IDENTIFICATION OF *SALMONELLA ENTERICA* SER. TYPHIMURIUM GENES NECESSARY FOR REGULATION OF FLAGELLIN PRODUCTION UNDER SPI-2-INDUCING GROWTH CONDITIONS

An Undergraduate Research Scholars Thesis

by

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Submitted to Honors and Undergraduate Research
Texas A&M University
in partial fulfillment of the requirements for the designation as

UNDERGRADUATE RESEARCH SCHOLAR

Approved by
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May 2013

Major: Genetics
Biochemistry

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ABSTRACT

Identification of *Salmonella enterica* ser. Typhimurium genes necessary for regulation of flagellin production under SPI-2-inducing growth conditions. (May 2013)

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Human food-borne pathogen *Salmonella enterica* ser. Typhimurium has evolved a sophisticated way to avoid the host immune response. *Salmonella* Typhimurium possesses various virulence factors to ensure successful colonization and persistence in the host, including but not limited to, fimbria, Type 3 Secretion Systems (T3SS-1 and T3SS-2), lipopolisaccharides (LPS), and flagella. Unlike many other bacteria, *Salmonella* can not only survive inside macrophages, but also replicate within these host cells. This strategy allows bacteria to stay invisible to the host immune system. Intracellular *Salmonella* can be detected by the host through flagellin recognition by Nod-like receptors (NLRs). NLRs in turn trigger Caspase-1 activation, which ultimately triggers pyroptosis of the host cell (Miao *et al.*, 2011). While sufficiently replicated intracellular *Salmonella* utilizes pyroptosis to be released from the macrophage to infect other host cells, premature activation of pyroptosis could be detrimental for intracellular *Salmonella in vivo*. In defense, *Salmonella* downregulates expression of flagellins through activation of T3SS-2 encoded on SPI (*Salmonella* Pathogenicity Island)-2. The goal of this project was to identify *Salmonella* single deletion mutants that can produce flagella despite SPI-2 activation in order to

expand our knowledge of genetic determinants involved in evasion of premature pyroptosis by *S.*Typhimurium .Screening of a library containing 2639 mutants, resulted in identification of 214 mutant candidates. We demonstrated that one of the mutants, identified and confirmed in our screen, *ΔSTM1697*, was expressing high levels of flagellin, despite expression of active T3SS-2. *ΔSTM1697*, predicted to encode a protein with putative diguanylate cyclase/phosphodiesterase domain 2, could play a role in c-di-GMP (cyclic di-GMP) metabolism. C-di-GMP is bacterial second messenger involved in control of motility/sessility lifestyle of *S.* Typhimurium.

Identification of gene products, such as *STM1697*, involved in protection of *Salmonella* Typhimurium against premature detection by host innate immune system during growth in macrophages will stimulate a search for therapeutic targets to fight typhoid fever.

DEDICATION

This report is dedicated to the Andrews-Polymenis laboratory, all of who have taught me an incredible amount about the world of scientific research in the past two and a half years.

ACKNOWLEDGEMENTS

I would like to acknowledge the Andrews-Polymenis laboratory for all of their enthusiastic guidance and support throughout this project. Specifically, I would like to thank Dr. Helene Andrews-Polymenis for opening the doors of her laboratory to me and allowing me the opportunity to learn under her expertise. Thank you to Dr. Johanna Eflenbein for a careful and critical reading of this manuscript. Also, thank you to Marissa Talamantes for helping me with supplies and preparation. I would especially like to thank my research advisor, Dr. Lydia Bogomolnaya, for gifting me with her project and imparting an incredible wealth of knowledge in bacterial science.

NOMENCLATURE

SCV	*Salmonella*-containing vacuole
T3SS	Type III Secretion System
SPI-1	*Salmonella*-pathogenicity-island-1
SPI-2	*Salmonella*-pathogenicity-island-2
NLR	Nod-like receptor
STM	*Salmonella* Typhimurium
NLRC4	NLR family CARD domain containing protein 4
NLRP3	NLR family pyrin domain containing protein 3
WT	Wild Type
SGD	Single gene deletion
LB	Luria-Bertani
MES	2-(N-morpholino)ethanesulfonic acid
OD_{600}	Optical density measured at 600 nanometer wavelength
PBS	Phosphate Buffered Saline
SDS	Sodium Dodecyl Sulfate
TCA	Trichloroacetic acid
SDS-PAGE	SDS-Polyacrylamide gel electrophoresis
TEMED	(N,N,N',N')-Tetramethylethylenediamine
V	Volts
PVDF	Polyvinylidene fluoride
AP	Alkaline phosphatase

CHAPTER I

INTRODUCTION

Salmonella enterica subspecies *enterica* is a gram-negative, motile, rod-shaped bacterium from the family *Enterobacteriaceae*. Non-typhoidal *Salmonella enterica* ser. Typhimurium is a leading cause of bacterial food-borne gastroenteritis in the United States and worldwide, resulting in approximately 1 million cases in the United States alone each year. It is a leading food-borne cause of death, particularly among elderly, young, and immunocompromised patients (Voetsch *et. al*. 2004; CDC, 2011).

Salmonella Typhimurium is an intracellular pathogen, and replication occurs within a *Salmonella*-containing vacuoule (SCV) within macrophages. The *Salmonella* Typhimurium genome encodes two Type III Secretion Systems (T3SS). *Salmonella*-pathogenicity-island-1 (SPI-1) encodes the T3SS-1 necessary for invasion of host cells. Survival within the host macrophage requires expression of a group of virulence genes found within the *Salmonella*-pathogenicity-island-2 (SPI-2) locus (Agbor and McCormick, 2011). Upon entrance into the host cell, expression of SPI-1 is downregulated and SPI-2 upregulated (Miao *et. al.* 2010). SPI-2 encodes the T3SS-2 which secretes effector molecules that regulate maturation of the SCV and prevent host detection (Coombes *et. al.*, 2004). An important component of *Salmonella* virulence is the SPI-2-mediated downregulation of flagellin that enables evasion of the host innate immune system, however the specific role of the T3SS-2 in this process remains poorly understood (Miao *et. al*., 2010).

