Salmonella enterica Coordination of Virulence Gene Expression and Carbon Metabolism during Infection

Undergraduate Research Thesis

Presented in partial fulfillment of the requirements for graduation
with honors research distinction in Microbiology in the
College of Arts and Sciences at The Ohio State University

by

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The Ohio State University
May 2016

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Acknowledgments

I would like to thank Dr. Brian Ahmer for his guidance and support in the twenty months I have been in his laboratory. His knowledge and insight have been invaluable as I have progressed through this project. I would also like to thank Dr. Anice Sabag-Daigle for her work on this project before I joined the laboratory that gave me a foundation to continue building upon. I would like to thank her for her excellent mentorship as well. She has taught me so much in terms of microbiology knowledge and laboratory techniques that have not only prepared me for classes but also for my future endeavors. I appreciate all the advice and encouragement she has given me as I have grown more independent. In addition, I would like to thank my fellow past and present laboratory members Katherine Miller, Henry Blunk, Bailyn Hogue, Andrew Dempsey, Juan Gonzalez, and Fabien Habyarimana for their help and support. It has been a privilege for me to work in Dr. Ahmer's lab as an undergraduate alongside so many incredible laboratory members.

Anice Sabag-Daigle performed most of the experiments for the initial stages of this project until I started working on this project in October 2014. Anice designed the primers used in this study and the control promoters that were sent for construction by GeneScript. She also constructed the wild type, *sirA* mutant, and *csrA52* mutant strains of *Salmonella* used for integrating the mCherry translational fusion plasmids onto the *Salmonella* chromosome.

I performed the initial work constructing the base plasmid pASD1106 using the pCE70 vector that Anice linearized, and she completed the construction of pASD1106. I PCR-amplified and TOPO-cloned the promoters of interest, found in Table 3, and cloned the promoters of interest and the control promoters into pASD1106 by creating translational fusions with

mCherry. Anice and I worked together to successfully integrate the mCherry translational fusion plasmids, seen in Table 4, onto the *Salmonella* chromosome of the three different strains. After each step of constructing pASD1106, cloning the promoters into pASD1106, and integrating the plasmids onto the *Salmonella* chromosome, I verified the constructs and strains by PCR. I assayed the *Salmonella* strains *in vitro*, and Anice developed the method to normalize the data and created the graphs using Prism. I further analyzed the data on Prism by using a one-way ANOVA statistical test.

Abstract

Salmonella enterica, which consists of zoonotic, rod-shaped, Gram-negative bacteria, is the cause of about 78 million cases of foodborne illness each year and is the leading cause of death by bacterial foodborne illnesses in the world. The category of foodborne illnesses itself is responsible for about $152 billion of annual economic losses. When *Salmonella enterica* serovar Typhimurium infects its host, the CsrA and BarA-SirA system regulates the expression of metabolic and virulence genes. Our goal is to determine the activity of the CsrA and BarA-SirA system during infection and how this activity coordinates carbon metabolism and the expression of virulence genes. We hypothesize that CsrA activity is low during intestinal infection and high during systemic infection. To test this hypothesis, genes of interest were cloned into translational fusion vectors containing a mCherry fluorescent reporter. These vectors were inserted at flippase recognition target (FRT) sites in the *Salmonella* chromosome of wild type, *sirA* mutant, and *csrA* mutant strains. During growth in the presence or absence of glucose and under conditions that induce *Salmonella* Pathogenicity Islands 1 and 2, these strains were assayed *in vitro* to replicate the intestinal and systemic environments in which these pathogenicity islands are expressed so that in the future, the results can be further analyzed *in vivo*. Preliminary findings show that CsrA activity is present *in vitro* based on the regulation of the genes of interest by CsrA. The outcomes of this study will not only enhance our understanding of the interaction between host and pathogen, but they will also be a stepping stone to the development of therapeutics against an illness that affects so many around the world.

Chapter 1 – Introduction

1.1 – Infection and Virulence

Salmonella enterica serovar Typhimurium is a rod-shaped, Gram-negative bacterium that possesses flagella and is facultatively anaerobic.[10] *S.* Typhimurium infects the gastrointestinal (GI) tract which can be detrimental to infants and the immunocompromised because it can lead to dehydration or systemic infection.[10] As seen in Figure 1, among foodborne illnesses, non-typhoidal *Salmonella* is responsible for the highest number of Disability Adjusted Life Years (DALYs) in the world.[15] Every year, approximately 78 million people worldwide contract *Salmonella*, and in the U.S. alone about 19,000 people are hospitalized and 380 people die from salmonellosis.[15, 22] The category of foodborne illnesses itself is the cause for around $152 billion of annual economic losses.[9] Symptoms of salmonellosis include nausea, vomiting, diarrhea, fever, and abdominal cramps that can last two to seven days.[10]

S. Typhimurium typically enters the body through the consumption of contaminated food, and because *Salmonella* is zoonotic, a major source of this contaminated food is derived from animals like chickens, turkeys, pigs, and cows.[3] Shown in Figure 2, after ingestion of the bacteria, *Salmonella* enter the intestine where they invade the epithelial cells and stimulate macropinocytosis, or the uptake of molecules via vesicles by a cell.[11] The bacteria multiply intracellularly in the epithelial cells of the intestinal mucosa.[10] The invasion of the epithelial cells causes the host cells to release pro-inflammatory cytokines which induce an acute inflammatory response, resulting in the symptoms of salmonellosis.[10]

To cross the intestinal epithelium, *Salmonella* enter through M cells in the follicle-associated epithelium separating the gut-associated lymphoid tissue (GALT) from the lumen and

surrounding the Peyer's patches, which are a collection of lymphoid follicles.[4] The type 3 secretion system 1 (T3SS-1), encoded in the *Salmonella* pathogenicity island 1 (SPI-1), is used to induce pinocytosis of the bacteria by the epithelial cells.[4] The T3SS-1 is similar to a syringe in the sense that it injects effectors into the epithelial cells which allow for the uptake of the bacteria, thus making SPI-1 important in the invasion by *Salmonella* into the host cells.[13, 20] Because SPI-1 is involved in the interaction between the bacteria and mucosal surfaces, its virulence gene expression is activated by low oxygen and high osmolarity, and it plays a role in inflammation and onset of salmonellosis symptoms like diarrhea.[13]

After crossing the intestinal epithelium, immune cells like macrophages phagocytose the *Salmonella*.[4] Once in the macrophage phagosome, low phosphate, low Mg^{2+}, and acidic conditions stimulate the expression of the TTSS-2 genes, encoded by SPI-2.[8, 12] The proteins released by the TTSS-2 prevent the phagosome from maturing by disrupting vesicular trafficking, which in turn prevents the destruction of *S.* Typhimurium from reactive oxygen species (ROS).[13] This allows for intracellular survival of the bacteria and their proliferation.[12] By surviving in the macrophages, *Salmonella* can be transported to other areas of the body, leading to systemic infection.[13]

1.2 – The CsrA and BarA-SirA System and Regulation

The *Salmonella* pathogenicity islands are regulated by the carbon storage regulator (CsrA) and BarA-SirA system.[1] Through the regulation of carbon metabolism, the system can also regulate virulence genes, motility, and biofilm formation.[1]

CsrA is a homodimeric protein that regulates genes post-transcriptionally by binding to the transcribed mRNA of a gene.[1] Depending on the gene it is controlling, CsrA can act as an

activator or repressor.[1, 17] As an activator, CsrA stabilizes the mRNA, thus protecting the transcript and increasing its translation.[17] CsrA represses translation by binding to a site that overlaps with the Shine-Dalgarno (S-D) sequence or by activating an endonuclease that degrades the mRNA.[1, 17]

CsrA activates glycolysis, metabolism of acetate, and formation of flagella but represses gluconeogenesis, biofilm formation, and the expression of SPI-1 and SPI-2 genes.[1, 18] The Csr system incorporates the small noncoding regulatory RNAs (sRNAs), CsrB and CsrC, which have CsrA binding sites that allow them to sequester the CsrA protein.[1] This prevents CsrA from binding to its target mRNA and decreases the amount of free CsrA present since an overabundance of CsrA protein present can inhibit SPI-1 genes.[1, 16] Via the activation of glycolysis, which leads to the production of short chain fatty acids like acetate, the BarA-SirA system is activated.[16] This in turn activates genes involved in *Salmonella* virulence and also *csrB*.[16] Consequently, the combination of CsrA and the BarA-SirA system allows CsrA to regulate itself through the expression of *csrB* and *csrC* when activated by SirA.[12]

