinformatic analyses to narrow down the region upstream of *hrpL* bound by HrpR/HrpS. I also verified role of CorR in the activation of *hrpL* in the presence and absence of HrpRS. Finally, I performed *in vitro* DNA binding assays on the *hrpL* promoter region with purified wild-type and mutant HrpR and HrpS proteins. Overall, my work will be useful for identifying other targets of HrpRS in *P. syringae*.

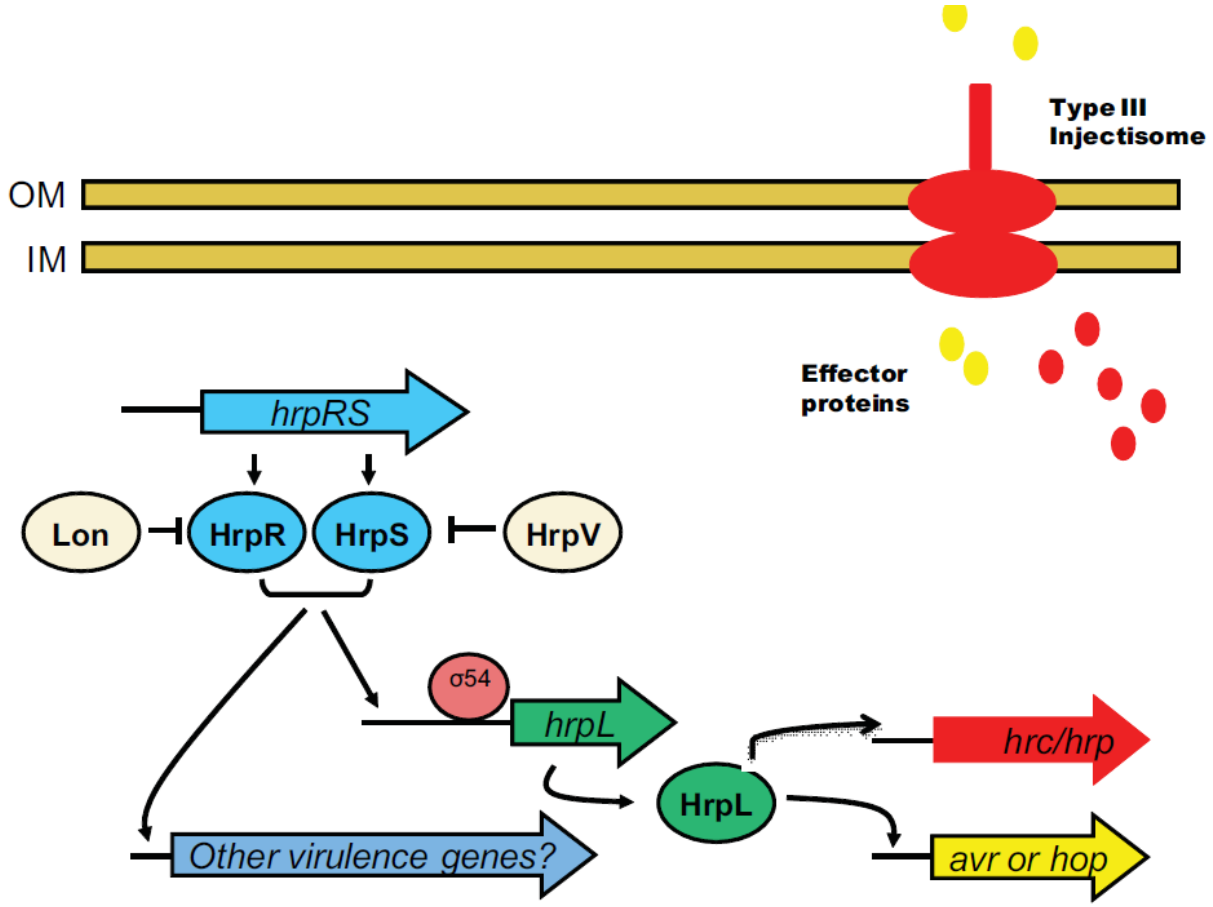


Figure 1: Summary of type III secretion system gene regulation. *hrp* genes are activated by HrpL and *hrpL* is activated by HrpRS in sigma-54 dependent manner. HrpR and HrpS are post-translationally regulated by Lon protease and HrpV

8.3) Methods and materials

Bacterial strains, plasmids and media

List of all the strains and plasmids that were used in this study are shown in the **Table 1** of this chapter. *E. coli* cultures were grown in LB media (1% Tryptone, 1% Yeast extract, 0.5% NaCl and 1mM NaOH) at 37^0C (BERTANI, 1951). *Pst*DC3000 strains were grown in KB media (2% proteose peptone, 10mL glycerol, 1.5g K_2HPO_4, 1.5g $MgSO_4.7H_20$) for genomic DNA isolation and other DNA manipulations (20). For GUS assays, β-Galactosidase assays and RNA preparations, *Pst*DC3000 was grown at room temperature in *hrp*-derepressing minimal (HDM) medium. This medium consists of 10mM fructose, 50mM potassium phosphate, 7.6mM ammonium sulfate, 1.7mM $MgCl_2$ and 1.7mM NaCl and is at pH6 (Sreedharan et al., 2006).

DNA manipulations

a) Construction of HrpR and HrpS expression vectors

hrpR was amplified with primers that add BamHI site at 5' end and XhoI site at 3' end (P1F/R). This fragment was cloned into a pET28a vector (Novagen) at the same sites that are located 3' to a His.T7 region. HrpR expressed from this vector (pR284) has a His-T7 tag on its N-terminal end. *hrpS* is cloned into pET30a vector (Novagen) at BamHI and XhoI sites downstream to a His.S region to make pS308 plasmid. pR284 and pS308 expressed His-T7-HrpR and His-S-HrpS proteins. *his.t7.hrpR* and *his.s.hrpS* regions from pR284 and pS308 vectors were cloned into pDUET (Novagen) vector at NcoI and XhoI sites

respectively. This vector (pRSFDUET) is used to co-express His-T7-HrpR and His-S-HrpS.

b) Construction of *lacZ* transcriptional fusions

752bp, 253bp, 169bp, 110bp and 47bp regions upstream to *hrpL* translation start site were generated using primers that add BamHI sites at the ends (P7F, P8F, P9F, P10F, P11F, and P12R). They were later cloned into pRG970 *lacZ* reporter vector (28) at the same site to obtain pL752, pL253, pL169, pL110, pL47. *his.t7.hrpR* and *his.s.hrpS* from pET vectors were cloned into pUCP24 (Schweizer, 1991) at KpnI and XhoI sites, and into pBBR1-MCS2 (Kovach et al., 1995) at EcoRI and SpeI sites respectively to obtain pHR24 and pBS12. These broad host range constructs were used to perform β-Galactosidase assays both in *E.coli* and *Pst*DC3000.

c) Construction of gene deletion vectors

Δ*hrpR,* Δ*hrpS* and Δ*hrpRS Pst*DC3000 mutants were generated using pK18*mobsacB* (Km^r) suicide vector in this study (Schäfer et al., 1994). 1.0 kb upstream region of the target gene is generated in a PCR reaction with primers that add a 3' 25bp region, which overlaps with 5' region of *nptII* gene. The downstream flanking region is generated with a 5' 25bp region that overlaps with 3' region of *nptII* gene. All the three fragments were ligated by performing a long flanking homology (LFH) PCR reaction where the flanking regions serve as mega primers (Swingle, Bao, Markel, Chambers, & Cartinhour, 2010). The product from the above PCR is gel purified and cloned into pK*18mobsacB*.These vectors were electroporated into *Pst*DC3000 and plated on KB-Kanamycin plates to select for

colonies that have pK18*mobsacB* integrated into the chromosome through recombination. Colonies from this plate are patched on a 10% sucrose KB plates to cure chromosome integrated pK18*mobsacB*. At this point 50% of colonies retain the target gene and 50% of colonies have the target gene replaced by *nptII*. Mutants were screened by performing a PCR on the genomic DNA and loss of gene expression was confirmed by RT-PCR (**Figure 3a**) (C. F. Wei et al., 2007). Δ*hrpR,* Δ*hrpS* and Δ*hrpRS* mutants of *Pst*DC3000 were used to perform RT-qPCR. Δ*hrpR,* Δ*hrpS* and Δ*hrpRS* mutants of SCH791 (**table 1, this chapter**) have been used to perform GUS assays.

d) Construction of vectors to express modified HrpR proteins

*hrpR*Δ*C* was amplified from pR284 and cloned into pET28a at XbaI and XhoI sites to obtain pRNC. The reverse primer (P14R) used here adds a stop codon before the region that codes for C-terminal tail of HrpR. 971bp 5' region of *his.t7.hrpR* from pR284 has been amplified with primers P14F and P15R. P15R adds a 25bp 3' overhang that overlaps with 5' region of *hrpS* HTH region. P16F/R were used to amplify *hrpS.HTH* region. Both the fragments were ligated in a PCR reaction where the overlapping regions extend to obtain *hrpR.HTH*$_s$. Later this fragment was cloned at XbaI and XhoI sites in pET28a to obtain pRS. *hrpR.HTH*$_s$*.C*$_R$ is obtained by ligating 971bp 5' region of *his.t7.hrpR* and *hrpS.HTH.hrpRC. hrpS.HTH.hrpRC* is obtained by amplifying *hrpS.HTH* with P16F and P17R that adds a *hrpR* C-terminal tail fragment at 3' end. *hrpR.HTH*$_s$*.C*$_R$ is cloned at XbaI and XhoI sites in pET28a to obtain pRSC.