Macrophages use Nod-like receptors (NLRs) of the innate immune system to detect the presence of intracellular *S.* Typhimurium T3SS-1 rod protein, PrgJ, and flagellin. Timely NLRC4 (NLR family CARD domain containing protein 4) detection is critical to host survival, and NLRC4 knockout mice are susceptible to infection with both wild type (WT) and constitutively expressing flagellin mutant *S*. Typhimurium, while NLRC4 +/+ mice efficiently clear off flagellin-expressing mutants (Miao *et. al*., 2010).

Specifically, NLRC4 recognizes PrgJ and flagellin by their translocation into host cells and in response, activates Caspase-1. NLRP3 (NLR family pyrin domain containing protein 3) also detects *S.* Typhimurium and triggers Caspase-1 activity but by unknown mechanisms (Broz *et. al.*, 2010). Caspase-1 triggers cytokine-independent pyroptosis of the macrophage, which causes a pro-inflammatory release of cytosolic contents for capture and degradation by neutrophils. *Salmonella* can evade detection by NLRC4 by downregulating SPI-1 expression and upregulating SPI-2, whose T3SS rod protein, SsaI, is not recognized by NLRC4 (Miao *et. al.*, 2011). SPI-2 has also been shown to downregulate flagellin expression, and constitutively expressing flagellin mutants are avirulent in WT mice (Miao *et. al*., 2010).

While it has been shown that pyroptosis can be a mechanism to release *Salmonella* after sufficient intracellular replication, premature pyroptosis due to host detection results in a clearance of *Salmonella in vivo* (Miao *et. al*., 2010). Identifying *S*. Typhimurium genes that are critical for the appropriate downregulation of flagellin upon SPI-2 expression can help elucidate the signaling pathway behind *Salmonella* evasion of the host during the intracellular replication period. Ultimately, this knowledge could potentially offer novel therapeutic options.

To identify *S*. Typhimurium genes involved in SPI-2-mediated downregulation of flagellin, we have conducted a screening of our laboratory's collection of *S.* Typhimurium non-polar single gene deletion (SGD) mutants (containing 2639 out of approximately 5000 genes present in the *S*.Typhimurium genome) for mutants which are motile in SPI-2-inducing swimming media (Santiviago *et. al*, 2009; Andrews-Polymenis, unpublished data). SPI-2-inducing media is designed to mimic the SCV environment, and it has been used extensively to study induction of SPI-2 in *Salmonella* (Coombes *et. al*., 2004). We identified 214 candidate mutants with motility under these conditions and quantitatively confirmed 74 candidates in a secondary screening process. Last, we confirmed flagellin expression on the cell surface and expression of SPI-2 apparatus by Western blotting analysis with anti-flagellin and anti-SPI-2 effector antibodies in five mutants.

CHAPTER II

METHODS

Strains and growth conditions

The strains used in this study were derived from *Salmonella enterica* serovar Typhimurium ATCC14028. A spontaneous nalidixic acid resistant derivative of ATCC14028, HA420 (Bogomolnaya *et al*., 2008), was used as wild type. All deletion mutants were generated by a previously described method (Datsenko and Wanner, 2009). Mutants in *ΔSTM0557* (Bogomolnaya *et al*., 2008) and *ΔmacAB* (Nishino *et al*., 2006) were used as positive controls for motility under SPI-2 conditions based on previous observations from our laboratory (unpublished data). All strains were routinely grown in Luria-Bertani (LB)-broth supplemented with 50 mg/L Nalidixic acid or 50 mg/L Kanamycin where appropriate or in SPI-2-inducing media (see below) at 37°C.

Pilot study to determine methodology for SPI-2 motility assay

Motility of *S*. Typhimurium WT, *ΔSTM0557* (a super-swimmer), and *ΔmacAB* (previously observed to be expressing flagellins in SPI-2 conditions, unpublished data) strains was determined to verify motility in the nutrient-limited conditions of SPI-2-inducing media. First, colonies were transferred from LB agar to SPI-2 agar, grown overnight, and then transferred to a fresh SPI-2 agar dish and grown overnight.

Primary screening of S. Typhimurium mutants for motility in SPI-2-inducing conditions

A collection of 2639 *S.* Typhimurium SGD mutants arranged in 96-well format was screened for motility in SPI-2-inducing conditions. Colonies were transferred from solid LB agar to SPI-2 agar (1.5% agar, 10 mM KCl, 15 mM $(NH_4)SO_4$, 1 mM K_2SO_4, 160 mM $MgCl_2$, 674 mM KH_2PO_4, 160 mM 2-(N-morpholino)ethanesulfonic acid (MES), 0.6% glycerol, 0.2% casamino acids, diluted in ddH_2O and adjusted to pH 5.8) with a 96-pin metal replicator and grown overnight at 37°C. This process was repeated once to ensure maximal expression of SPI-2. Colonies were then transferred to SPI-2 swimming plates (0.3% agar in SPI-2 media), and allowed to grow for approximately 3 hours at 37°C. Mutants with motility halos on at least three of the four plates were designated as a candidate for secondary screening. Wild type (WT) and *ΔSTM0557* were added to each plate as negative and positive controls, respectively. Also, mutants that were unable to maintain growth on SPI-2 agar were recorded.