During systemic infection as opposed to infection concentrated in the intestine, *Salmonella* replicates within vacuoles of macrophages.[3] Glucose is transported into *Salmonella* by the phosphotransferase system (PTS).[3] It has been shown that the irreversible step of glycolysis catalyzed by phosphofructokinase is important for intracellular replication in macrophages, making glycolysis necessary for this intracellular replication and glucose a critical carbon source for growth in macrophages and successful systemic infection.[3]

BarA-SirA makes up a two-component regulatory system with BarA as the sensor kinase and SirA as its response regulator, and both are required for SPI-1 gene expression to be

activated.[23] BarA is membrane-bound and after sensing certain environmental signals such as the greater presence of metabolic products like acetate rather than propionate and butyrate, it phosphorylates SirA in the cytoplasm.[8, 16] Seen in Figure 3, SirA activates *csrB*, which is involved in the repression of *csrA*, and indirectly activates *hilA* which is important for activation of promoters of the SPI-1 genes and the T3SS-1.[23] The occurrence of glycolysis and the presence of its products in the distal ileum of the small intestine allow for the activity of *Salmonella*.[16]

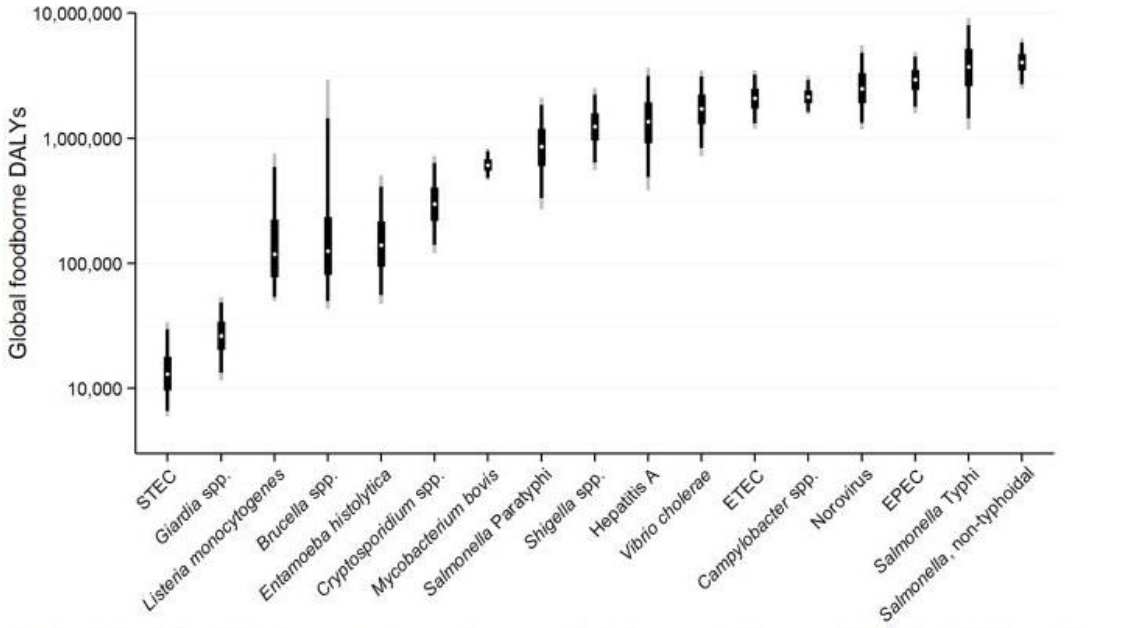
1.3 – *ssaJ*: The Selected Target Promoter

The promoter of the virulence gene *ssaJ* was chosen as a target to study regulation by CsrA. *ssaJ*, which is at the 5' end of an operon, encodes a structural component of the T3SS-2, making it important for intracellular survival.[8, 14] As a SPI-2 promoter, it is positively regulated by SsrB (Figure 3) and is activated in acidic conditions representative of the phagosome.[8] *ssrB* is directly regulated by HilD which is positively controlled by SirA and negatively controlled by CsrA.[18] The exception is when *Salmonella* is growing in conditions of low Mg^{2+} and phosphate.[18]

1.4 – Objectives

The goal of this study was to determine the activity of the CsrA and BarA-SirA system during infection and how this activity coordinates carbon metabolism with the expression of virulence genes. It is hypothesized that CsrA activity is low during intestinal infection and high during systemic infection.

1.5 – Figures



Disability Adjusted Life Years for each pathogen acquired from contaminated food ranked from lowest to highest with 95% Uncertainty Intervals, 2010. Note figure is on a logarithmic scale. The figure shows the median (white dot); Inter-Quartile Range = 50%UI = 25%/75% percentiles (thick black line); 90% UI = 5%/95% percentiles (thin black line); 95% UI = 2.5%/97.5% percentiles (thin grey line). Note, figure does not include four foodborne intoxications due to *Clostridium botulinum*, *C. perfringens*, *S. aureus*, and *Bacillus cereus* due to a lack of data for global estimation. In addition, data for non-typhoidal *Salmonella enterica* infections and invasive non-typhoidal *S. enterica* have been combined.

Figure 1: 2010 data from the World Health Organization showing global foodborne Disability Adjusted Life Years (DALYs) from lowest to highest for a variety of pathogens. Non-typhoidal *Salmonella*, which encompasses *S. enterica*, is shown to have the highest number of DALYs in the world.[15]

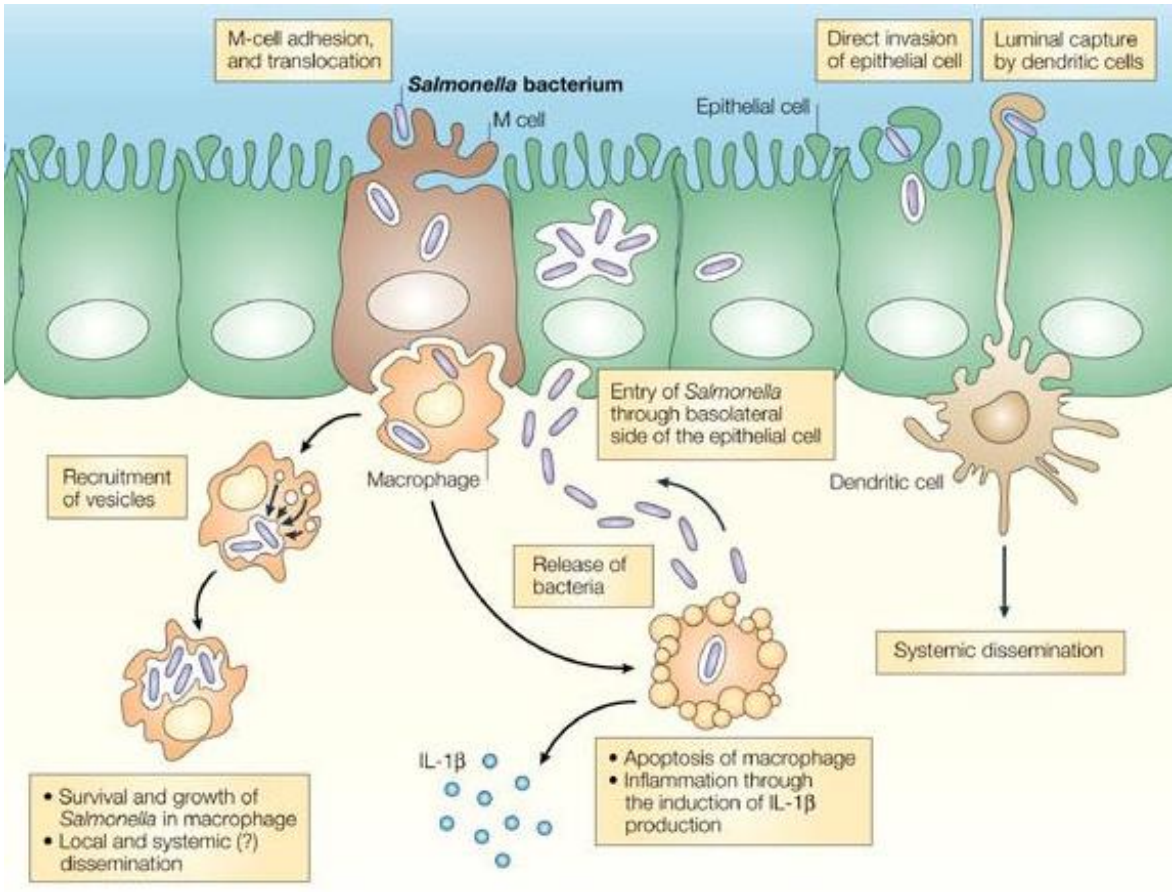


Figure 2: The bacteria can be translocated by the epithelial cells of the host and taken up by macrophages afterwards. Once in the macrophages, the bacteria can survive within the vesicles which may lead to local and systemic infection, or they can cause apoptosis of the macrophages, thus releasing the bacteria. *Salmonella* may also be taken up by dendritic cells which can spread the bacteria to other parts of the body.[21]