All the primers used in this study were ordered from Integrated DNA Technologies (IDT) and are listed in the **Table 2** of this chapter. PCR reactions were setup according to the protocols supplied with Phusion® High-Fidelity polymerase (Thermo Scientific). All the DNA fragments that were cloned into plasmids were sequenced at DNA sequencing facility at University of Missouri-Columbia.

β-Galactosidase assays

E. coli MC4100 cultures were started at 0.1 OD_{600} from an overnight culture. They were induced with IPTG and grown until they reached OD_{600} 0.5. *Pst*DC3000 cultures were started and induced at 0.2 OD_{600} and grown for 24hrs. 200µL of culture was added to 800µL of Z- buffer (16.1 g $Na_2HPO_4.7H_2O$, 5.5g $NaH_2PO_4.H_2O$, 0.75 g KCl, 0.246 g $MgSO_4.7H_2O$, 2.7mL β-Mercaptoethanol, H_2O to make final volume to 1L) and lysed with 40µL of 0.1 % SDS and 80µL of chloroform. 0.2 mL of ONPG (Ortho-Nitrophenyl-β-galactoside), which is a substrate of β-Galactosidase was added to above lysed cells and incubated in a water bath at 30^0C for 10 minutes or until the color changes to yellow due to the release of Ortho-Nitrophenol. This reaction was stopped by adding 0.5mL Na_2CO_3. Final OD was recorded at wavelength 420nM and all the readings were substituted in the below formula.

$$\text{Miller Units} = 1000 \times \frac{OD_{420}}{\text{Time(S)} \times \text{Vol (ml)} \times OD_{600}}$$

Rest of the assay is performed as mentioned by Miller and coworkers (Miller, 1971) . *Pst*DC000 strains for β-Galactosidase assay are grown in HDM media and *E. coli* strains were grown in LB media.

β-glucuronidase (GUS) assays

*Pst*DC3000 strains were grown overnight and washed in 10mM $MgCl_2$ three times and suspended in 150µL of 10mM $MgCl_2$. New cultures were started in HDM at 0.2 OD_{600} and 50µL cultures were collected every 2 hrs in 96 well plate for 24 hrs and frozen at -80^{0}C until assayed. 150µL of GUS extraction buffer [50mM $NaHPO_4$ (Ph 7.0), 10mM EDTA (pH8.0), 0.1% sarcosyl, 0.1% Triton-X 100, 10mM β-Mercaptoethanol] was added to each well to lyse the cells and release β-glucuronidase. 8ul of these lysed cells was transferred to an opaque 96-well plate and 50µL of GUS reaction buffer [GUS extraction buffer + 1mg/ml 4-methylumbeliferyl β-glucuronide (MUG)] was added and incubated for 10 minutes. β-glucuronidase releases a fluorescent compound 4-methylumbeliferyl (MU) from MUG and its fluorescence was measured in Perkin-Elmer plate reader. The readings obtained from the plate reader are converted to pmoles of MU released by using a standard curve. All the values from above are substituted in [GUS specific activity = pmol/(min*ml*OD600)] to calculate GUS activity (Gallagher, 1992; Stauber et al., 2012).

RNA Extraction

*Pst*DC3000 cultures were started at 0.2 OD_{600} in HDM and shaken at RT for 2 hours. RNA was extracted using an RNA extraction kit (Zymo Research) followed by DNAse I treatment (Mo Bio).

RT-PCR

For cDNA synthesis 2.5µg of RNA suspended in 13µL RNase free water was mixed with 2µL of 100ng/µL random hexamers, 1µL 10mM RNAse free

dNTPS and heated for 5 minutes at 65^0C followed by addition of 4µL first strand-buffer, 1µL 0.1M DTT and 1µL RNase inhibitor RNasOUT (Invitrogen) and heated for 2 minutes at 42^0C. Later 1µL of SuperScriptIII reverse transcriptase (Invitrogen) was added to the reaction and incubated for 55^0C for 60 minutes to synthesize the cDNA. SuperScript enzyme was denatured by heating the reaction at 95^0C for 2 minutes. The reaction is cooled down to RT and 1µL of RNase was added to remove RNA from the reaction. The cDNA synthesized is later used as template in a PCR reaction to amplify target region in the genome.

RT-qPCR

WT and mutant *Pst* DC3000 cultures were started at 0.2 OD_{600} and grown for 2 hours in HDM and harvested for RNA isolation. cDNA was synthesized as mentioned above. 3.5 µL of 1/100 dilution of cDNA, 7.5µL of Bio-Rad's 2X SYBR® green (DNA polymerase, dNTPs, MgCl2, SYBR®Green I dye, enhancers, stabilizers, and fluorescein) and 2µL each of 1.5pm forward and reverse primers were mixed together. The reaction was heated to 95°C for 3 minutes to activate the DNA polymerase followed by 39 cycles of denaturation (95°C for 10 seconds), primer annealing (60.5°C for 30 seconds) and extension (72°C for 30 seconds) in Bio-Rad C1000 thermal cycler. Fluorescence emitted by SYBR green bound to dsDNA was recorded after every cycle. $2^{-\Delta\Delta C_T}$ method was used to determine relative expression of the target gene compared to the calibrator. Bio-Rad C1000 thermal cycler determines the cycle number (C) at which the target gene amount reaches a threshold level. ΔC_{Tq} values were determined by subtracting C value of an internal control from the C value of the

target gene. ΔC_{Cb} is determined by subtracting C value of an internal control from the C value of calibrator. $\Delta\Delta C_T$ is calculated by subtracting ΔC_{Cb} from ΔC_{Tq}. $2^{-\Delta\Delta C_T}$ value gives the relative fold expression of target gene compared to the calibrator (Livak & Schmittgen, 2001). *gapdh* is used as an internal control in this study. 1 in 10 dilution of cDNA was used as the template. Primers used to amplify a portion the target genes (<200bp) are shown in the **Table 2** of this chapter.

Protein Preparation and Analysis

E. coli BL21 (DE3) strains harboring vectors (**Table1**, **this chapter**) that activate genes under T7 promoter control were used to express proteins. BL21 strains produceT7 polymerase when induced by IPTG. T7 polymerase activates genes that are under the control of T7 promoter. *his-t7-hrpR* and *his-s-hrpS* in pET28a, pET30a and pDUET vectors are under T7 promoter control. 500mL bacterial cultures were grown at 37^0C until they reached 0.5 OD_{600} and kept on ice for 15 minutes. They were induced with 0.1mM IPTG and shaken at 16^0C for 6 hours. Bacteria were pelleted and lysed by incubating in 30 mL lysis buffer [20mM Tris.HCl (pH8), 500mM KCl, 20% glycerol, 1mM DTT, 10mM Imidazole, 0.25% Triton X, 2mg/mL lysozyme (Gold Bio Labs)] for 30 minutes at RT followed by sonication with Fischer Scientific 550 Sonic Dismembrator (20S pulses at level 2 for 5 times with a 30S interval). This lysate is centrifuged at 15,000g to remove debris and mixed with 500uL of Ni-NTA resin (Novagen) that was equilibrated with equilibration buffer (20mM Tris.HCl (pH8), 500mM KCl, 20% glycerol, 1mM DTT, 10mM Imidazole) and incubated at 4^0C for 1 hour. The lysate-resin mixture later was loaded into a protein column (Biorad) and unbound

protein was allowed to drain. The resin in the column that bound the His-Tag protein was washed with 10mL each of wash buffer 1(20mM Tris.HCl (pH8), 500mM KCl, 20% glycerol, 1mM DTT, 50mM Imidazole) and wash buffer 2 (20mM Tris.HCl (pH8), 500mM KCl, 20% glycerol, 1mM DTT, 100mM Imidazole). The proteins were eluted 4X with 1mL of elution buffer (20mM Tris.HCl (pH8), 500mM KCl, 20% glycerol, 1mM DTT, 250mM Imidazole). Second and third elutions were transferred into a 3mL dialysis bag (Thermo Scientific) and dialyzed overnight in 2 L dialysis buffer (20mM Tris.HCl (pH8), 500mM KCl, 20% glycerol, 1mM DTT) at 4°C (25, Mindy Steiniger lab protocols). Dialyzed proteins were centrifuged in 30K centrifugal filters (Millipore) to remove any IHF (21KDa) contaminant.

Presence of proteins was checked by running purified proteins on a 10% SDS-PAGE followed by coomassie gel staining and Western blot analysis. For coomassie gel staining, SDS PAGE was soaked in coomassie stain (40% Methanol, 7% acetic acid 0.025% Brilliant Blue R250) for 30-minutes and de-stained in water overnight. For western blots, proteins were transferred from 10% SDS-PAGE gel onto a Nitrocellulose membrane in a semi dry blotter. The nitrocellulose membrane was blocked in 5% milk overnight. The membrane is soaked in 1:10000 dilution of T7-antibody (Novagen) for 1hr while probing for His.T7.HrpR and other modified HrpR proteins, and 1: 5000 S-antibody (Novagen) for 30 minutes to probe His-S-HrpS protein.Horse radish peroxidase conjugated anti-goat secondary antibodies are used at 1:30000. All the antibodies were diluted in TBST [20mM Tris.HCl (pH 7.5) 150mM NaCl, 0.1%

Tween20]. After antibody treatment, membrane was washed 3X in TBST and soaked in 10 ml of SuperSignal west pico chemiluminescent substrate (Thermo scientific) for 10 minutes followed by exposure onto an X-ray film. The X- ray film was developed and fixed in 20% developer and 20% fixer solutions respectively from Kodak (Rodriguez, Schechter, & Lee, 2002).