SPI-2 media sensitivity confirmation

Mutants in the primary screen unable to grow in SPI-2 conditions were confirmed before being removed from the screening process. SPI-2 media sensitive mutants were streaked in triplicate on LB, SPI-2, and M9 Minimal (1.6% agar, 1 mM $MgSO_4$, 0.2% glucose, 0.1 mM $CaCl_2$, 10% 10x M9 salts (0.25 M $Na_2HPO_4 \times 2H_2O$, 0.22 M KH_2PO_4, 0.085 M NaCl, 0.187 M NH_4Cl) diluted in ddH_2O) agar plates and incubated overnight at 37°C. Growth was recorded and mutants that were sensitive to SPI-2 media were removed from the screening process.

Secondary screening to quantify motility halo diameters of primary candidates

A collection of 214 mutants underwent a quantitative secondary screening to verify motility in SPI-2 inducing conditions, as well as identify mutants displaying a significant phenotype. Each candidate was inoculated in both 5 mL LB-broth (supplemented with 50 mg/L Nalidixic acid or 50 mg/L Kanamycin where appropriate), as well as 5 mL SPI-2 liquid media and grown overnight at 37°C. The OD_{600} of each culture was measured, and samples were diluted to a constant cell density using Phosphate Buffered Saline (PBS). A 5 µL aliquot of each LB culture was spotted on LB swimming agar, and a 5 µL aliquot of each SPI-2 culture was spotted on SPI-2 swimming agar. WT and *ΔmotA* (non-motile mutant) were added as positive and negative controls, respectively, to each LB agar plate, while *ΔSTM0557* and WT were added as positive and negative controls, respectively, to each SPI-2 agar plate. LB agar plates were incubated at 37°C for 3 hours, while SPI-2 agar plates were incubated at 37°C for 6 hours. The diameter of each motility halo was recorded.

Western blot of select confirmed mutants

Five mutants confirmed in the secondary screen process were selected for Western blotting analysis. Each mutant was inoculated in both 5 mL LB-broth (supplemented with 50 mg/L Nalidixic acid or 50 mg/L Kanamycin where appropriate) as well as SPI-2 liquid media and grown overnight at 37°C.

Cell concentration for each culture was normalized by reading the OD_{600}. LB and SPI-2 cultures were centrifuged at maximum speed (Eppendorf 5415) for 10 minutes and resuspended in 1 mL PBS. Bacteria were vortexed for 5 minutes each to generate a sheared flagella fraction, and cells

were separated by centrifugation for 5 minutes at maximum speed. The flagella-containing fractions were collected and Trichloroacetic acid (TCA) was added to make a final concentration of 6%. Samples were kept at 4°C overnight, followed by centrifugation at maximum speed for 15 minutes at 4°C. Precipitates were washed with 300 μL acetone twice. Resulting samples (flagella fraction), were resuspended in 100 μL of 2X Sodium Dodecyl Sulfate (SDS) sample buffer. Samples were boiled for 10 minutes and stored at -20°C until use. A whole cell lysate fraction was also prepared with the original bacterial pellet. After the removal of the sheared flagella fraction, the remaining pellet was resuspended in 200 μL 2X SDS sample buffer and boiled for 10 minutes. Whole cell lysates were stored at -20°C until use. A sample of SPI-2 culture supernatant was prepared to verify functioning SPI-2 machinery by removing the supernatant of the SPI-2 liquid cultures prior to flagella and whole cell fraction preparation. This supernatant was incubated with 6% TCA on ice for 30 minutes, followed by centrifugation for 30 minutes at 4000 rpm at 4°C (Sorvall Legend RT). The samples were washed twice with 300 μL acetone, and centrifuged briefly at maximum speed to generate a pellet. This pellet was resuspended in 100 μL 2X SDS sample buffer and boiled for 10 minutes. SPI-2 supernatant samples were stored at -20°C until use.

SDS-Polyacrylamide gel electrophoresis (SDS-PAGE) was performed using a 12% polyacrylamide gel (1.25 mL 4x lower Tris-Cl/SDS buffer , pH 8.8 (Sambrook and Russell, 2001), 2 mL of 30% acrylamide/bis solution, 37.5:1 (Bio-Rad), 1.725 mL H_2O, 30 μL of 10% ammonium persulfate, and 10 uL (N,N,N',N')-Tetramethylethylenediamine (TEMED)) with a stacking layer (0.3875 mL 4x upper Tris/SDS, pH 6.8 (Sambrook and Russell, 2001), 0.234 mL of 30% acrylamide/bis solution, 37.5:1, 0.93 mL H_2O, 18 uL of 10% ammonium persulfate, and

6 uL TEMED). All samples were loaded, including WT, *ΔSTM0557* (a super-swimmer), *ΔfljB*, *ΔfliC* (mutants that does not produce flagella), *ΔssaK* (mutant lacking SPI-2 machinery), and *ΔsseD* (mutant in SPI-2 effector) all prepared under LB and SPI-2 conditions. The gel underwent electrophoresis for 1 hour at 150 Volts (V). Proteins were transferred to an Immobilon-P polyvinylidene fluoride (PVDF) membrane (Millipore) for 1 hour at 100V at room temperature with stirring. After transfer, the membrane was blocked for 1 hour in 5% milk/0.1% Tween/PBS (blocking buffer) at room temperature.