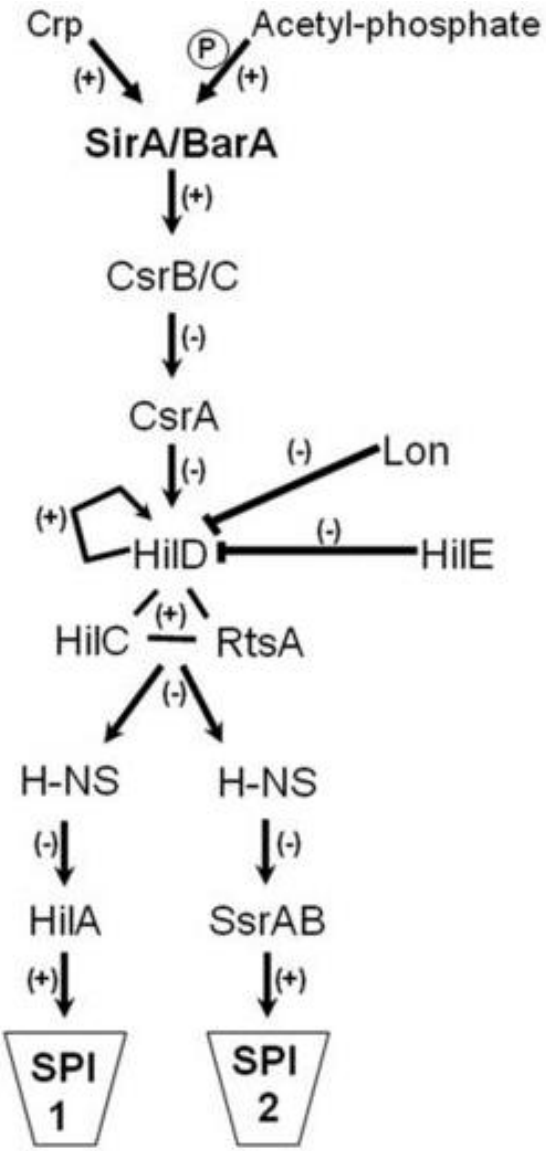


Figure 3: The CsrA and BarA-SirA regulation system. The BarA-SirA system activates *csrB*/*C* which repress *csrA*. CsrA represses *hilD* which down-regulates the global repressor H-NS. H-NS represses the genes that control SPI-1 and SPI-2.[18]

Chapter 2 – Materials and Methods

2.1 – Bacterial Growth

Unless otherwise stated, all bacteria cultures were grown overnight in Luria-Bertani broth (LB) + 50 µg/mL kanamycin (kan) using the 37°C roller drum. Colonies were grown on LB agar plates + 50 µg/mL kan and incubated overnight at 37°C. The wild type *Salmonella* strain 14028 was grown on LB agar without kan and incubated overnight at 37°C for further use.

2.2 – Cloning of *ssaJ*

The promoter of interest, *ssaJ*, was PCR amplified using Phusion polymerase (New England Biolabs), and TOPO TA cloning (Invitrogen) was used to clone the fragments into pCR 2.1 TOPO vectors that were chemically transformed into competent One Shot TOP10 *Escherichia coli* DH5-α cells (Invitrogen). The transformants were plated on LB agar plates with kan and 10 mg/mL X-gal added. White colonies with the *lacZ* gene interrupted were selected for plasmid purification using the Qiagen Miniprep kit. The presence of the *ssaJ* fragment in the plasmid was confirmed by restriction enzyme digestion using the enzymes *Eco*RI and *Nco*I, and the DNA sequence was verified at The Ohio State University's Plant-Microbe Genomics Facility (PMGF).

2.3 – Construction of the Base Plasmid pASD1106

As seen in Figure 4A, the gene for mCherry in the pUC57 plasmid was PCR amplified using Clontech CloneAmp HiFi PCR Premix. Primers with a multiple cloning site (MCS) at the end were added. The amplified mCherry insert had fifteen extra base pairs at the ends that were homologous to the ends of the linearized pCE70 vector. Using the Clontech In-Fusion HD

Cloning kit, the mCherry insert was fused with the pCE70 vector at the homologous ends to create the final base plasmid pASD1106 (Figure 4B) that was transformed into One Shot PIR1 competent *E. coli* cells (Invitrogen).

2.4 – Control Promoters

Four control promoters were synthesized and cloned into pUC57 by GeneScript. These control promoters were used to observe regulation by CsrA. λP_R was the constitutive background control for mCherry expression while λP_R*pgaAΔrut*, λP_R*pgaAΔrutΔcsrA*$_{BS-1}$, and λP_R*pgaAΔrutΔcsrA*$_{BS-2}$ were used to study the CsrA regulation of the *pgaA* leader. Because the Rho utilization site (rut) was deleted, Rho was unable to bind and terminate transcription which negated Rho as a confounding variable and allowed for the ability to observe regulation by CsrA specifically. λP_R*pgaAΔrutΔcsrA*$_{BS-1}$ contained point mutations from GG to CC in the CsrA binding site, and λP_R*pgaAΔrutΔcsrA*$_{BS-2}$ had substitutions in the S-D and translation initiation sequences of *csrA*.

2.5 – Translational Fusions

The minipreps of *ssaJ*, the control promoters, and pASD1106 were digested with *Eco*RI and *Nco*I. The linearized fragments were gel purified using a Qiagen Gel Extraction kit and ligated together to form the final plasmid containing the promoter fused with mCherry at the multiple cloning site (MCS) in pASD1106. The ligations were transformed into *E. coli* BW20767 competent cells that require *pir* for propagation of the plasmid. The plasmids were PCR-verified using the forward primer of the promoter and the reverse primer of mCherry.

2.6 – Electroporation into *Salmonella*

The plasmids containing their respective promoter were electroporated into three different *Salmonella* strains with pCP20—wild type (ASD1214), *sirA* mutant (ASD150), and *csrA52* mutant (ASD151)—in order to integrate the promoter and mCherry reporter onto the *Salmonella* chromosome at the flippase recognition target (FRT) site. Figure 5 displays the mechanism of the electoporations. The three strains were grown in LB broth + 200 µg/mL ampicillin (amp) for a maximum of eighteen hours at 30°C while shaking. The electroporation had a one hour outgrowth at 37°C while shaking before being plated on LB + kan and incubated overnight at 42°C. Each strain was verified by PCR amplification using the forward primer downstream of the *pagC* region in *Salmonella* and the reverse primer of mCherry.

2.7 – *in vitro* Assays

ssaJ and the control promoters in their respective *Salmonella* strains and *Salmonella* 14028, as the control, were assayed *in vitro* in four conditions: LB broth + 50 mM MOPS buffer (intestinal conditions), LB broth + 50 mM MOPS buffer + 5 mM D-glucose (systemic conditions), LB + 1% NaCl (SPI-1 inducing conditions), and mLPM (low phosphate, low magnesium-containing minimal medium and SPI-2 inducing conditions). Liquid overnight cultures were started for all sixteen strains in each of the four conditions and grown for a maximum of eighteen hours in the 37°C roller drum. The cultures were started from isolated colonies when grown in intestinal, systemic, and SPI-1 conditions but from the primary streak when grown in SPI-2 conditions.

The following day, the sixty-four liquid cultures were aliquoted into opaque, white 96-well plates and into clear 96-well plates. Each culture and each type of media were aliquoted

three times to allow for three technical triplicates. The white plates were read from the top using the Tecan SpectraFluor Plus microplate reader (PMGF). mCherry was excited at a wavelength of 590 nm, and the emission of mCherry was measured at a wavelength of 635 nm. Each culture was then aliquoted into the clear 96-well plates, and the SpectraMax M5 spectrophotometer, set at an absorbance of 600 nm, and SoftMax Pro software were used to determine the optical density (OD).

The *in vitro* assays were repeated two more times for a total of three biological replicates and nine technical replicates. Data was analyzed using Prism software. The fluorescence measurements of the 14028 strain grown in each type of medium were subtracted from the respective measurements of *ssaJ* and the control promoters to account for fluorescence by the *Salmonella* and medium itself. After calculating the mean fluorescence of each strain in the respective condition, the data were normalized by dividing by the mean OD of each strain in each condition.

2.8 – Tables and Figures

Table 1: Strains and plasmids used in this study.