Electrophoretic mobility shift assay (EMSA)

LightShift® Chemiluminescent EMSA Kit (Thermo Scientific) was used to perform gel retardation assays. Proteins were incubated with 20fmoles of 5' biotin labeled DNA fragment in 2µL 10X binding buffer [100mM Tris (pH7.5), 500mM KCl, 10mM DTT] in 20µL reaction volume for 20 minutes and loaded on to a 4% native gel (3mL 5X TBE, 4mL 30% acrylamide, 3mL glycerol, 112.5µL 20% APS, 30µL TEMED, 19.895mL ddH_20). The gel was run in 0.5X TBE buffer at 70V for 3 hrs. DNA in the gel was transferred on to a nylon membrane (Sigma Biobond) in a semi dry blotter. The DNA was UV-crosslinked to the membrane at 120mJ/cm^2 for 2 minutes. The membrane was probed with Streptavidin-Horse Radish Peroxidase conjugate that binds 5' biotin labeled DNA followed by soaking in a SuperSignal west pico chemiluminescent substrate (Thermo scientific) for 5 minutes. The membrane later is exposed on to an X-ray film and developed. EMSA probe was obtained by amplifying 253bp *hrpL* promoter region with 5'Biotin labeled primers (P23F and P23R) (**Table 2** of this chapter).

DNaseI Footprinting

Proteins were incubated with DNA fragment that is 5' HEX and 3'FAM labeled in 20µL 5X TKMC buffer [10mM Tris-HCl (pH7.5), 10mM KCl, 25mM

$MgCl_2$, 25mM $CaCl_2$] and a total reaction volume of 90µL for 20 minutes. 10µl of 0.01 U/µL of DNasI (Promega) was added to it and incubated at 37^0C for 5 minutes. The reaction was stopped by adding 10µL 200mM EDTA and heating at 100°C for 2 minutes. DNA was precipitated using Wizard®SV Gel – PCR cleanup Kit (Promega) and sent to Mizzou for analysis (Zianni, Tessanne, Merighi, Laguna, & Tabita, 2006) (Special thanks to Dr. Bashkin's Lab, Dr. Dupureur's Lab). *hrpL* promoter fragment was amplified with a HEX labeled P23F and a FAM labeled P23R primers (**Table 2** of this chapter).

Magnetic Beads Assay

1µg of Biotin labeled DNA was incubated with 5µL Dynabeads® M-280 Streptavidin (Invitrogen) in 1 mL of wash buffer [5mM Tris-HCl (pH7.5), 0.5mM EDTA, 1M NaCl] for 30 minutes. Dynabeads were washed 3X with protein binding buffer [1mM Tris-HCl (pH7.5), 50mM KCl, 1mM DTT, 0.1% BSA] to remove any unbound DNA. 4µg of protein was added to Dynabeads bound with DNA and incubated for 20 minutes. Dynabeads were collected at the bottom of the tube using a magnet and supernatant protein binding buffer was pipetted out and this was done 3X. 50µL of Laemmlis sample buffer was added to the beads and heated at 95°C for 2 minutes to release the proteins from the DNA. Proteins were later detected by performing a western blot using specific antibodies. All the steps in this procedure were done as mentioned in the protocol that came with Dynabeads® M-280 Streptavidin (Invitrogen).

Bioinformatics analysis

hrpJ-hrpL intergenic regions from different *Pseudomonas syringae* species whose sequences are available were retrieved from NCBI. ClustalΩ (Goujon et al., 2010; McWilliam et al., 2013; Sievers et al., 2011) was used to align promoter regions of *hrpL* from different *Pseudomonas syringae* species to identify conserved regions. MEME software was used to generate a logo for the conserved region (T. L. Bailey et al., 2009).

Table 1

Strain	Description	Resistance	Source[a]
Pseudomonas Syringae* pv *tomato			
DC3000	Whole genome sequenced. Suitable for genetic manipulations	Rifampacin	(Cuppels, 1986)
SCH791	DC3000 with *hrpL::uidA* chromosomal transcriptional fusion. Used in GUS assays	Rifampacin	(Stauber et al., 2012)
CUCPB5114	Δ*hrp* gene cluster mutant. Used to perform β-Galactosidase assays	Rifampacin	(Fouts, Badel, Ramos, Rapp, & Collmer, 2003)
ST1012	Δ*hrpR* DC3000 mutant	Rifampacin	
ST1005	Δ*hrpS* DC3000 mutant	Rifampacin	

ST1015	Δ*hrpRS* DC3000 mutant	Rifampacin	
ST1023	Δ*hrpR* in *hrpL::uidA* DC3000 background	Rifampacin	
ST1021	Δ*hrpS* in *hrpL::uidA* DC3000 background	Rifampacin	
ST1017	Δ*hrpRS* in *hrpL::uidA* DC3000 background	Rifampacin	
E. coli			
BL21 (DE3)	F^- *ompT hsdSb* (rB- mB-) *gal dcm lon* (DE3)	None	Novagen
DH5α	F^- *endA1 recA1 hsdR17(rK- mK+) deoR* thi^{-1} *supE44* λ^- *gyrA96 relA1*	Nalidixic acid	Invitrogen

MC4100	*F- araD139 delta(argF-lac)U169 rpsL 150 (Strr) relA1 flbB5301 deoC1 tsF25 rbsR*	Streptomycin	(Casadaban, 1976)
Plasmids			
pET28A		Kanamycin	Novagen
pET30S		Kanamycin	Novagen
pUCP24		Gentamycin	(Schweizer, 1991)
pDUET		Kanamycin	Novagen
pBBRI-MCS2		Kanamycin	(Kovach et al., 1995)
pRG970		Spectinomycin	(van den Eede, Deblaere, Goethals, van Montagu, & Holsters, 1992)
pR284	*hrpR* of DC3000 cloned into pET28a	Kanamycin	
pS308	*hrpS* of DC3000 cloned into pET30a	Kanamycin	
pRSFDUET	*hrpR* and *hrpS* cloned into pDUET vector	Kanamycin	

pHR24	*his.t7.hrpR* cloned into pUCP24	Gentamycin	
pBS12	*his.s.hrpS* cloned into pBBR1-MCS2	Kanamycin	
pL752	752bp *hrpL* promoter region cloned into pRG970	Spectinomycin	
pL253	253bp *hrpL* promoter region cloned into pRG970	Spectinomycin	
pL169	169bp *hrpL* promoter region cloned into pRG970	Spectinomycin	
pL110	110bp *hrpL* promoter region cloned into pRG970	Spectinomycin	
pL47	47bp *hrpL* promoter region	Spectinomycin	

	cloned into pRG970		
pRK415	*corR* of DC3000 cloned into pRKCRS	Tetracycline	
pK18*mobsac*	Suicide vector harboring a *sacB* gene	Kanamycin	
pKR18R	1.0 kb upstream and downstream regions of *hrpR* flanking *nptII* cloned into pK18*mobsacB*	Kanamycin	
pKR18S	1.0 kb upstream and downstream regions of *hrpRS* flanking *nptII* cloned into pK18*mobsacB*	Kanamycin	
pKR18RS	1.0 kb upstream and downstream regions of *hrpRS*	Kanamycin	

	flanking *nptII* cloned into pK18*mobsacB*		
pRNC	*hrpR*Δ*C* cloned into pET28a	Kanamycin	
pRS	*hrpR*Δ*HTH*$_s$ cloned into pET28a	Kanamycin	
pRSC	*hrpR*Δ*HTH*$_s$Δ*C* cloned into pET28a	Kanamycin	

a) All the strains were generated in this study unless referenced.

Table 2

Primers[a]	Sequence (5' to 3')[b]	Description
P1F	TCGGATCCATGAGTACAGGCATCGATAAG	To Clone *hrpR* into pET28a
P1R	ACCTCGAGGACATCAACGTTGCATAAC	To Clone *hrpR* into pET28a
P2F	GTGGATCCATGAGTCTTGATGAAAGG	To Clone *hrpS* into pET30a
P2R	ATCTCGAGCAGCCTGCGAATCGGT	To Clone *hrpS* into pET30a
P3F	TATACCATGGGCAGCAGCCATCATCATC	To Clone *hrpR* into pDUET1
P3R	TTGCCCATGGACACCTGCGTTTCAGACCC	To Clone *hrpR* into pDUET1
P4F	CCGCTCGAGCACCATCATCATCATCATTC	To Clone *hrpS* into pDUET1
P4R	ATCTCGAGCAGCCTGCGAATCGGT	To Clone *hrpS* into pDUET1
P5F	GCGGTACCCCTCTAGAAATAATTTTG	To Clone *hrpR* into pUC24
P5R	ATCTCGAGCAGCCTGCGAATCGGT	To Clone *hrpR* into pUC24
P6F	GCGAATTCCCCTCTAGAAATAATTTTG	To Clone *hrpS* into

		pBBR1-MCS2
P6R	AAACTAGTGGTGGTGGTGGTGCTC	To Clone *hrpS* into pBBR1-MCS2
P7F	GCTAACGGATCCTTGGCGCTGTTGATC	To Clone 752bp *hrpL* promoter region into pRG970
P8F	CGGATCCACGATTTTCATAGGGCAGTTC	To Clone 253bp *hrpL* promoter region into pRG970
P9F	GGGCGGGATCCTTCACATTTTAAAATATCT	To Clone 110bp *hrpL* promoter region into pRG970
P10F	TGGATCCAAGCTGGCATGGTTATCG	To Clone 47bp *hrpL* promoter region into pRG970
P11F	AGCGGATCCATGTTTTTGTGCCAAAAGCTG	To Clone 169bp *hrpL* promoter region into pRG970
P12R	GGGATCCGGGCTTACCCTGATTTAGT	Reverse primer to clone all *hrpL* promoter regions of different

		length into pRG970
P13F	GCCAGGAAGCTTCGATTACAGGTCATTACAC	To clone *corR* into pRK415
P13R	GAATTGTTCTAGACTCTACGATGCCGCTCC	To clone *corR* into pRK415
P14F	CCCTCTAGAAATAATTTTGTTTAACTTTAA	To clone *hrpR*Δ*C* into pET28a
P14R	GTGCTCGAGTCAAACTCCCAGTTCCTT	To clone *hrpR*Δ*C* into pET28a
P15R	GGCTTACCGAATCCACACAATTGTCCCTGT GCCGCTTCAAGGCATCCTGG	To make 971bp 5' *his.t7.hrpR* fragment with a 20bp 3' overhang that overlaps overlap with *hrpS* HTH region.
P16F	GACAATTGTGTGGATTCGGTAAGCC	To amplify *hrpS* HTH region.
P16R	ATTCTCGAGTCAGATCTGCAATTCTTTGATGCGT	To amplify *hrpS* HTH region
P17R	ATTCTCGAGTCAGACCCCGGCCGTCGCAG CGATCGGCGCTGCGATCTGCAATTCT TTGATGCGTCG	To amplify *hrpS* HTH region with *hrpR* C-terminal tail at its 3' end