Rabbit anti-*fljB, -fliC* (Difco) and *sseD* (gift from John Brumell, University of Toronto) antibodies were used at 1:1,000; 1:10,000 and 1:10,000 dilution in blocking buffer, respectively, to confirm flagellar (*fljB* and *fliC*) and SPI-2 effector expression (*sseD*). The membrane was placed in 10 mL primary antibody solution in blocking buffer, and incubated for 1 hour at room temperature. The membrane was washed four times with 0.1%/Tween/PBS for 10 minutes each, followed by a 45 minute incubation at room temperature with anti-rabbit goat IgG antibody conjugated with alkaline phosphatase (AP) (Sigma) used at 1:10,000 dilution in blocking buffer. The membrane was washed 3 times in 0.1%/Tween/PBS for 10 minutes each at room temperature, followed by a single wash in PBS for 10 minutes. The membrane was pressed against blotting paper for 5 minutes, followed by incubation with Immun-Star AP substrate (Bio-Rad) for 5 minutes at room temperature. The membrane was again pressed against blotting paper for 5 minutes, followed by X-ray detection.

CHAPTER III

RESULTS

Pilot study to determine methodology for SPI-2 motility assay.

To optimize the conditions for SPI-2 activation we used *S*. Typhimurium WT, *ΔSTM0557*, and *ΔmacAB* strains. Wild type cells are not expressing flagellins on the surface when T3SS-2 is expressed and, therefore, cells are expected to be non-motile on SPI-2 motility plates. We used WT cells in this experiment as a control for SPI-2 induction. In previous work in the laboratory, it was shown that some mutants, namely, *ΔSTM557* and *ΔmacAB*, are still expressing flagellins on the cell surface even when T3SS-2 is active (unpublished data). We used these mutants as the positive controls for swimming plates to determine the time required for initiation.of swimming motility

Motility of *S*. Typhimurium *ΔSTM0557*, and *ΔmacAB* strains, but not WT strain was visible on SPI-2 swimming agar best when strains were grown on LB agar first, then transferred to SPI-2 agar twice consecutively as described in Chapter II (data not shown). This double transfer ensures stored nutrients are depleted, which is necessary for SPI-2 induction. From this experiment, we also concluded that SPI-2-swimming plates should be incubated for 3 hours at 37 ^{0}C before motility halos become visible in 96-well format and 6 hours when mutants are individually spotted on swimming plates.

Primary screening of S. Typhimurium mutants for motility in SPI-2-inducing conditions

From the collection of 2639 *S.* Typhimurium SGD mutants screened for motility in SPI-2-inducing conditions, 214 mutants created visible motility halos on at least three out of four SPI-2

agar plates within the three hour incubation period. A representative image of a motility halo from the primary screen is shown in Figure 1. These 214 mutants were classified using the Clusters of Orthologous Groups (COGs) online database, shown in Table A1. The COGs classifications varied significantly across the 214 mutants, lending no single majority class. However, some classifications were overrepresented, including transcription (8.5%), as well as amino acid and carbohydrate transport and metabolism (11% and 7.5% respectively). The entire breakdown for all clusters identified is shown in Figure 2. These classifications aligned with known functions of some mutants. For example, the *STM3860* is encoding for a homolog of *E.coli* transcriptional regulator, SgrR, involved in sugar phosphate stress (Horler and Vanderpool, 2009), and this gene was appropriately classified in the COGs database as involved in the amino acid transport and metabolism cluster. Also, the *STM2912* is a predicted LysR-type transcriptional regulator in *Salmonella* important for virulence (Yoon et al, 2011), and this gene was classified as a part of the transcription cluster by COGs.

SPI-2 media sensitivity confirmation

During the primary screening of the SGD library, 48 mutants were found to be sensitive to SPI-2-inducing media. The mutants did not grow, or displayed significant growth defects, on SPI-2-inducing media in the 96-well format. These mutants were confirmed in triplicate by streaking on LB, M9 minimal media, and SPI-2 media prior to being removed from the screening process. Mutants confirmed to be sensitive to either M9 minimal, SPI-2, or both medias are listed in Table A3. All mutants were able to grow in LB media. Due to the similarity in M9 minimal and SPI-2 media composition, most mutants sensitive to the M9 minimal environment were also sensitive to SPI-2-inducing conditions. However, *ΔSTM4415*, *ΔSTM4101*, and *ΔSTM3526* were

uniquely sensitive to SPI-2-inducing conditions only, suggesting these mutants are sensitive to either the low pH of SPI-2 media (pH 5.8) or an ingredient only found in SPI-2-inducing media. The nature of these mutants' sensitivity to SPI-2 –inducing media conditions is intriguing and will be investigated in the future.

Secondary screening to quantify motility halo diameters of primary candidates

Seventy-four SGD mutants found in the primary screen were individually placed on LB and SPI-2 motility media and incubated for 3 or 6 hours, respectively. Representative plates from the secondary screen are shown in Figures 3 and 4. Following incubation, the diameter of the resulting motility halo was measured for each SGD mutant, and this data was reported as the SGD mutant diameter's percentage of the WT motility halo for each preparation of media (Table 1).

Of the 74 mutants screened in the secondary process, some were confirmed to be producing motility halos in SPI-2-inducing conditions. Student's T-Test was used to determine whether motility halos were larger than WT by a statistically significant amount. Five mutants displaying large SPI-2 motility halos were selected for Western blotting analysis. These five mutants, *ΔSTM1444* (*slyA*), *ΔSTM1697*, *ΔSTM2151*, *ΔSTM1055(gtgE)*, and *ΔSTM3792*, produced motility halos that were on average 263%, 231%, 209%, 180%, and 170% of the WT motility halo size, respectively (Table 1). Three of these mutants, *ΔSTM1444*, *ΔSTM2151*, and *ΔSTM1055*, were confirmed to produce motility halos in SPI-2-inducing conditions that were of a statistically significant difference from the WT halo, as determined by Student's T-Test ($p<0.05$). Also, *ΔSTM1697* was found to produce a motility halo of a statistically significant diameter different

from WT on LB media. *ΔSTM1697 and ΔSTM3792* was also producing a significant motility halo on SPI-2 plates (Figure 4) but there was a lot of variation between plates that resulted in high standard deviation. Therefore, we decided to include both *ΔSTM1697* and *ΔSTM3792* mutants in the list of candidates selected for Western blotting analysis. *ΔSTM1444*, or Δ*slyA*, was expected to be motile in SPI-2-inducing conditions, because of the known function of *slyA* in SPI-2 activation (Fabrega and Vila, 2013). Without *slyA*, the SPI-2 apparatus cannot be activated, and therefore flagellin expression is not downregulated.