***Salmonella* Strain**	**Genotype**	**Description**
14028	Wild type *Salmonella enterica* serovar Typhimurium	American Type Culture Collection
ASD1214	14028 ΔIG(*pagC*-STM14_1502)::FRT + pCP20	14028 with a FRT site inserted downstream of the *pagC* stop codon; electroporated with pCP20
ASD150	14028 *sirA4*::hygromycin ΔIG(*pagC*-STM14_1502)::FRT + pCP20	14028 *sirA4*::hygromycin with a *pagC*::cam insertion electroporated with pCP20
ASD151	14028 Δ*csrA52* ΔIG(*pagC*-STM14_1502)::FRT + pCP20	14028 with cam marker flipped out of *csrA52*::cam so *csrA* is unmarked; *pagC*::cam insertion; electroporated with pCP20
SLZ200	14028 ΔIG(*pagC*-STM14_1502)::FRT::pSLZ100	mCherry with the lambda right promoter was electroporated into ASD1214; Kan^R; PCR-verified insertion of the suicide vector.
SLZ201	14028 *sirA4*::hygromycin ΔIG(*pagC*-STM14_1502)::FRT::pSLZ100	mCherry with the lambda right promoter was electroporated into ASD150; Kan^R; PCR-verified insertion of the suicide vector.
SLZ202	14028 Δ*csrA52* ΔIG(*pagC*-STM14_1502)::FRT::pSLZ100	mCherry with the lambda right promoter was electroporated into ASD151; Kan^R; PCR-verified insertion of the suicide vector.
SLZ203	14028 ΔIG(*pagC*-STM14_1502)::FRT::pSLZ101	mCherry with the lambda right promoter and *pgaA* leader was electroporated into ASD1214; Kan^R; PCR-verified insertion of the suicide vector.
SLZ204	14028 *sirA4*::hygromycin ΔIG(*pagC*-STM14_1502)::FRT::pSLZ101	mCherry with the lambda right promoter and *pgaA* leader was electroporated into ASD150; Kan^R; PCR-verified insertion of the suicide vector.
SLZ205	14028 Δ*csrA52* ΔIG(*pagC*-STM14_1502)::FRT::pSLZ101	mCherry with the lambda right promoter and *pgaA* leader was electroporated into ASD151; Kan^R; PCR-verified insertion of the suicide vector.
SLZ206	14028 ΔIG(*pagC*-STM14_1502)::FRT::pSLZ102	ASD1214 was electroporated with mCherry containing the *pgaA* promoter and mutations in the CsrA binding site; Kan^R; PCR-verified insertion at *pagC* insertion site.
SLZ207	14028 *sirA4*::hygromycin ΔIG(*pagC*-STM14_1502)::FRT::pSLZ102	ASD150 was electroporated with mCherry containing the *pgaA* promoter and mutations in the CsrA binding site; Kan^R; PCR-verified insertion at *pagC* insertion site.

Salmonella Strain	Genotype	Description
SLZ208	14028 Δ*csrA52* ΔIG(*pagC*-STM14_1502)::FRT::pSLZ102	ASD151 was electroporated with mCherry containing the *pgaA* promoter and mutations in the CsrA binding site; Kan^R; PCR-verified insertion at *pagC* insertion site.
SLZ209	14028 ΔIG(*pagC*-STM14_1502)::FRT::pSLZ103	ASD1214 was electroporated with mCherry containing the *pgaA* promoter and mutations in the S-D and translation initiation sequences; Kan^R; PCR-verified insertion at *pagC* insertion site.
SLZ210	14028 *sirA4*::hygromycin ΔIG(*pagC*-STM14_1502)::FRT::pSLZ103	ASD150 was electroporated with mCherry containing the *pgaA* promoter and mutations in the S-D and translation initiation sequences; Kan^R; PCR-verified insertion at *pagC* insertion site.
SLZ211	14028 Δ*csrA52* ΔIG(*pagC*-STM14_1502)::FRT::pSLZ103	ASD151 was electroporated with mCherry containing the *pgaA* promoter and mutations in the S-D and translation initiation sequences; Kan^R; PCR-verified insertion at *pagC* insertion site.
SLZ215	14028 ΔIG(*pagC*-STM14_1502)::FRT::pSLZ105	ASD1214 electroporated with mCherry containing the *ssaJ* promoter; Kan^R; PCR-verified insertion at the *pagC* insertion site
SLZ216	14028 *sirA4*::hygromycin ΔIG(*pagC*-STM14_1502)::FRT::pSLZ105	ASD150 electroporated with mCherry containing the *ssaJ* promoter; Kan^R; PCR-verified insertion at the *pagC* insertion site.
SLZ217	14028 Δ*csrA52* ΔIG(*pagC*-STM14_1502)::FRT::pSLZ105	ASD151 electroporated with mCherry containing the *ssaJ* promoter; Kan^R; PCR-verified insertion at the *pagC* insertion site.
Plasmid	**Genotype**	**Description**
pASD1106	FRT-mCherry oriR6K (Kan^R); FRT orientation A	mCherry replaced *tnpR*, *lacZ* and *lacY*; FRT sequence intact; MCS for translational fusion using *Nco*I and *Eco*RI; terminator between the FRT sequence and the MCS to prevent read-through transcription; Kan^R; *pir*-dependent.
pASD7000	pUC57 λP_R	λ right promoter (P_R) with the leader and ATG of the *cro* gene from λ phage.
pASD7001	pUC57 λP_R-*pgaAΔrut*	λ right promoter without leader fused to the *pgaA* leader with three codons of *pgaA*; rut sites are deleted to remove Rho-dependent regulation.
pASD7002	pUC57 λP_R-*pgaAΔrutΔcsrA*$_{BS-1}$	λ right promoter without the leader fused to the *pgaA* leader with three codons of *pgaA*; rut sites are deleted; the CsrA binding sites are mutated with point mutations from GG to CC.

Plasmid	Genotype	Description
pASD7003	pUC57 λP_R-*pgaAΔrutΔcsrA*$_{BS-2}$	λ right promoter without the leader fused to the *pgaA* leader with three codons of *pgaA*; rut sites are deleted; the *csrA* S-D and translation initiation sequences contain substitution mutations.
pSLZ100	λP_R-mCherry	mCherry in pASD1106 with lambda P_R promoter cloned into *Eco*RI and *Nco*I restriction sites; Kan^R.
pSLZ101	λP_R-*pgaAΔrut*-mCherry	mCherry in pASD1106 with *pgaA* promoter cloned into *Eco*RI and *Nco*I restriction sites; Kan^R.
pSLZ102	λP_R-*pgaAΔrutΔcsrA*$_{BS-1}$-mCherry	mCherry in pASD1106 with the *pgaA* promoter; rut sites were deleted; CsrA binding sites mutated (GG to CC); cloned into *Eco*RI and *Nco*I restriction sites; Kan^R.
pSLZ103	λP_R-*pgaAΔrutΔcsrA*$_{BS-2}$-mCherry	mCherry in pASD1106 with the *pgaA* promoter; rut sites were deleted; *csrA* S-D and translation initiation sequences mutated; cloned into *Eco*RI and *Nco*I restriction sites; Kan^R.
pSLZ105	pASD1106 *ssaJ*	mCherry in pASD1106 with the *ssaJ* promoter cloned into *Eco*RI and *Nco*I restriction sites; Kan^R.
pSLZ118	pCR 2.1 TOPO P_{ssaJ}	PCR-amplified promoter region of *ssaJ*; *Eco*RI and *Nco*I sites for translational fusion with fluorescent FRT plasmids; Kan^R.
pCE70	FRT-*tnpR-lacZY* oriR6K (Kan^R); contains wild-type *tnpR* Shine-Dalgarno; FRT orientation A	Inverse PCR-amplified product was fused with the mCherry insert to generate pASD1106.[19]
pCP20	*cI857* λP_R *flp* pSC101 oriTS (Amp^R Cam^R)	(5)

Table 2: Oligonucleotide primers used for PCR amplification and verification purposes.