P18F	GAGCCGCACGAATTCGTTTTTGCC AGTGATCCACGCCA	To amplify 1 kb upstream *hrpR* region
P18R	GCGTGCAATCCATCTTGTTCAATCA TCGTTCACTCTCATGGTGGGTGG	To amplify 1 kb upstream *hrpR* region with a 25bp *nptII* overlap at its 3' end
P19F	ATCGCCTTCTTGACGAGTTCTTCT GAAACGCAGGTGTGGTTATGC	To amplify 1kb downstream region of *hrpR* with a 25bp *nptII* overlap at its 5' region
P19R	TACCAGCGTTCTAGATTGGTAC TCACTAGGTGGCAGC	To amplify 1kb downstream region of *hrpR*
P20F	TATGGTGATGGATCCATGATAG TAATTCTCAACTTTGTGATCTT	To amplify 1 kb upstream *hrpS* region
P20R	GCGTGCAATCCATCTTGTTCAATC ATCCCATGACCCCCAGGAC	To amplify 1 kb upstream *hrpS* region with a 25bp *nptII* overlap at its 3' end
P21F	ATCGCCTTCTTGACGAGTTCTTCTG ATTTTTTGCAAAGACGCTGGAA	To amplify 1kb downstream region of *hrpS* with a 25bp *nptII* overlap at its 5' region

P21R	AACTGGGCCTTGAAGCTTTCCATG AACTGGGCGACTTTTT	To amplify 1kb downstream region of *hrpS*
P22F	ATGATTGAACAAGATGGATTGCACG	To amplify *nptII*
P22R	TCAGAAGAACTCGTCAAGAAGGCG	To amplify *nptII*
P23F	ACGATTTTCATAGGGCAGTTCTAAG	To make a Biotin labeled 253bp *hrpL* promoter region
P23R	GGGCTTACCCTGATTTAGTGGTG	To make a Biotin labeled 253bp *hrpL* promoter region
P24F	CCAAGGCTACCGGCAGGACC	RT-PCR primer for *gapdh*
P24R	AACGGGCCGTGTACAGTGTCG	RT-PCR primer for *gapdh*
P25F	TTTCCCCGCACAACAGCAAGTC	RT-PCR primer for *hrpR*
P25R	TGAATGCACCGTTGACCACACC	RT-PCR primer for *hrpR*

P26F	GCTTCCTCCGCTACGTAACCAGTC	RT-PCR primer for *hrpS*
P26R	GGCACGTCCAGTTCCAGGCTTAC	RT-PCR primer for *hrpS*

a) F = Forward primer, R= Reverse primer
b) Sequences underlined with black line are restriction sites and those underlined with red line are overlapping regions

8.4) Results

Regulation of *hrpL* in *Pst* DC3000

In a previous study, both HrpR and HrpS were shown to be necessary for the activation of *hrpL* when all these *Pst* DC3000 genes were ectopically expressed in *E. coli* (Hutcheson et al., 2001). As a first step in identifying HrpR/S binding site in the *hrpL* promoter region, I made different lengths of *hrpL* promoter fragments. I cloned these fragments into pRG970, which is a *lacZ* reporter plasmid (**Figure 2**). LacZ is produced when the *hrpL* promoter is active and β-galactosidase activity of these strains is directly proportional to *hrpL* promoter activity. Hence, β-galactosidase is only observed when the reporter vector with *hrpL* promoter fragment that includes HrpR/S binding site is present. His.T7.HrpR expressed from pHR24 and His.S.HrpS expressed from pBS12 were electroporated individually and together into *E.coli* MC4100 strains harboring different *hrpL* reporter vectors (**Table 3** of this chapter). I observed that *hrpL* was active only in the presence of both HrpR and HrpS in *E.coli* like previous studies (Hutcheson et al., 2001) (**Table 3** of this chapter). My results also indicated that tagged HrpR and HrpS proteins are active. In addition, I observed that *lacZ* was expressed in the reporter vector that harbored the 169bp *hrpL* promoter region, but not in the vector that has the 47bp *hrpL* promoter. Therefore, I hypothesized that the upstream activating sequence (UAS) bound by HrpR and HrpS was between -47bp and -169bp upstream of the *hrpL* translation start site. During this time, *Jovanovic et al* published similar results in *E.coli* (Jovanovic et al., 2011).

HrpR and HrpS are native to *Pseudomonas syringae* species and might function differently in *E.coli*. So, I decided to test how HrpR and HrpS activate *hrpL* in *Pst* DC3000. First, I created Δ*hrpR,* Δ*hrpS* and Δ*hrpRS* mutants of *Pst* DC3000 (**Methods and materials** section of this chapter). *hrpRS* is transcribed from a single promoter and their protein products interact with each other (Hutcheson et al., 2001). Δ*hrpR,* Δ*hrpS* mutants I generated were non-polar. This was verified by performing RT-PCR for both *hrpR* and *hrpS* in Δ*hrpR* and Δ*hrpS*. *hrpR* is absent but not *hrpS* in Δ*hrpR* and *hrpS* is absent but not *hrpR* in Δ*hrpS* (**Figure 3a**). RT-PCR of *gapdh* was done in all mutants to serve as a loading control and no reverse transcriptase reactions were setup to check if there is any background genomic DNA contamination (**Figure 3a**). I then performed β-galactosidase assays on Δ*hrpR,* Δ*hrpS*, Δ*hrpRS* and Wt *Pst* DC3000 harboring *hrpL* reporter vectors (**Table 4** of this chapter). I observed that *hrpL* was active only in the presence of HrpR and HrpS and β-galactosidase activity was seen only in the strains that had 169bp *hrpL* reporter vector but not 110bp *hrpL* reporter vector.

We further validated that both HrpR and HrpS are required for *hrpL* expression in DC3000 by performing a GUS assay and RT-qPCR. We performed GUS assays in Δ*hrpR,* Δ*hrpS* and Δ*hrpRS* mutants of SCH791, a DC3000 strain carrying a *hrpL::uidA* transcriptional fusion. We observed GUS activity only in Wt SCH791 (**Figure 3b**). In RT-qPCR analysis, we assessed *hrpL* expression in Δ*hrpR,* Δ*hrpS*, Δ*hrpRS* and Wt DC3000 by comparing with *hrpL* expression in Δ*hrpRS* DC3000. We observed that *hrpL* expression is negligible in Δ*hrpR,*

Δ*hrpS* mutants and ≈15-fold higher in Wt DC3000 compared to the Δ*hrpRS* mutant (**Figure 3c**).

Previous studies showed that *hrpL* expression was decreased and delayed in a Δ*corR* DC3000 when grown in HDM. They also showed that CorR binding site is located between -664bp and -752bp upstream of *hrpL* translation start site (**Figure 2**) (Sreedharan et al., 2006). I hypothesized that HrpRS dependent *hrpL* activation is more in the presence of CorR. But our results suggested that CorR did not activate *hrpL* when ectopically expressed in *E. coli* in the presence or absence of HrpRS (**Table 3** of this chapter) or in WT and Δ*hrpRS Pst* DC000 (**Table 4 of** this chapter). Although this data contradicts with the previous study (Sreedharan et al., 2006), this does not leave out the possibility that CorR might activate *hrpL* in other conditions.