Western blot of select confirmed mutants

Flagellin expression in *ΔSTM1055*, *ΔSTM1444*, *ΔSTM1697*, *ΔSTM2151*, and *ΔSTM3792* was determined by Western blot analysis probing against the minor *Salmonella* flagellin, FljB, and the major *Salmonella* flagellin, FliC. Strains prepared in LB media were able to express both flagellins when allowed sufficient exposure to X-ray film prior to detection (data not shown).. The exceptions to this are the antibody controls, *ΔfljB* and *ΔfliC*, as expected, as well as *ΔSTM1444*, which is likely due to dilution error during sample preparation. As seen in Figure 5a, significant FljB expression was observed for *ΔSTM0557*, as well as *ΔSTM1697*. Previous experiments in the laboratory have shown that in *ΔSTM0557* population, FljB and FliC are expressed at approximately equal amounts (unpublished data). Significant FliC expression is observed in all strains, as shown in Figure 5b, except in the antibody controls once again, as well as *ΔSTM1444* due again to a dilution error. In our WT (S.Typhimurium ATCC14028s) strain, FliC is the predominant from of flagellin (unpublished data), and this is consistent with higher expression of FliC than FljB for WT sample. Interestingly, *ΔSTM1697* has high expression of both flagellins compared to any of the other mutants.

Strains prepared in SPI-2 media were also used to evaluate flagellin expression. As expected, WT cells under SPI-2 conditions were producing neither FliC nor FljB (Figures 6a and 6b). In agreement with previous data from the lab (unpublished data), both FljB and FliC were expressed on the cell surface of *ΔSTM0557* mutant. In addition, FljB expression was very high in *ΔSTM1697* (Figure 6a). When allowed a longer exposure time to X-ray film prior to detection, FljB expression could be seen for *ΔSTM1055*, *ΔSTM2151*, and *ΔSTM1444* (data not shown).. FliC expression was very high in *ΔSTM1697* (Figure 6b), while a longer exposure time was required to show FliC expression in *ΔSTM1055*, and *ΔSTM1444* (data not shown).

Supernatant of SPI-2 samples was also collected to probe against T3SS-2 effector molecule, SseD, in an attempt to determine if SPI-2 activation occurred for each strain. An operating T3SS-2 apparatus will secrete SseD effector molecules, which can be found in the supernatant of liquid cultures. Western blot with anti-*sseD* antibodies revealed that all mutants except *ΔSTM1444* were activating T3SS-2 machinery in SPI-2-inducing conditions (Figure 7). *ΔsSTM1444*, as mentioned previously, lacks the ability to activate SPI-2, and therefore flagellin is not receiving downregulation signals. Included samples were an antibody control, *ΔsseD*, and *ΔssaK*, a mutant that cannot form SPI-2 machinery (Fabrega and Vila, 2013), neither of which showed presence of SPI-2 effector, SseD, as expected. Therefore, motility for these mutants as verified by flagellin expression is occurring simultaneously with SPI-2 activation.

ΔSTM1697 shows the most significant phenotype of the five mutants selected for Western analysis. *STM1697*, predicted to encode a protein with putative diguanylate

cyclase/phosphodiesterase domain 2, could play a role in c-di-GMP (cyclic di-GMP) metabolism (Jonas et al, 2010). C-di-GMP is an important bacterial second messenger involved in regulation of many cellular processes including transition from motility to sessility (Romling *et. al*, 2013). This is consistent with our findings, and suggests that *ΔSTM1697* may overexpress flagellin or lack an ability to regulate flagellin expression, regardless of SPI-2 effector presence.

CHAPTER IV

CONCLUSIONS

This study initiated a screen of our laboratory's SGD mutant library for *Salmonella* Typhimurium mutants that are motile under SPI-2-inducing conditions. While we are excited to continue the screening of our library, we currently report five mutants, *ΔSTM1055*, *ΔSTM1444*, *ΔSTM1697*, *ΔSTM2151*, and *ΔSTM3792*, which produce significant levels of flagellin expression under SPI-2-inducing conditions. Only one of these mutants, *ΔSTM1444,* is defective in T3SS-2 activation. One mutant,, *ΔSTM1697,* shows a significant phenotype compared to the other mutants, and should be considered a novel therapeutic target. Exploring how *ΔSTM1697* is able to bypass SPI-2 flagellin downregulation may reveal novel signaling pathways helpful in understanding *Salmonella* virulence.