Primer	Sequence (5' – 3')	Description
BA3137	TTGAATTCGCCATCGCGGATGTCGCCTGTCTTATCTACC	Forward primer of the *ssaJ* insertion fragment for translational fusion to a fluorescent reporter using FRT insertion on chromosome; *Eco*RI restriction site.
BA3138	CCATGGCGAGCATATCCACTAATTGTGCAATATCC	Reverse primer of the *ssaJ* insertion fragment for translational fusion to a fluorescent reporter using FRT insertion on chromosome; *Nco*I restriction site.
BA3199	GCATGCGATTTAAGCAATCAATGTCGGATGC	Forward primer to inverse PCR-amplify pCE70 for making the *csrA* translation vector.
BA3200	TCTAGAAAGTATAGGAACTTCCCGATCCCAAGC	Reverse primer to inverse PCR-amplify pCE70 for making the *csrA* translation vector.
BA3201	CCTATACTTTCTAGAGAATAGGAACTTCAAAAACCCGCTTCG GCGGGTTTTTTTCCGAATTCGATATCGGATCCCCATGG	Forward primer to PCR amplify mCherry with the FRT terminator and MCS for HD In-Fusion cloning with inverse PCR-amplified pCE70.
BA405	GCAATGTAACATCAGAGATTTTGAG	Forward primer of the mCherry reporter.
BA3202	GCTTAAATCGCATGCTTATTTATACAGTTCGTCCATA	Reverse primer to PCR amplify mCherry for HD In-Fusion cloning with the inverse PCR-amplified pCE70.
BA1554	CGGCACGGTAAAGGCGACATTTAAA	Primer for the confirmation of the knock in downstream of *pagC* in *Salmonella*

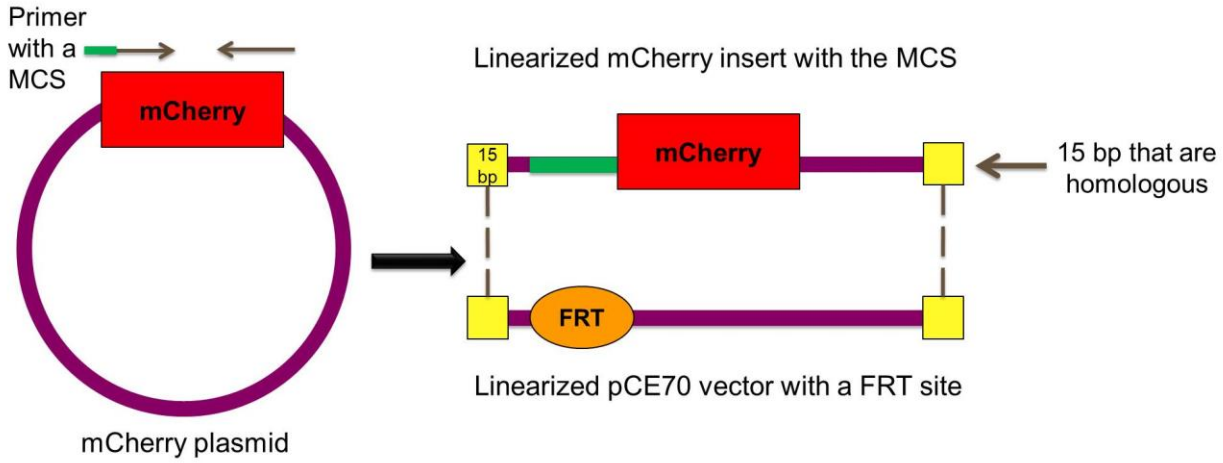


Figure 4A: Construction of the pASD1106 base plasmid using Clontech In-Fusion HD Cloning.

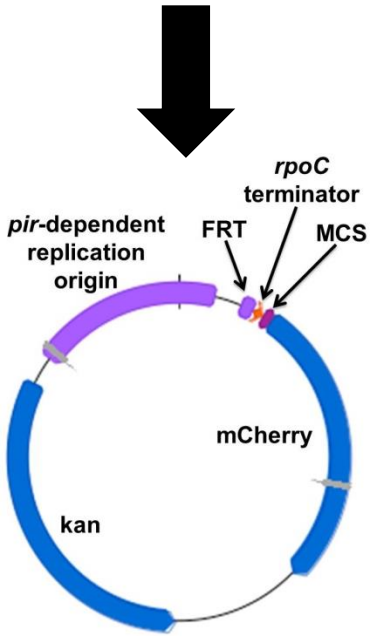


Figure 4B: Map of the final pASD1106 base plasmid which was used to create translational fusions with the mCherry reporter after the promoters were cloned into the multiple cloning site.

Table 3: Promoters for the genes of interest and their progress of being cloned into pASD1106. The promoter that is studied for the purpose of this thesis is *ssaJ*, outlined by a box.

Target Promoters for Assaying	PCR Amplified	TOPO Cloned and Transformed	Sequenced	Cloned into pASD1106
csrA	✔	✔	✔	✔
barA	✔	✔	✔	✔
sirA	✔	✔	✔	✔
csrB	✔	✔	✔	✔
hilA	✔	✔	✔	✔
invF	✔	✔	✔	
sopB	✔	✔	✔	✔
fimA	✔	✔	✔	✔
ssrB	✔	✔	✔	✔
ssaJ	✔	✔	✔	✔

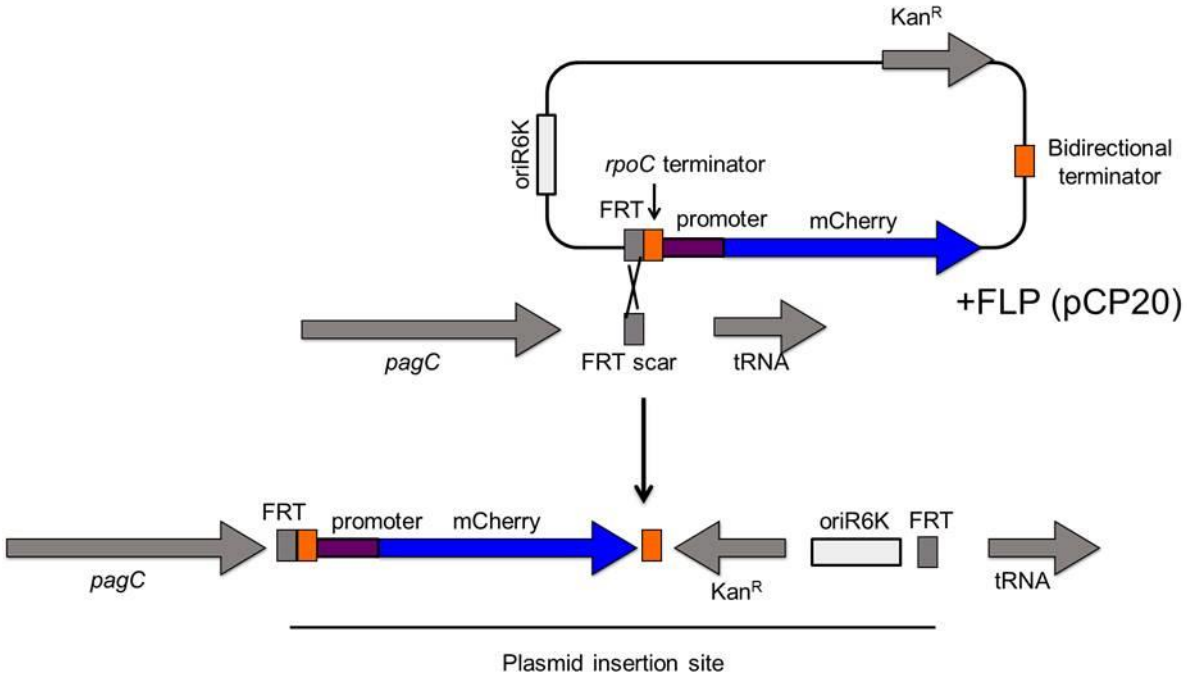


Figure 5: Recombination of the plasmids onto the *Salmonella* chromosome at the flippase recognition target (FRT) site. The vectors containing the promoter and mCherry reporter were electroporated into *Salmonella* wild type, *sirA* mutant, and *csrA52* mutant strains where they integrated into the chromosome at the FRT site. The *rpoC* terminator prevents read-through transcription of *pagC* so that its expression does not potentially affect that of the mCherry fusions.

Table 4: Control promoters and promoters for the genes of interest and their progress of being electroporated into the three *Salmonella* strains. The strains analyzed for the purpose of this thesis are the electroporations of the control promoters and *ssaJ*, outlined by a box.