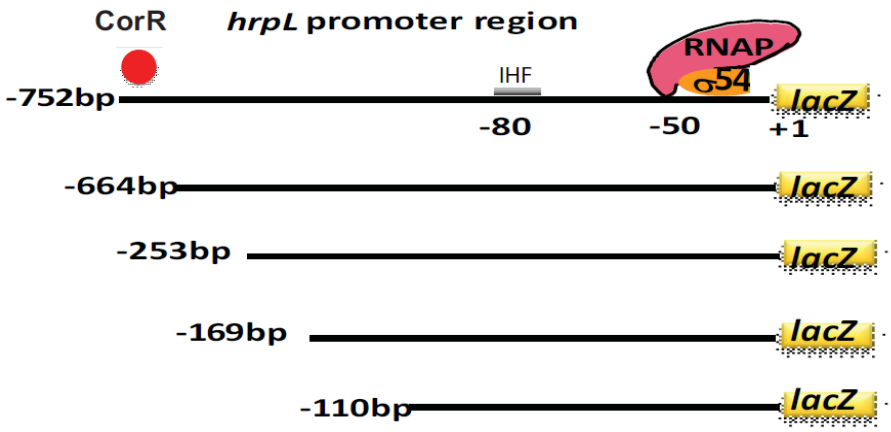


Figure 2: *hrpL::lacZ* fusion constructs. Different length *hrpL* promoter regions are fused to *lacZ*. Top strand shows RNAP-σ54 holoenzyme and CorR binding regions. Putative IHF binding site is also shown

Table 3

β-Galactosidase assays in *E. coli* MC4100						
hrpL **promoters**	**R**	**S**	**RS**	**RS_{WT}**	**CorR**	**RS_{wt}+Cor R**
752 bp	ND	ND	25.75±4.03	47.65±1.48	5.67±0.28	76.59±5.96
664 bp	ND	ND	21.678±0.23	ND	ND	58.75±1.31
253 bp	*1.48 ±0.13*	0.68±0.05	65.17±0.94	62.32±0.67	4.81±0.11	65.36±1.86
169 bp	ND	ND	21.49±0.23	57.42±2.66	3.72±0.13	74.26±3.42
47 bp	ND	ND	0.908±0.05	1.29±0.03	ND	ND

R = His.T7.HrpR, S = His.S.HrpS, RS = His.T7.HrpR+ His.S.HrpS, RS_{Wt} = $HrpRS_{Wt}$.

Table 4

β-Galactosidase assays in *Pst*						
***hrpL* promoters**	***Pst***	**Δ*hrpR***	**Δ*hrpR*+R**	**Δ*hrpS***	**Δ*hrpS*+S**	**Δ*hrp***
752 bp	*59.26±3.64*	4.24±*0.59*	110.231±*13.59*	4.40±*0.19*	24.55±*1.01*	3.86±*0.28*
169bp	*56.60±2.87*	3.04±*1.37*	94.32±*7.15*	4.76±*0.030*	20.57±*1.25*	5.13±*0.46*
110bp	*0.041±0.03*	0.71±*0.02*	0.47±*0.06*	0.78±*0.10*	0.29±*0*	0.62±*0.15*
Empty Vector	*0.92±0.14*	ND	ND	ND	ND	ND

Pst = *Pseudomonas syringae* DC3000, Δ*hrpR* = Δ*hrpR* DC3000, R = His.T7.HrpR, Δ*hrpS* =Δ*hrpS* DC3000, S = His.S.HrpS, Δ*hrp* = DC3000 lacking most of the *hrp* PAI.

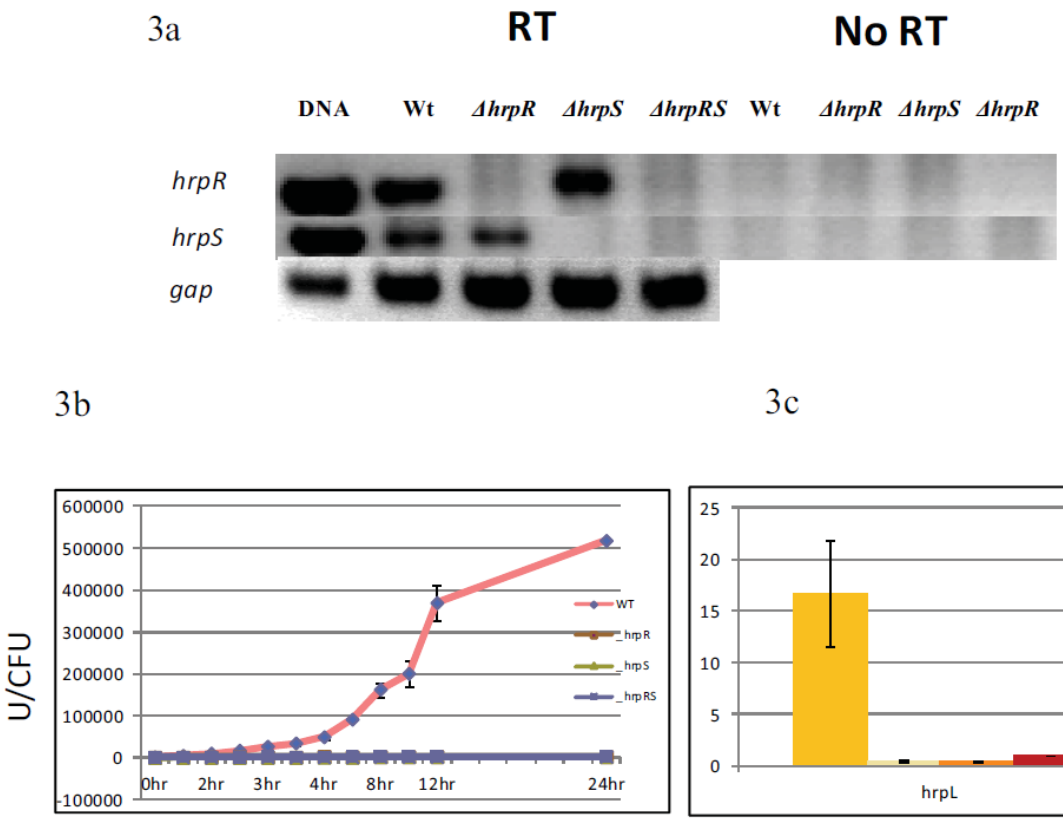


Figure 3: Regulation of *hrpL* by HrpR and HrpS in Wt and mutant DC3000.3a) RT-PCR gel that confirmed loss of gene expression in study to RT-PCR gels that confirmed the loss of gene Δ*hrpR,* Δ*hrpS* and Δ*hrpRS Pst* DC3000. *hrpR, hrpS* and *gap* on the left side of the gel indicate the primers used. GUS specific activity (Units/CFU) in Wt, Δ*hrpR*, Δ*hrpS* and Δ*hrpRS hrpL::uidA* DC3000and *hrpL* expression in Wt DC3000 compared with mutants (3b,3c)

HrpS and HrpRS binds *hrpL* promoter

Data from my previous experiments strongly suggest that the HrpRS binding site could be between -110bp and -169bp upstream of *hrpL* translation start site. His.T7.HrpR (38.1 KDa) and His.S.HrpS (38.7 KDa) have been expressed from pET vectors in *E. coli* BL21and purified on a Ni-NTA column that bind His tagged proteins. Because the previous study showed that HrpR and HrpS are active in a hetero-hexameric state (Jovanovic et al., 2011), I also co-expressed tagged proteins from a pDUET vector. Thus, HrpR and HrpS could also be purified as a complex. The His.S.HrpS expressed from the pDUET vector is 40.7 KDa since it has longer N-terminal region upstream of the His tag (**Figure 4a, lane9**). We analyzed the purified protein samples by running them on an SDS-PAGE gel followed by Coomassie staining and Western blot analysis (**Figure 4a, 4b**) (**Methods and materials** from this chapter**).** I observed that the proteins were of expected sizes.

Jovanovic et al showed that HrpR, HrpS and HrpRS complex bind *hrpL* promoter region (Jovanovic et al., 2011). My results initially showed a single shift with HrpR and two shifts with HrpS and HrpRS complex (data not shown). I followed up this experiment by DNaseI footprinting of *hrpL* in the presence of purified HrpR, HrpS and HrpRS. I observed that the footprints overlapped with putative IHF binding site (data not shown) (Hales, Gumport, & Gardner, 1994) (**Figure 2, Figure 9a**). I concluded that my protein preparations were contaminated with *E.coli* IHF. I expected a second DNaseI sensitive region in *hrpL* in the presence of HrpS and HrpRS but failed to observe it. This could be because of weak

binding of HrpS and HrpRS. To avoid IHF (21KDa) contamination, after purification, I passed His-T7-HrpR through Millipore 30K centrifugal filters to filter out proteins that are less than 30KDa. I observed that IHF free His.T7.HrpR did not shift *hrpL* (**Figure 5a**). Both His.T7.HrpS and His.T7.HrpR/His.S.HrpS complex shifted *hrpL* DNA (**Figure 5b, 5c).**

IHF stabilizes super helixes of dsDNA and promotes gene activation (Joly & Buck, 2011). I hypothesized that HrpR might not be binding to linear *hrpL* promoter regions in the EMSA due to absence of super helixes. So, I performed an EMSA experiment with His-T7-HrpR and *hrpL* in the presence of purified IHF (Gift from Dr. Steven Goodman). However, His.T7.HrpR didn't bind *hrpL* even in the presence of IHF (**Figure 5d**).