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APPENDIX

Table A1. COGs classification for primary screen candidates

		COGs Classification[a] (see key in Table A2)																					
STM	**Gene**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**	**21**	**22**
csrB																							22
ryeB																							22
STM0011	*yaaI*																						22
STM0044	*yaaY*																						22
STM0108	*tbpA*			3																			
STM0170	*hpt*						6																
STM0301	*safC*												12		14								
STM0304	*sinR*								8														
STM0315	*prfH*																						22
STM0370	*prpD*																				20		
STM0375	*ampH*																			19			
STM0386	*proC*		2																				
STM0390	*aroM*																						22
STM0392	*rgdC*										10												
STM0413	*tsx*															15							
STM0441	*cyoG*																	17					
STM0466											10												
STM0526	*ylbA*																				20		
STM0548	*fimF*																						22
STM0549	*fimZ*								8										18				
STM0557																							22
STM0585	*fepA*			3																			
STM0595	*entC*	1						7															
STM0597	*entB*							7															
STM0637	*dacA*															15							
STM0654	*ybeQ*																				20		
STM0665	*gltI*		2																				
STM0665	*gltI*																		18				
STM0702	*kdpE*																		18				
STM0712	*ybgJ*		2																				
STM0764									8														
STM0785	*ybhE*				4																		
STM0788	*hutG*		2																				
STM0791	*hutH*		2																				
STM0811	*ybhN*																					21	
STM0817	*ybhF*																			19			
STM0826	*ybiN*																				20		
STM0836	*ybiR*			3																			
STM0940	*ybjX*[b]																					21	
STM0955	*aat*											11											
STM1055																							22

STM1072								8														
STM1103	*hpaD*																					22
STM1128			2																	20		
STM1152	*yceK*																					22
STM1159	*yceO*																					22
STM1222	*potD*		2																				
STM1260															15							
STM1312	*celA*				4																		
STM1325	*ydiZ*																					22
STM1341											11											
STM1344	*ydiV*																	18				
STM1351	*ydiT*																17					
STM1357	*ydiF*				5																	
STM1391	*ssrB*							8									18				
STM1427	*cfa*														15							
STM1444	*slyA*							8														
STM1547								8														
STM1551																						22
STM1562	*hdeB*																					22
STM1600																						22
STM1614																						22
STM1660.S	*fnr*												13									
STM1686	*pspE*		3																			
STM1697																		18				
STM1775	*hemK*								9													
STM1798	*ycgR*																					22
STM1802	*dadX*														15							
STM1803	*dadA*		2																				
STM1815	*minD*															16						
STM1816	*minE*															16						
STM1818	*fadD*				5		7															
STM1839																						22
STM1853	*pphA*																	18				
STM1865																						22
STM1879	*ptrB*		2																				
STM1883	*purT*					6																
STM1884	*eda*			4																		
STM1886	*zwf*			4																		
STM1888	*pykA*			4																		
STM1892.S	*znuC*																					22
STM1911																						22
STM1932	*ftnB*		3																			
STM1947	*uvrY*																	18				
STM1992	*dcm*									10												
STM2009	*amn*					6																
STM2028	*cbiG*	1																					
STM2045	*pduJ*																					22

STM2072	*hisD*		2																				
STM2100	*wcaL*															15							
STM2110	*wcaF*																				20		
STM2112	*wcaD*																						22
STM2151	*stcB*												12		14								
STM2167	*dld*																						
STM2168	*pbpG*															15							
STM2184	*sanA*																					21	
STM2231																							22
STM2256																		17					
STM2273		1																					
STM2289					4																		
STM2298	*pmrF*															15							
STM2306	*menC*	1																					
STM2307	*menB*	1																					
STM2312	*elaA*																				20		
STM2330	*lrhA*								8														
STM2367	*dedA*																					21	
STM2369	*usg*		2																				
STM2395	*pgtE*																						22
STM2399	*pgtP*				4																		
STM2405		1	2																				
STM2426																							22
STM2440	*cysM*		2																				
STM2454	*eutR*								8														
STM2465	*eutM*																						22
STM2486																					20		
STM2499.S	*purM*																						22
STM2509	*nupC*										10												
STM2554	*hcaT*		2	3	4																20		
STM2609																							22
STM2613																							22
STM2614																							22
STM2650	*yfiP*																					21	
STM2692																15							
STM2765																							22
STM2796	*yqaE*																					21	
STM2848	*hycF*																	17					
STM2856	*hypC*											11											
STM2907	*pphB*																		18				
STM2912									8														
STM2916					4																		
STM2923																							22
STM2927	*surE*																				20		
STM2939	*ygcH*																						22
STM2944	*ygcB*																				20		
STM2995.S																							22

STM3043	*dsbC*										11												
STM3044	*xerD*									10													
STM3046	*ygfX*																						22
STM3062	*serA*	1	2																				
STM3093	*endA*									10													
STM3108	*yggL*																					21	
STM3122				3																			
STM3124									8										18				
STM3135					4																		
STM3168	*ygiR*																	17					
STM3191																							22
STM3215	*ygjI*								8														
STM3241	*tdcE*																	17					
STM3290	*argG*		2																				
STM3323	*yhbJ*																				20		
STM3330	*gltB*		2																				
STM3469	*pabA*	1	2																				
STM3505	*feoA*			3																			
STM3506	*feoB*			3																			
STM3525	*glpE*			3																			
STM3614	*dctA*																	17					
STM3616	*yhjL*																				20		
STM3623	*yhjT*																						22
STM3648	*yiaG*								8														
STM3663	*bax*																				20		
STM3685	*mtlA*				4																		
STM3692	*lidP*																	17					
STM3700	*gpxA*																	17					
STM3702	*grxC*										11												
STM3704	*pmgl*				4																		
STM3708	*tdh*		2																		20		
STM3709	*kbl*	1																					
STM3726	*mutM*									10													
STM3734	*rph*									9													
STM3744	*recG*								8		10												
STM3746	*gltS*		2																				
STM3766																							22
STM3770																							22
STM3771																							22
STM3781					4																		
STM3789	*uhpB*																		18				
STM3792					4																		
STM3801	*dsdX*		2		4																		
STM3806																							22
STM3860			2																				
STM3879	*yieN*																				20		
STM3922	*rffG*														15								