	Promoter in pASD1106	Wild Type	*sirA* Mutant	*csrA52* Mutant
Control Promoters	λP_R	✔	✔	✔
	$\lambda P_R pgaA\Delta rut$	✔	✔	✔
	$\lambda P_R pgaA\Delta rut\Delta csrA_{BS-1}$	✔	✔	✔
	$\lambda P_R pgaA\Delta rut\Delta csrA_{BS-2}$	✔	✔	✔
Virulence Gene Promoters	*csrA*	✔		✔
	barA	✔	✔	✔
	sirA	✔	✔	✔
	csrB			
	hilA			
	invF			
	sopB			
	fimA	✔	✔	✔
	ssrB	✔	✔	✔
	ssaJ	✔	✔	✔

Chapter 3 – Results and Discussion

3.1 – CsrA Regulation in the Control Promoters

The constitutive λP_R::mCherry fusion acts as a negative control for the expression of the mCherry reporter to confirm non-differential expression despite being in a *sirA* or *csrA52* mutant background. This can be seen in Figures 6 through 9 based on the fact that the λP_R::mCherry control has consistent expression of mCherry as this gene is not normally found in *Salmonella* and thus is not regulated by the CsrA and BarA-SirA system. Consequently, the normalized fluorescence levels of mCherry show no statistical difference between the wild type, *sirA* mutant, and *csrA52* mutant. However, when the strains were grown in intestinal and SPI-1 inducing conditions (Figures 6 and 8), there is a statistically significant decrease in mCherry expression from the wild type to the *sirA* mutant which shows regulation by CsrA and SirA. This may suggest that there are other gene products regulated by CsrA that are activated in these specific conditions that also regulate λP_R.

pgaA is a well-characterized leader, allowing for it serve as a positive control for CsrA regulation. Nevertheless, a second level of regulation by Rho was found and thus serves as a confounding variable to CsrA regulation because as seen in Figure 10, Rho can terminate transcription by binding to the Rho utilization (rut) site.[2] This may be mistaken for decreased activity of the promoter since its respective gene does not become translated into a functional protein, so as a result, the rut site was deleted to remove the confounding variable. Because *pgaA* has been shown to be post-transcriptionally repressed by CsrA, this was used to demonstrate the presence of CsrA activity *in vitro*.[25] Without the confounding variable rut, CsrA regulation of *pgaA* could be studied specifically.

The λP_R*pgaAΔrut* construct should be regulated by CsrA directly and by SirA indirectly. To test this, the construct was grown in four conditions to see if this regulation would be observed. Figures 6 through 9 show the wild type strain having the greatest expression. However when *sirA* is deleted, expression of mCherry, and hence the *pgaA* leader, significantly decreases as expected. Because *pgaA* is repressed by CsrA, when *sirA* is deleted, CsrB is not produced; therefore, CsrA continues to be produced which in turn leads to reduced expression of *pgaA*.[25] Expression of mCherry in the *csrA52* mutant is not as high as expected. It is thought that without CsrA activity present, expression of the *pgaA* leader would be greater than that of the wild type since *pgaA* would not be repressed by CsrA, but this is not observed in Figures 6 through 9. There is a possibility that the mutation in the *csrA52* mutant may still allow for some of the original functionality of CsrA and repress *pgaA* in moderation. In addition, trends of the expression levels of mCherry in the λP_R*pgaAΔrut* construct follow a similar trend as the λP_R construct. This may further indicate that λP_R was a poor selection as a negative control for mCherry expression since there is still regulation of the λP_R promoter observed when there should not be. The regulation of λP_R by CsrA in intestinal and SPI-1 conditions introduces a confounding variable for the λP_R*pgaAΔrut* construct and makes it difficult to determine if CsrA and SirA are regulating only *pgaA*.

The λP_R*pgaAΔrutΔcsrA*$_{BS-1}$ and λP_R*pgaAΔrutΔcsrA*$_{BS-2}$ constructs contain different mutations that eliminate CsrA regulation, either by preventing CsrA from binding or from being translated, and this shows *pgaA* expression levels when CsrA is not present. As seen in Figures 6 through 9, these mutations appear to have partially eliminated regulation by CsrA and SirA. However, this may be due to the uneven activity of the λP_R promoter in the different backgrounds and may not indicate regulation by CsrA or SirA.

To take into account for the CsrA and SirA regulation happening in the λP_R::mCherry fusion, the normalized relative fluorescence units of the λP_R*pgaAΔrut*, λP_R*pgaAΔrutΔcsrA*$_{BS-1}$, and λP_R*pgaAΔrutΔcsrA*$_{BS-2}$ strains can be divided by the normalized relative fluorescent units of the corresponding λP_R strains since each of these promoters also have λP_R incorporated into the fusion. That way, the excess fluorescence from the λP_R::mCherry fusion can be omitted and the regulation of *pgaA* by CsrA and SirA can be observed in isolation from these confounding effects of λP_R regulation by CsrA and SirA in addition to regulation by Rho.

3.2 – Regulation of *ssaJ* by CsrA

As seen in Figure 11, the expression of *ssaJ* follows a similar trend when the strains are grown in intestinal, systemic, and SPI-1 inducing conditions. The wild type and *sirA* mutant strains do not show significant differences in expression levels of *ssaJ*, but its expression increases greatly in the *csrA52* mutant strain. As a promoter that plays a role in the T3SS-2, SsaJ has the highest level of activity in SPI-2 inducing conditions particularly for the wild type strain. Like for the control promoter λP_R*pgaAΔrut*, *ssaJ* expression decreases in the *sirA* mutant and is greater in the *csrA52* mutant than the *sirA* mutant. This suggests that CsrA represses *ssaJ* because without SirA indirectly repressing *csrA*, there is more CsrA and thus less *ssaJ* expression and more *ssaJ* expression when CsrA is not present. This makes sense because CsrA represses the SPI-2 promoter *ssrB* that activates *ssaJ*.[18]

3.3 – Conclusions and Future Directions

Based on the *in vitro* assays of the control promoters and *ssaJ*, it can be seen that CsrA regulation is occurring *in vitro*. These strains will be used to determine when and where

regulation by CsrA takes place *in vivo*. The goal is to observe the activity of the control promoters and *ssaJ* in tissue culture and ultimately in a murine model.

Epithelial cells and mouse macrophages will be infected with the respective strains of *Salmonella*. The multiplicity of infection (MOI) for *Salmonella* has yet to be determined. The infection of epithelial cells will be incubated for a shorter period of time to allow for the expression of SPI-1 genes that permit the invasion of the epithelial cells by *Salmonella*. The infection of the macrophages will be incubated longer so that SPI-2 genes may be expressed to allow for the proliferation of *Salmonella*, thus mimicking systemic infection.

After incubation, the excess *Salmonella*, which have yet to infect the cells, will be killed off using an antibiotic that does not penetrate eukaryotic cells. To look at CsrA regulation, the expression of the promoters between infected and uninfected cells will be observed by quantifying the intensity of mCherry emission using confocal microscopy. The parameters for measuring the intensity of mCherry emission will need to be defined by quantifying the emission of the lowest and highest fluorescing control promoters before working with *ssaJ*.

It is suspected that *ssaJ* activity will be the highest in the macrophage infection since it codes for a structural component of the T3SS-2 which allows for intracellular survival and proliferation of *Salmonella*.[12, 14] Some regulation of *ssaJ* by CsrA is expected to occur during infection of the epithelial cells but more greatly pronounced during infection of macrophages considering that these immune cells can play a role in carrying *Salmonella* to other parts of the body and therefore possibly leading to systemic infection.[12] Moreover, the activity of the BarA-SirA system is greater in the intestines where the epithelial cells are located due to the higher concentration of short chain fatty acids that can activate this system.[16] From the activation of the

BarA-SirA system, *csrB* is expressed resulting in lower activity of CsrA.[16] These assumptions regarding infection of the epithelial cells and macrophages may also apply to the mouse model. Activity of CsrA and expression of *ssaJ* will be lower in cells of the small intestine. However, regions in the mouse to where macrophages can carry *Salmonella*, like the mesenteric lymph nodes, liver, and spleen, will have greater activity of CsrA and expression of *ssaJ*.[4]

In the end, the outcomes of this study will enhance the understanding of the interaction between host and pathogen. This may be a stepping stone to the development of therapeutics against an illness that affects so many around the world.