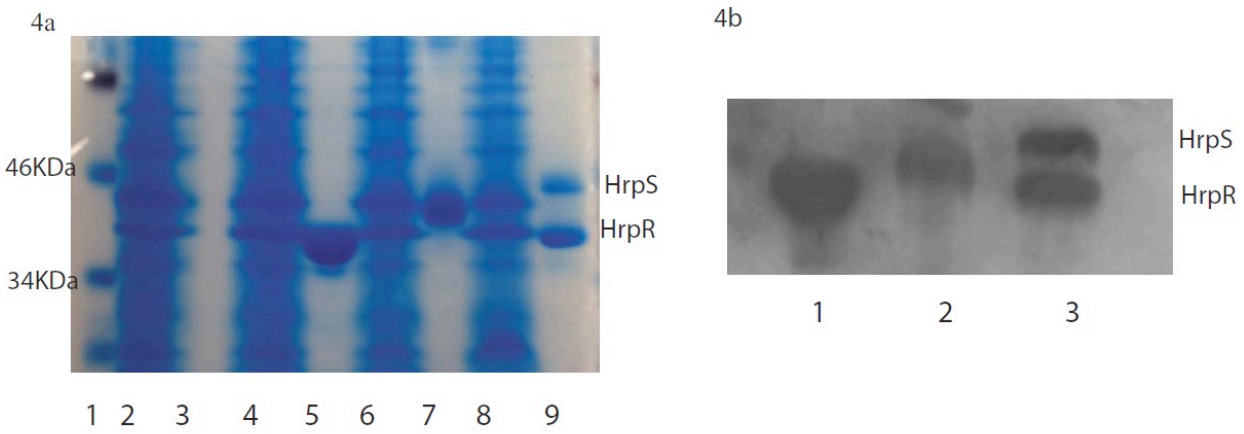
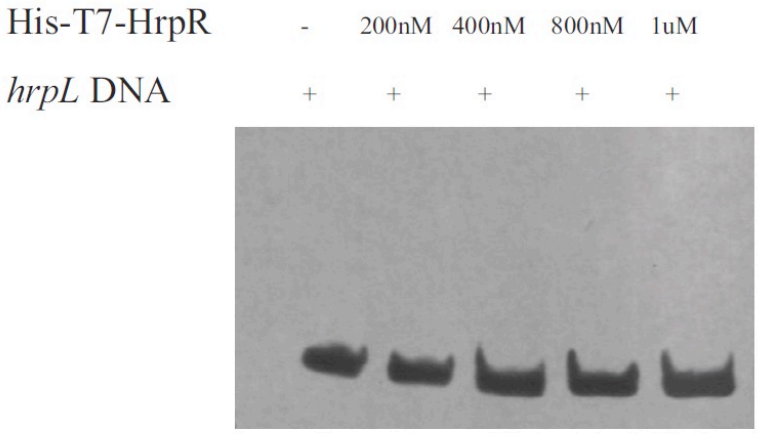
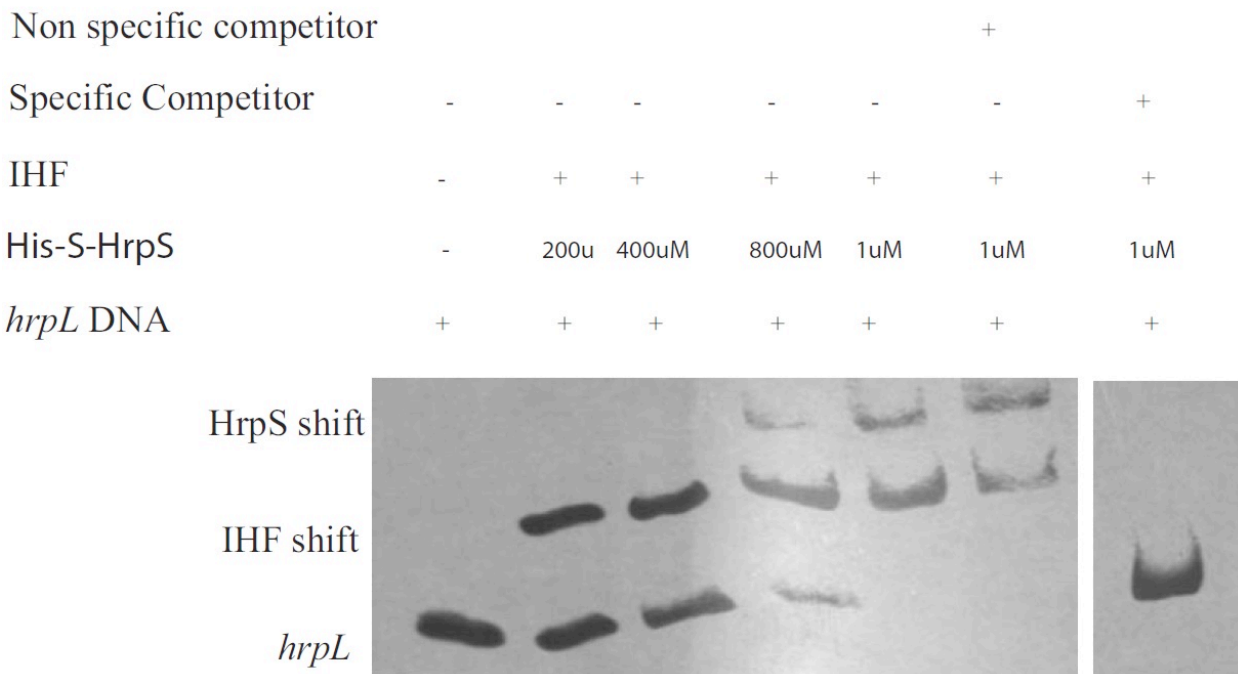


Figure 4: Analysis of tagged proteins. 4a) Coomassie gel of whole lysates and purified proteins. Lane 1: Protein Marker, lane 2: Empty vector lysate, lane 3: Empty vector elution, lane 4: His-T7-HrpR lystae, lane 5: His-T7-HrpR elution, lane 6: His-S-HrpS lysate, lane 7: His-S-HrpS elution, lane 8: Co-expressed His-T7-HrpR and His-S-HrpS lysate, lane 9: Co-expressed His-T7-HrpR and His-S-HrpS elution. 4b) Western blot to analyze tagged HrpR and proteins. His-T7-HrpR is identified using anti-T7and His-S-HrpS is identified using anti-S. Lane 1: His-T7-HrpR, lane 2: His-S-HrpS, lane 3 : Co-expressed His-T7-HrpR and His-S-HrpS

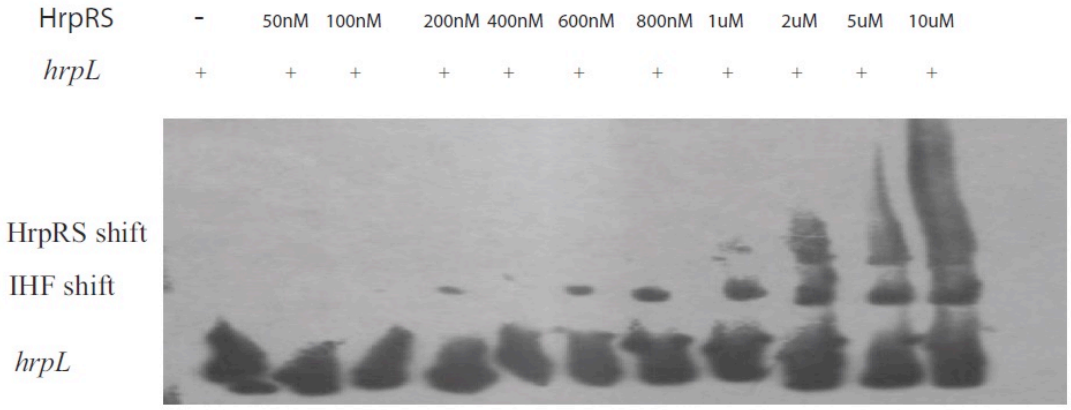
5a


His-T7-HrpR - 200nM 400nM 800nM 1uM
hrpL DNA + + + + +

5b


Non specific competitor +
Specific Competitor - - - - - - +
IHF - + + + + + +
His-S-HrpS - 200u 400uM 800uM 1uM 1uM 1uM
hrpL DNA + + + + + + +
HrpS shift
IHF shift
hrpL

5c



5d

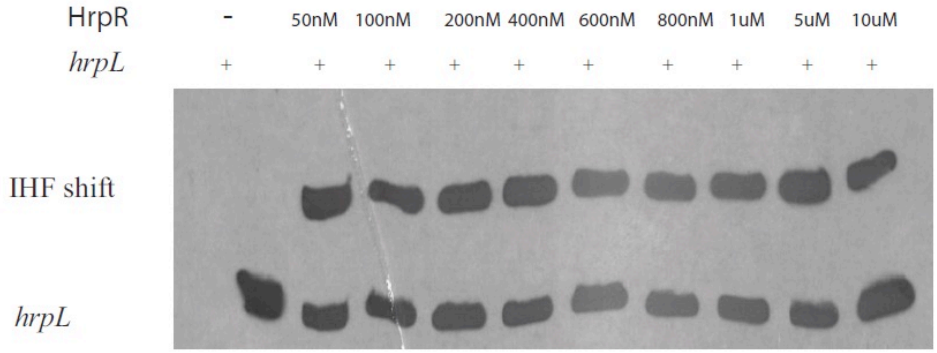


Figure 5: EMSA of *hrpL* with purified His-T7-HrpR and His-S-HrpS proteins. 5a) EMSA of *hrpL* in the presence of 200nM to 1uM of His-T7-HrpR. 5b) EMSA of *hrpL* in the presence of 200nM to 1uM of His-S-HrpS. Last 2 lanes *hrpL* shift in the presence of non-specific and specific competitor (un-labled 253bp *hrpL* promoter region). 5c) EMSA of *hrpL* in the presence of 50nM-10uM of His-T7-HrpR/His-S-HrpS complex. 5d) EMSA of *hrpL* by His-T7H presence of IHF.

HrpR binds *hrpL* in the presence of HrpS

hrpL shift in the presence of HrpRS (**Figure 5c**) could be due to HrpS alone. To check this, I performed a Super-shift assay using anti-T7 for His-T7-HrpR and anti-S for His-S-HrpS. But, I did not observe any super shift. This could be because the T7 and S tags that are located C-terminal to His tag might not be available for the antibodies. So, I designed an experiment with Dynabeads® M-280 Streptavidin (Invitrogen). I incubated 0.5μM HrpRS with streptavidin beads coated with *hrpL* promoter region DNA. We eluted protein from the beads after washes and performed a western blot to detect HrpR and HrpS proteins using anti-T7 and anti-S antibodies respectively (Explained in detail in **Methods and materials** of this chapter). Both HrpR and HrpS were bound to the beads with 253bp *hrpL* promoter region fragment (**lane 4, Figure 6**) but not to beads without DNA or the 47bp *hrpL* promoter fragment (**lane 2 and lane 3 in Figure 6**). These results confirm that HrpR binds to the *hrpL* promoter region in the presence of HrpS.