STM3943	*cyaY*			3																			
STM3969	*yigN*																					21	
STM3973	*tatA*														14								
STM3979	*fre*	1																17					
STM3983	*fadB*					5																	
STM3994	*mobA*	1																					
STM4019					4																		
STM4041																							22
STM4068									8														
STM4073	*ydeW*								8														
STM4114	*pflD*																	17					
STM4120	*argE*		2																				
STM4121	*argC*		2																				
STM4189	*yjbB*			3																			
STM4194	*yjbD*																						22
STM4266	*soxR*								8														
STM4278																							22
STM4296	*adi*		2																				
STM4307																					20		
STM4308																					20		
STM4318									8												20		
STM4395	*yifZ*		2		4																20		
STM4400	*sodC*				4																20		
STM4500	*yjhP*							7													20		
STM4505																							22
STM4506								7															
STM4536																							22
STM4547	*yjjQ*								8										18				
STM4560	*prfC*									9													

[a]The Clusters of Orthologous Groups (COGs) classification places genes into probable generic functional groups and pathways. Primary screen candidates are listed with their COGs classification(s); the key is provided in Table A2.
[b]Also referred to as *somA*

Table A2. Color key for COGs classification

COGs Color Key[a]	
1	Coenzyme transport and metabolism
2	Amino acid transport and metabolism
3	Inorganic ion transport and metabolism
4	Carbohydrate transport and metabolism
5	Lipid transport and metabolism
6	Nucleotide transport and metabolsim
7	Secondary Metabolites Biosynthesis, Transport, and Catabolism
8	Transcription
9	Translation, Ribosomal Structure and Biogenesis
10	Replication, Recombination, and Repair
11	Posttranslational Modification, Protein Turnover, and Chaperones
12	Cell Motility
13	Aerobic, Anaerobic Respiration; Osmotic Balance (CRP family)
14	Intracellular Trafficking, Secretion, and Vesicular Transport
15	Cell Wall/Membrane/Envelope Biogenesis
16	Cell Cycle Control, Cell Division, and Chromosome Partitioning
17	Energy Production and Conversion
18	Signal Transduction Mechanisms
19	Defense Mechanisms
20	General Function Prediction (by COGs) Only
21	Function Unknown
22	Uncharacterized in COGs

[a]Color key for Table A1 shows the primary candidates' classification group titles

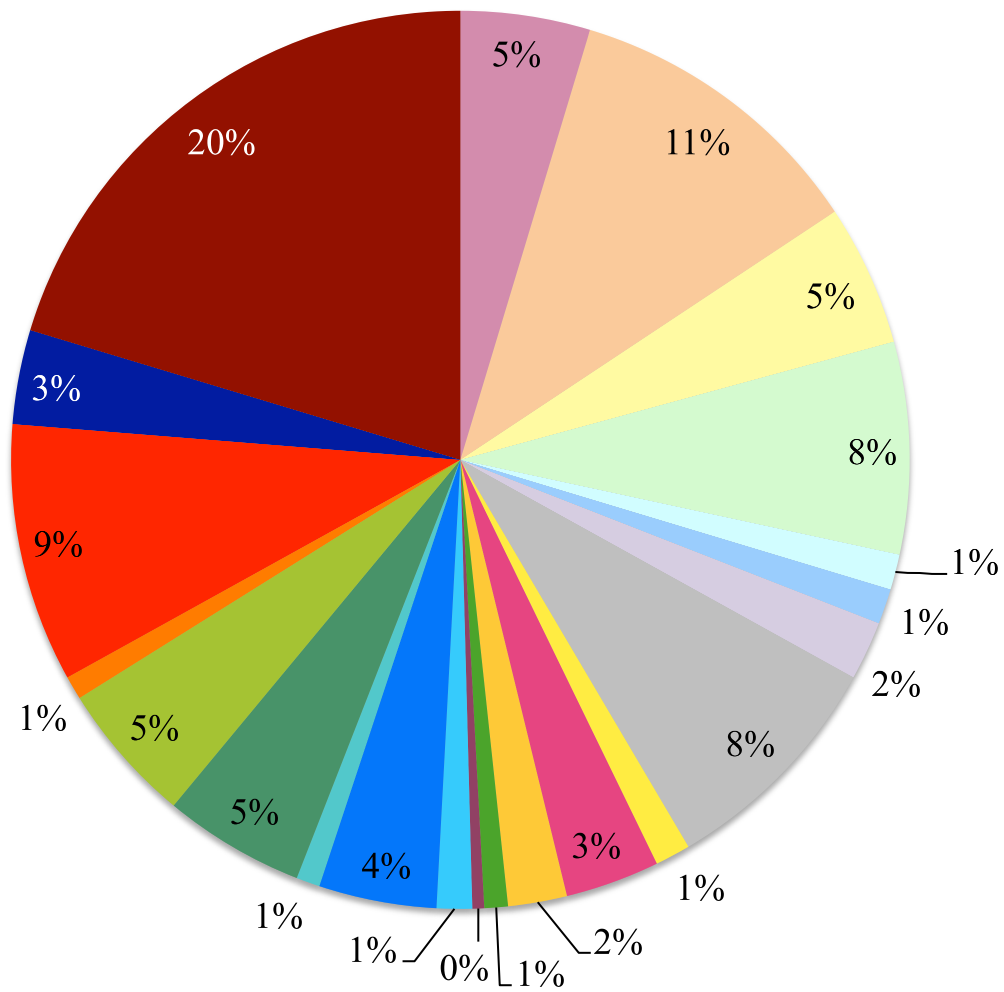

Figure A1. Graphic representation of COGs classification distribution for primary screen candidates highlights diversity of distribution. Color key is provided in Table A2.