3.4 – Figures

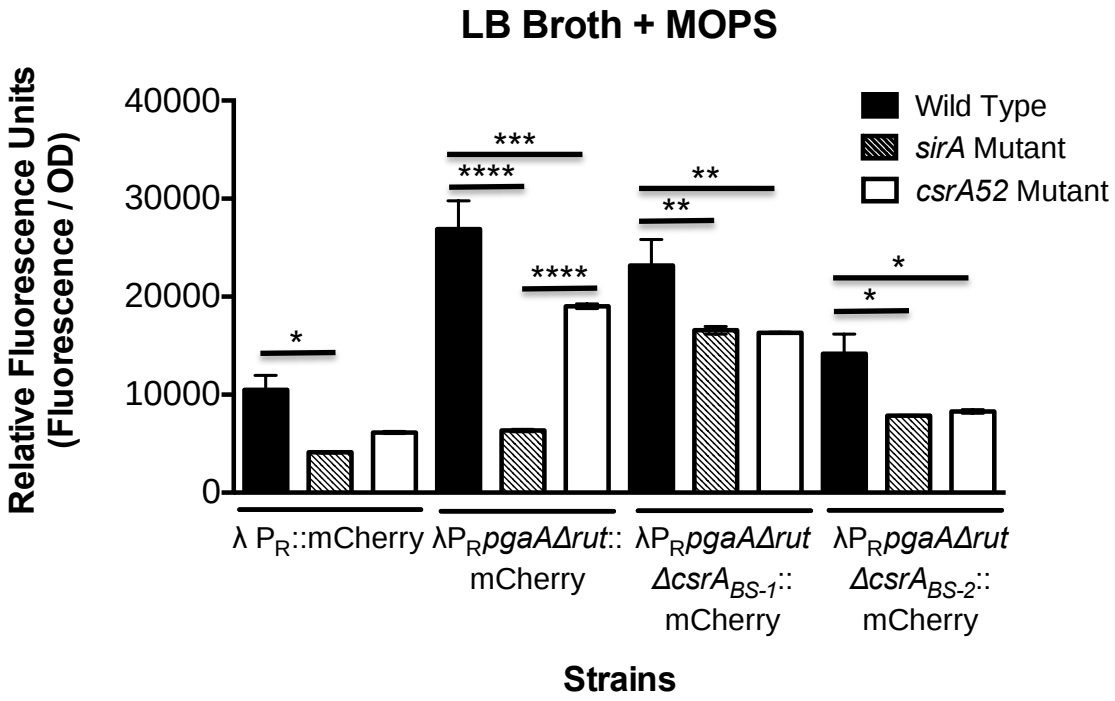


Figure 6: Expression from the control promoters by cells grown in intestinal conditions. The expression of mCherry was normalized by dividing the fluorescence by the OD for each respective strain. One-way ANOVA statistical analysis was performed on this data. p = 0.05, * ; p = 0.01, ** ; p = 0.001, *** ; p = 0.0001, ****

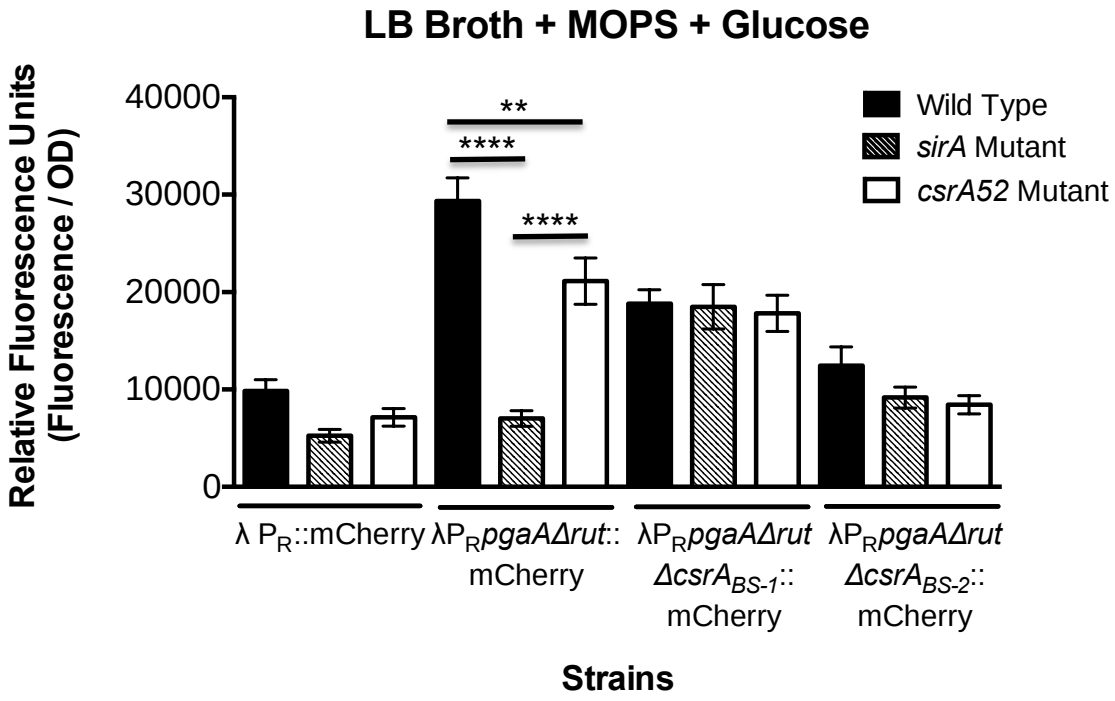


Figure 7: Expression from the control promoters of cells grown in systemic conditions. The expression of mCherry was normalized by dividing the fluorescence by the OD for each respective strain. The data was analyzed using one-way ANOVA statistical analysis. p = 0.05, * ; p = 0.01, ** ; p = 0.001, *** ; p = 0.0001, ****

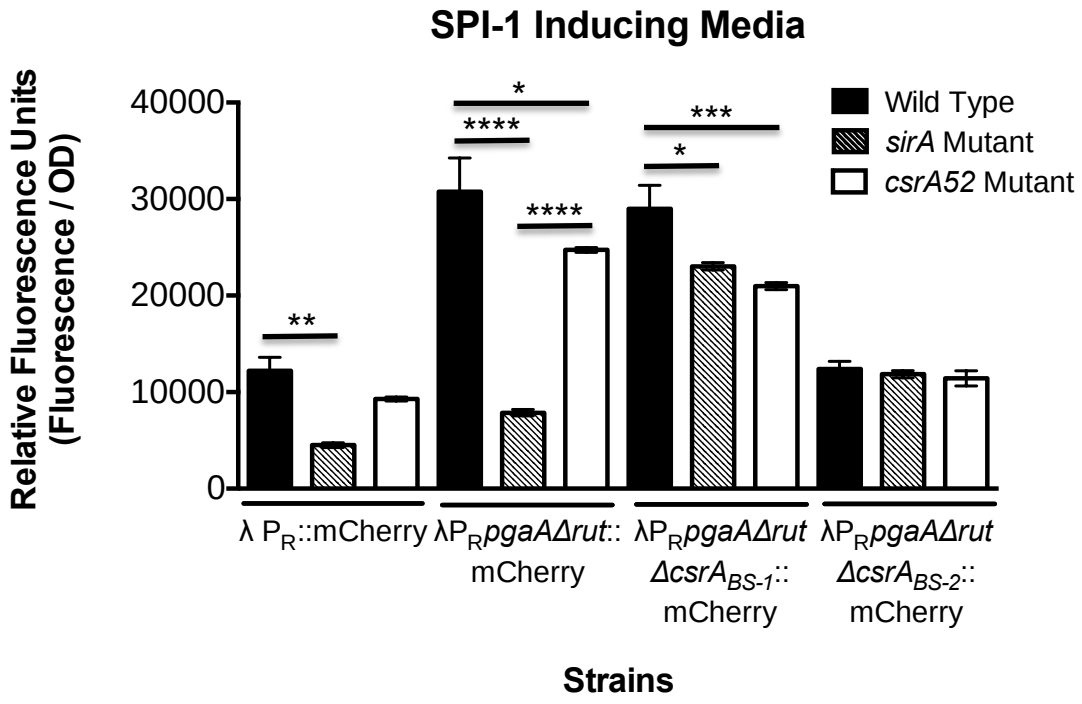


Figure 8: Expression of the control promoters by cells grown in SPI-1 conditions. The expression of mCherry was normalized by dividing the fluorescence by the OD for each respective strain. One-way ANOVA statistical analysis was used to analyze this data. p = 0.05, * ; p = 0.01, ** ; p = 0.001, *** ; p = 0.0001, ****