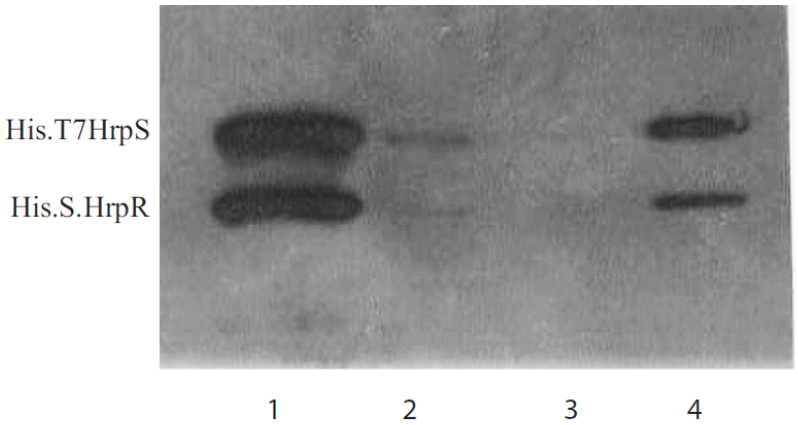


Figure 6: Pull down of proteins using streptavidin beads coated with *hrpL* promoter region. Lane 1 shows purified tagged HrpR and HrpS loaded directly. Lane 2 and 3 shows the protein bound to naked beads and to non-specific DNA on beads. Lane 4 shows protein bound to 253bp *hrpL* promoter region on the beads.

Modified HrpR proteins bind the *hrpL* promoter region

Since HrpR and HrpS differentially bound to *hrpL* promoter region, I decided to check for the differences in DNA binding domains of HrpR and HrpS. I aligned Helix-Turn-Helix (HTH) regions of HrpR and HrpS from few *Pseudomonas syringae* species. I observed three main differences between the HrpR and HrpS HTH regions. First, in most pathovars of *P.syringae* HrpR has an extra 10 amino acid C-terminal tail after the HTH region. The tail is shorter in few pathovars. Second, helix 1 or the stabilizing helix, is different in HrpR and HrpS. Third, helix 2 or the DNA binding helix of both HrpR and HrpS is identical except for two amino acids. They are R295 and I297 in HrpS and H297 and M299 in HrpR (**Figure 7**). To test whether these differences affect DNA binding, I made three modified HrpR proteins: 1) HrpR without the C-terminal tail (HrpRΔC), 2) HrpR with its HTH replaced by the HrpS HTH (HrpR.HTH$_S$) and no C-terminal tail, and 3) HrpR.HTH$_S$ with an added HrpR C-terminal tail (HrpR.HTH$_S$C$_R$) (**Figure 8a**). I expressed these proteins on pET vectors in *E.coli* BL21 and purified them. The purified samples were analyzed on a Coomassie stained SDS-PAGE gel and proteins of expected size were observed (**Figure 8b**). I later performed mobility shift assays on *hrpL* with all modified HrpR proteins. All the three modified proteins bound to *hrpL* (**Figure 8c**). These results suggest that the C-terminal tail in HrpR might prevent HrpR DNA binding. However, the C-terminal tail did not prevent HrpR.HTH$_S$C$_R$ from binding to *hrpL* DNA. It is possible that the inhibitory properties of the HrpR C-terminal tail require the HrpR HTH.

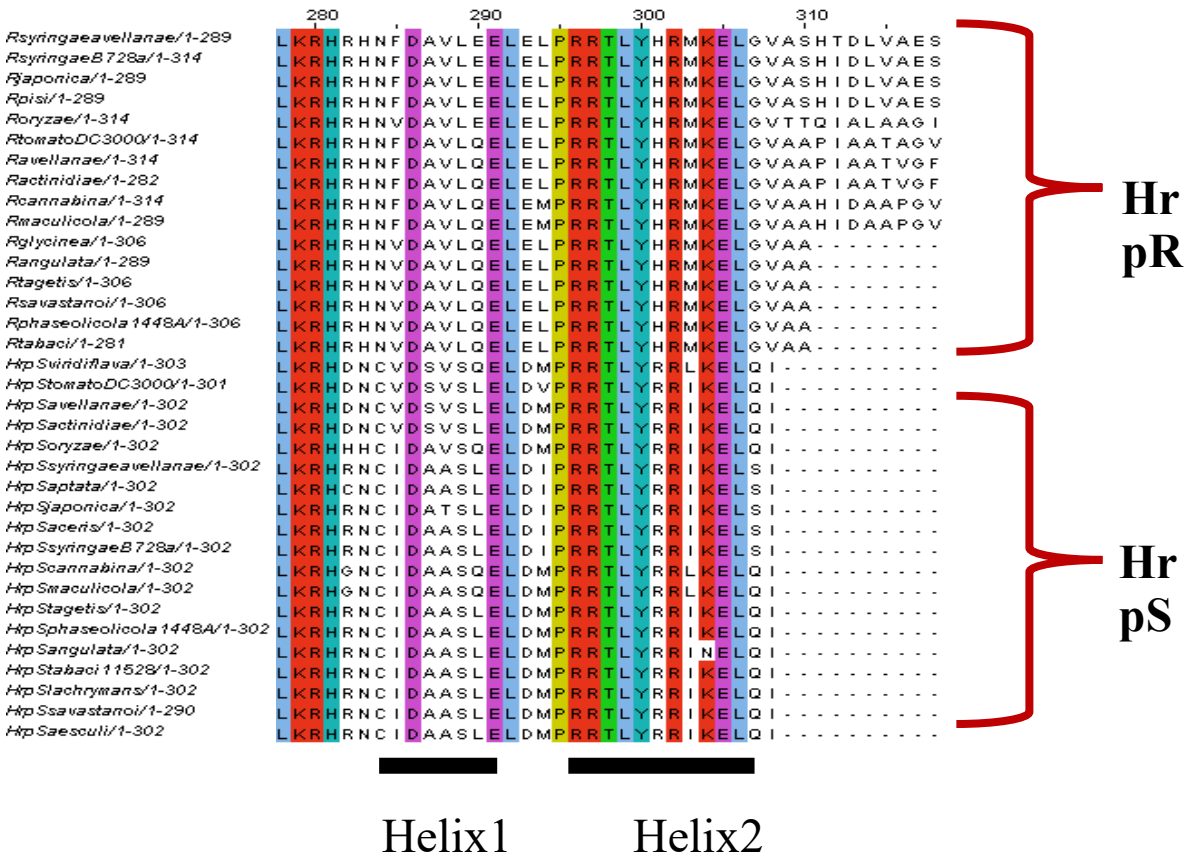
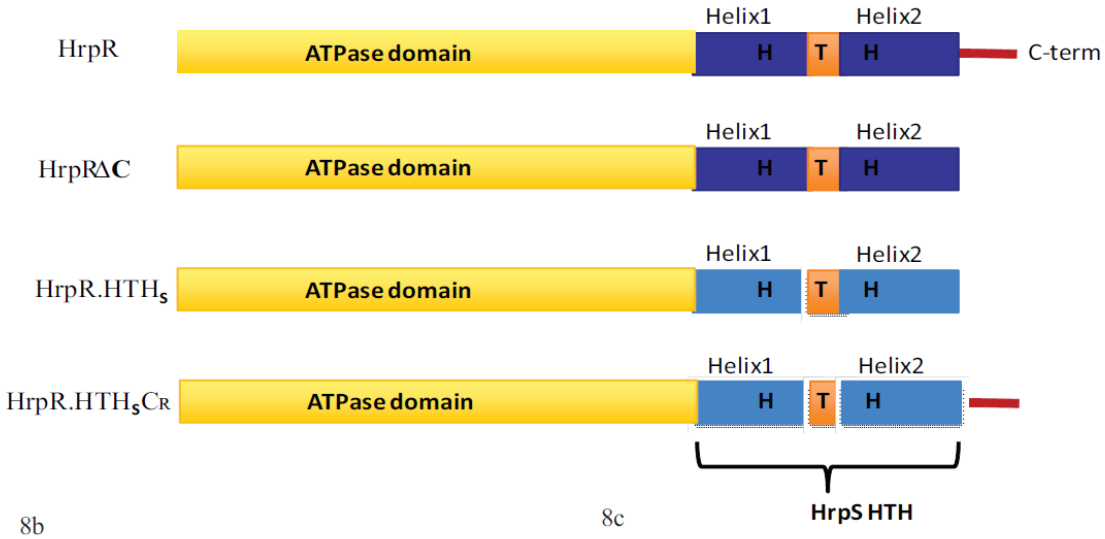
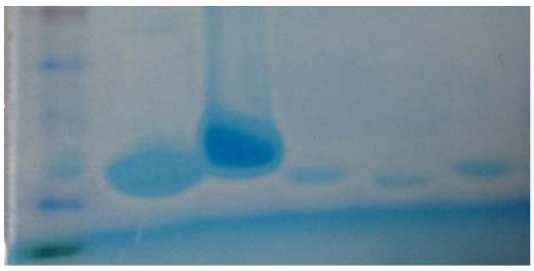


Figure 7: Alignment of Helix-turn-helix regions of HrpR and HrpS proteins from different *P. syringae* pathovars.