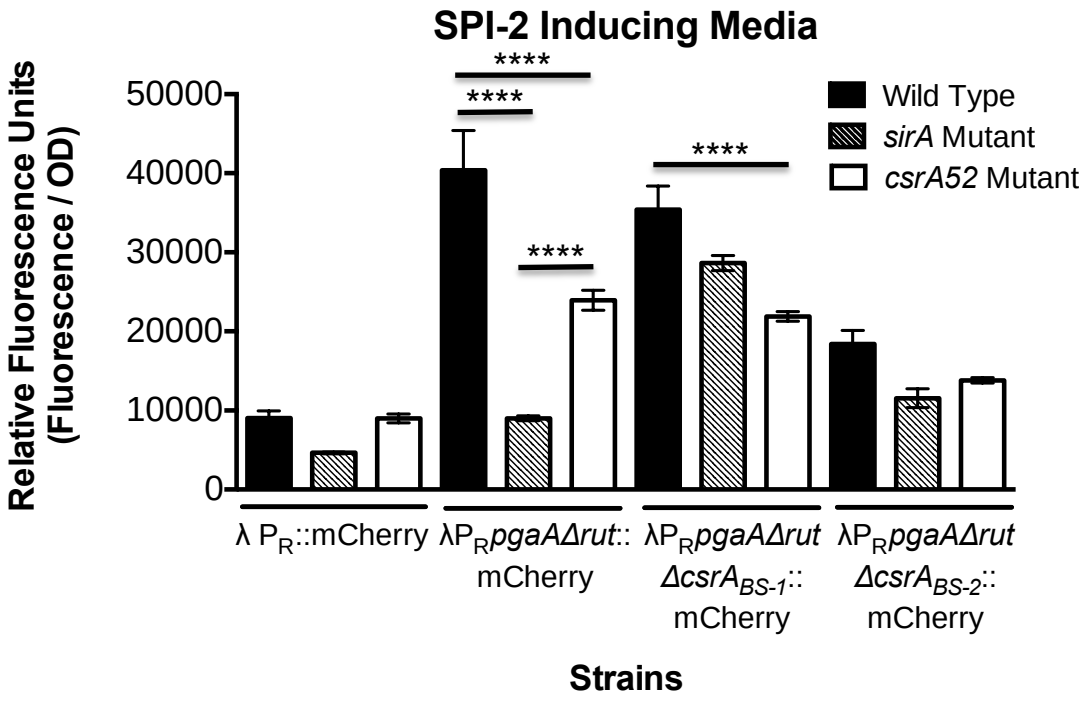


Figure 9: Expression of the control promoters by cells grown in SPI-2 conditions. The expression of mCherry was normalized by dividing the fluorescence by the OD for each respective strain. The data was studied using one-way ANOVA statistical analysis. p = 0.05, * ; p = 0.01, ** ; p = 0.001, *** ; p = 0.0001, ****

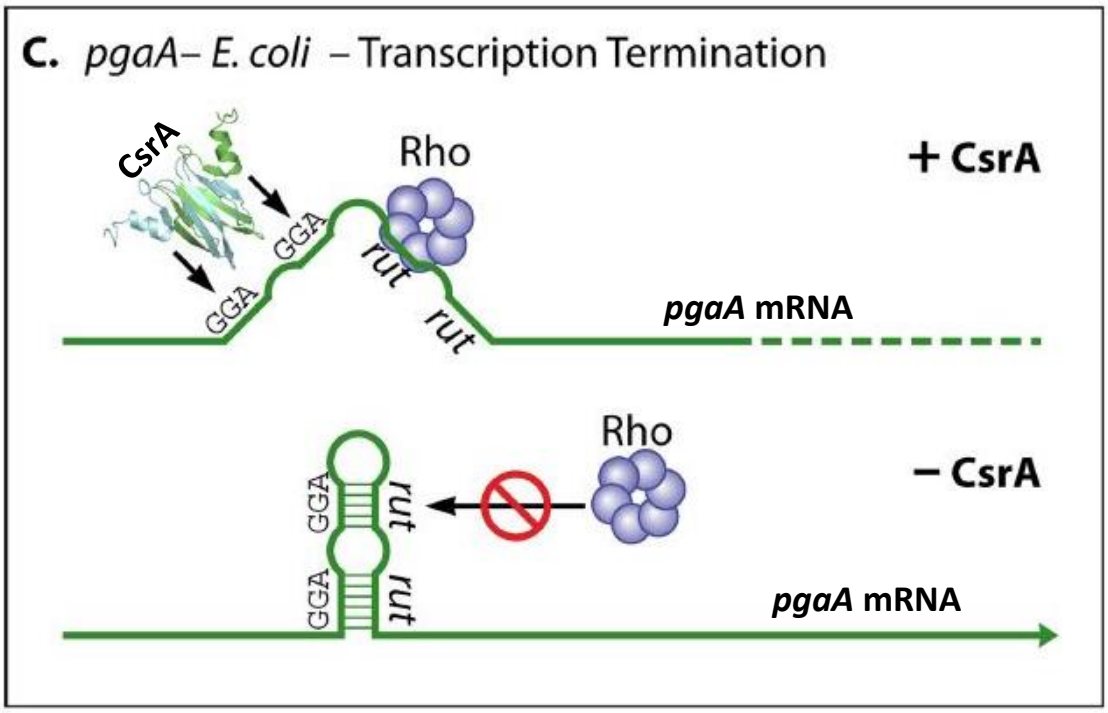


Figure 10: Rho termination of the transcription of *pgaA*. The binding of CsrA allows for Rho-dependent termination of *pgaA* transcription by preventing the formation of a hairpin loop and exposing the rut sites. Without CsrA, the rut sites are not available for Rho to bind, so transcription continues.[24]

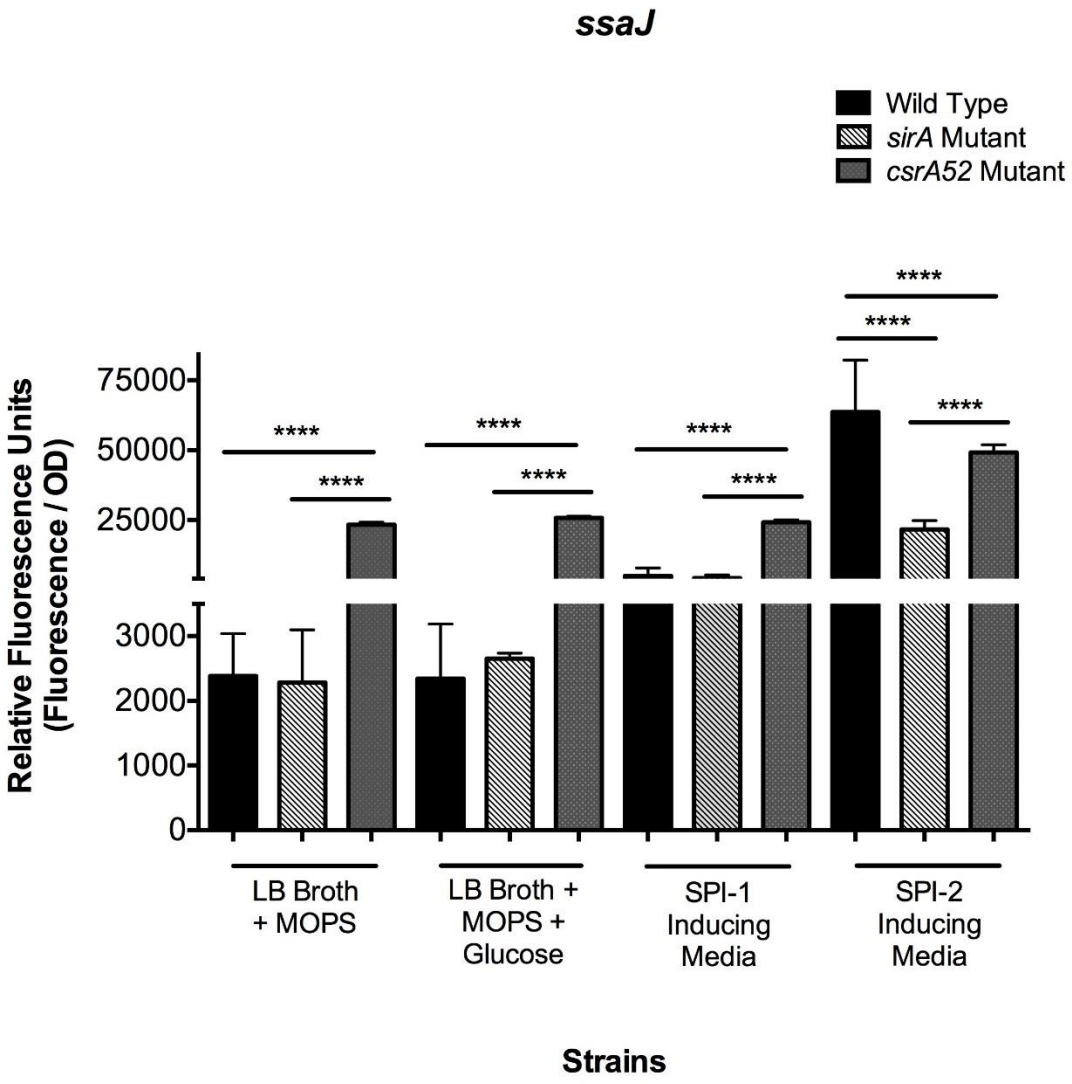


Figure 11: Expression of the target promoter *ssaJ* by cells grown in intestinal, systemic, SPI-1, and SPI-2 conditions. The expression of mCherry was normalized by dividing the fluorescence by the OD for each respective strain. The statistical analysis one-way ANOVA was used to analyze the data. p = 0.05, * ; p = 0.01, ** ; p = 0.001, *** ; p = 0.0001, ****

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