8a

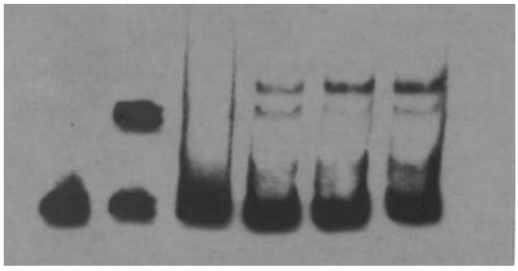


8b



1 2 3 4 5 6

8c



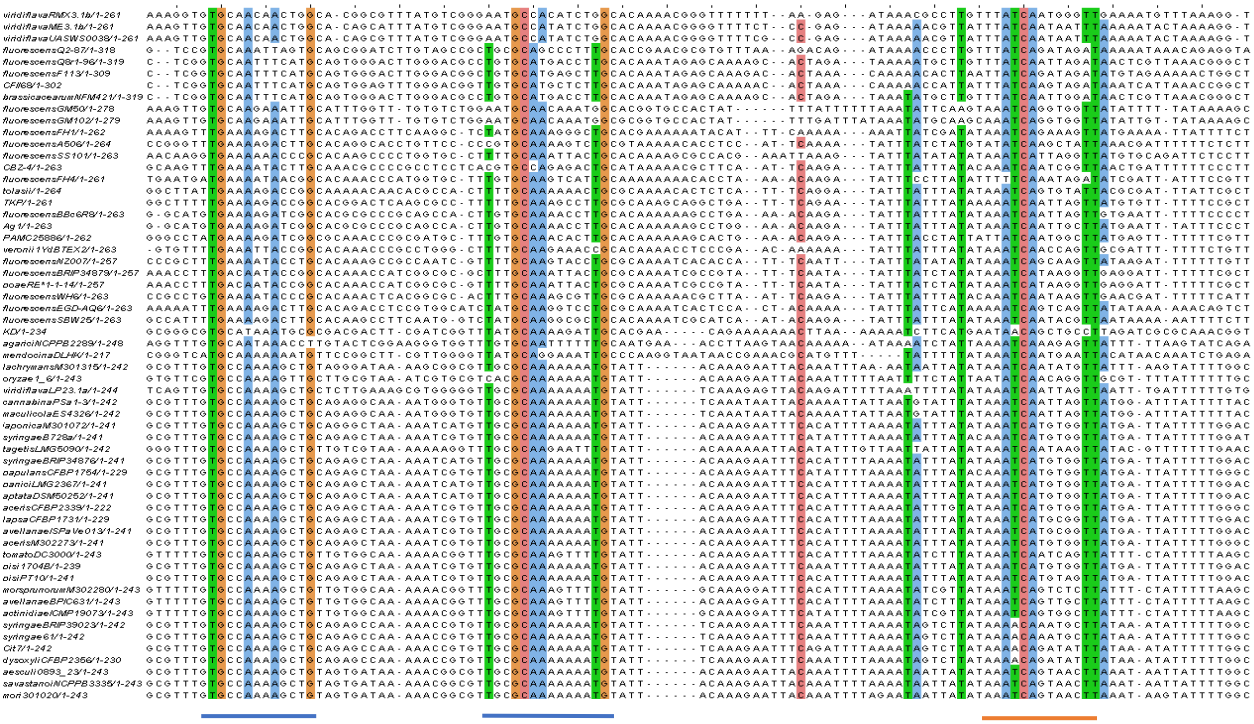
1 2 3 4 5 6

Figure 8: Construction of modified HrpR proteins and mobility shift assays of *hrpL* promoter with modified HrpR proteins. 8a) Wt HrpR and 3 modified HrpR proteins are shown. 8b) Coomassie stained-SDS gel of modified HrpR proteins. Lane 1: Protein ladder, lane 2: His-T7-HrpR, lane 3: His-S-HrpS, lane 4: His-T7-HrpRΔC , lane 5: His-T7-HrpR.HTHs, lane 6: His-T7-HrpR.HTHs.C$_R$. 8c) EMSA with modified HrpR proteins. Lane1: *hrpL* only, lane 2: *hrpL*+IHF, lane 3: *hrpL* +His-T7-HrpR, lane 4: *hrpL*+His-T7-HrpRΔC, lane 5: *hrpL*+ His-T7-HrpR.HTHs, lane 6: *hrpL*+ His-T7-HrpR.HTH.C$_R$

hrpL might have two UAS regions

I aligned *hrpL* promoter regions from 62 *Pseudomonas syringae* species using ClustalΩ (Goujon et al., 2010; McWilliam et al., 2013; Sievers et al., 2011). Some of these bacteria belong to different pathovars of *P.syringae* and several belong to different species among the *Pseudomonas* genus that are evolutionarily distant to *Pst* DC3000. All of these *P.syringae* pathovars or *Pseudomonas* species contain *hrpR* and/or *hrpS* genes. I observed two conserved regions which are ≈14bp long and ≈18bp apart (**Figure 9a**). These two conserved regions lie within 169bp *hrpL* promoter region. This corroborates with our β-Galactosidase results in **Table 4** of this chapter. We later aligned these sequences using MEME software and generated a weblogo for the conserved regions (**Figure 9b**).

9a



9b

Figure 9: Bioinformatic analysis of *hrpL* promoter regions from 62 different *Pseudomonas syringae* species. 9a) Clustal Ω analysis of *hrpL* regions identified 2 conserved regions (Indicated by blue lines) between -169bp and -110bp upstream of *hrpL* translation start site. Red line indicates putative IHF binding site. 9b) WebLogo derived after MEME analysis.

8.5) Discussion

My goal was to study how HrpR and HrpS regulated *hrpL*, identify the binding site of HrpR/S in the *hrpL* promoter region and use this to identify other HrpRS targets. In previous studies and in my work, both HrpR and HrpS were shown to be required to activate *hrpL* in *E. coli* (Hutcheson et al., 2001; Jovanovic et al., 2011). Here we also showed that both HrpR and HrpS are required to activate *hrpL* in their native organism *Pseudomonas syringae* pv *tomato*. Our data from *hrpL* reporter plasmid studies, GUS assays and RT-qPCR in Δ*hrpR*, Δ*hrpS* and Δ*hrpRS*-DC3000 mutants strongly support this conclusion

I also wanted to verify role of CorR in *hrpL* expression. I observed that CorR did not have any role in the *hrpL* activation both in the presence and absence of HrpRS when cultures were grown in HDM. Role of CorR in *hrpL* activation under other environmental studies still needed to be verified.

HrpR and HrpS enhancing binding proteins are active in hetero-hexameric state like other NtrC family enhancer binding proteins. *Jovanovic et al* showed that HrpR, HrpS and HrpRS complex bind *hrpL* promoter region (Jovanovic et al., 2011; Wyman, Rombel, North, Bustamante, & Kustu, 1997). In my Initial experiments, I got the same results. However, when I followed the EMSA studies up with DNaseI footprinting assay using purified HrpR, HrpS and HrpRS I derived different conclusions. The footprint overlapped with the IHF binding site consensus sequence WATCAANNNNTTR (Hales et al., 1994) (**Figure 9a**). IHF binds between the UAS and the promoter and bends the DNA to bring the enhancer binding proteins to the close proximity of RNA polymerase-σ^{54}

holoenzyme (Bush & Dixon, 2012). IHF was shown to be non-dispensable for the activation of *hrpL* by HrpR and HrpS (Jovanovic et al., 2011). I concluded that my protein preparations were contaminated with *E. coli* IHF proteins. To avoid IHF contamination in our subsequent protein preparations, I passed the proteins through centrifugal filters (Millipore) that filter out proteins less than 30KDa. I performed mobility shift assays with these proteins on *hrpL* and found that HrpS and HrpRS complex bind *hrpL* but not HrpR. This contradicts the earlier study and I suspect that their purified protein samples were contaminated with IHF.

Since HrpS binds *hrpL* but not HrpR, I hypothesized that a variation in DNA binding domain of HrpR was inhibiting its interaction with *hrpL* promoter region. All three HrpR proteins with modified HTH domain bound the *hrpL* promoter suggesting that the C-terminal tail prevents HrpR DNA binding. However, the confusing aspect of my results is that the C-terminal tail of HrpR did not prevent HrpR.HTH_SC_R from binding *hrpL.* This might be because the C-terminal tail inhibits *hrpL* binding only in the presence of HrpR HTH. These experiments should be further validated by reproducing them again.

From our study, it has been shown that *hrpL* is the direct target of HrpRS. Here, I narrowed down the HrpRS binding region in the *hrpL* promoter and obtained a WebLogo of the most conserved nucleotides in the region. This potential HrpRS binding sequence could be confirmed by DNaseI footprinting studies or mutational analysis.

A microarray study on Δ*hrpRS* DC3000 and Δ*hrpL* DC3000 had found many genes that are activated by *hrpRS* independent of *hrpL* (Lan et al., 2006). They

identified 60 *hrpRS* induced genes, and I hypothesize that at least some of these genes are direct targets of HrpRS. Induction of these genes could be beneficial for pathogenesis independent of T3SS. In the future other direct targets of HrpRS could be identified by generating a position weight matrix of the potential HrpRS binding site using RSAT software (Turatsinze, Thomas-Chollier, Defrance, & van Helden, 2008) and using it to search the intergenic regions of the *Pst* DC3000 genome. These potential targets could be confirmed by performing EMSA and reporter assays similar to the experiments I carried out with *hrpL.* Identifying direct targets of HrpRS will expand the current knowledge on pathogenesis of *Pst* DC3000 and other *P.syringae* pathovars.

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