Table A3. SPI-2 media sensitive mutant confirmation

		Media Sensitivity[a]	
STM	**Gene**	**M9**	**SPI-2**
STM0067	*carB*	Red	Red
STM0232	*accA*	Red	Red
STM0421	*yajO*	Red	Yellow
STM0533	*purK*	Red	Red
STM0745	*tolQ*	Yellow	Red
STM0746	*tolR*	Yellow	Red
STM0978	*aroA*	Red	Yellow
STM1163	*pyrC*	Red	Red
STM1347	*aroH*	Red	Red
STM1723	*trpE*	Red	Red
STM1726	*trpB*	Red	Red
STM1727	*trpA*	Red	Red
STM2309	*menD*	Red	Red
STM2324	*nuoF*	Red	Red
STM2362	*purF*	Red	Red
STM2565	*purG*	Red	Red
STM2566		Red	Red
STM2578	*pdxJ*	Red	Red
STM2580	*era*	Red	Red
STM2933	*cysC*	Red	Yellow
STM2935	*cysD*	Red	Yellow
STM2946	*cysH*	Red	Yellow
STM3401	*aroE_2*	Red	Red
STM3477	*cysG*	Red	Yellow
STM3526	*glpD*	Green	Red
STM3699	*cysE*	Red	Red
STM3733	*pyrE*	Red	Red
STM4008		Red	Red
STM4101	*metL*	Green	Yellow
STM4361	*hfq*	Red	Red
STM4415	*fbp*	Green	Red
STM4462	*yjgG*	Red	Red

[a]STM mutants identified as SPI-2 media sensitive were confirmed in three separate trials by streaking on LB, M9 Minimal, and SPI-2 media. Red represents unable to grow, yellow represents significant growth defect, and green represents normal growth ability.

FIGURES

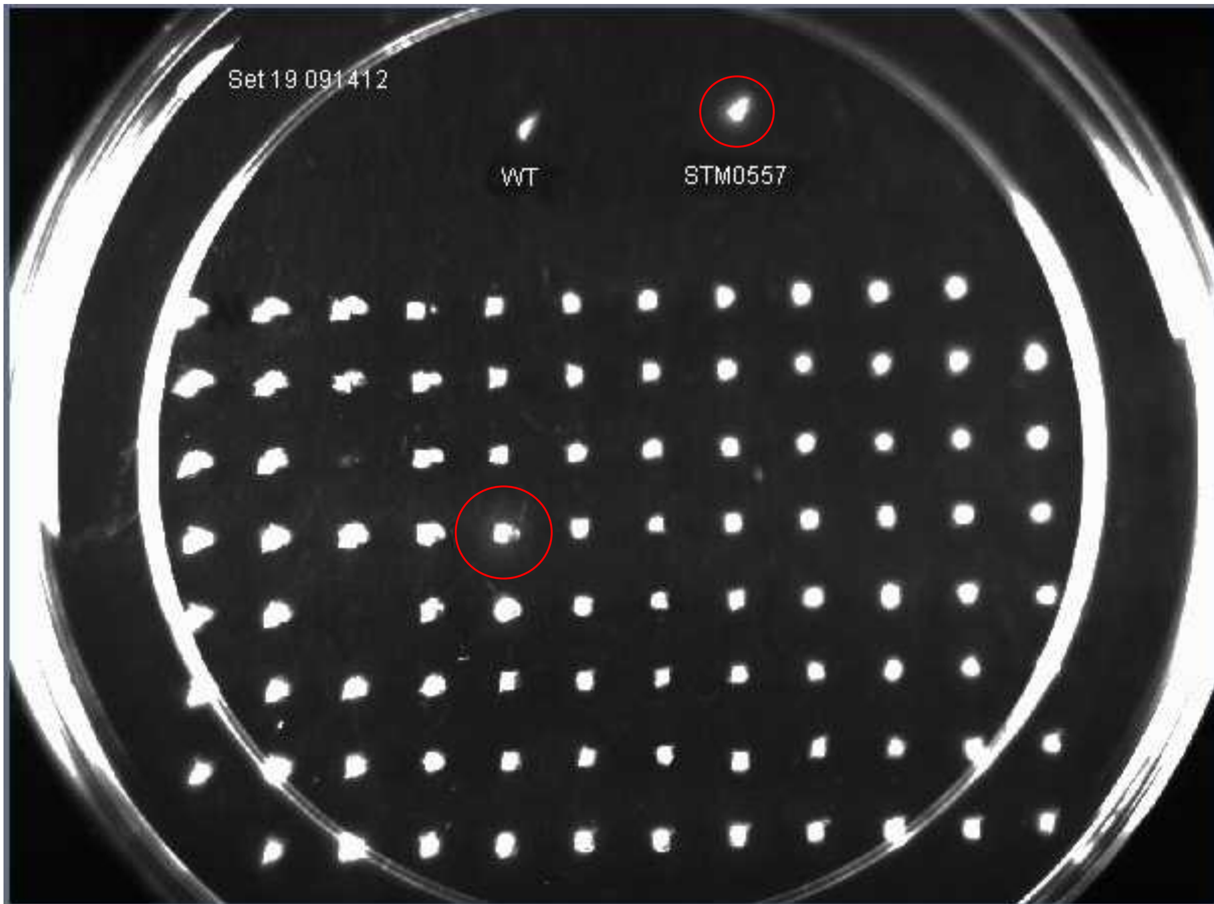

Figure 1. Representative picture of a SPI-2 motility plate from primary screening. Of the 96 mutants transferred to this SPI-2 motility plate from SPI-2 media, only one (highlighted in red circle) shows a motility halo comparable to the super-swimmer control, *ΔSTM0557*.

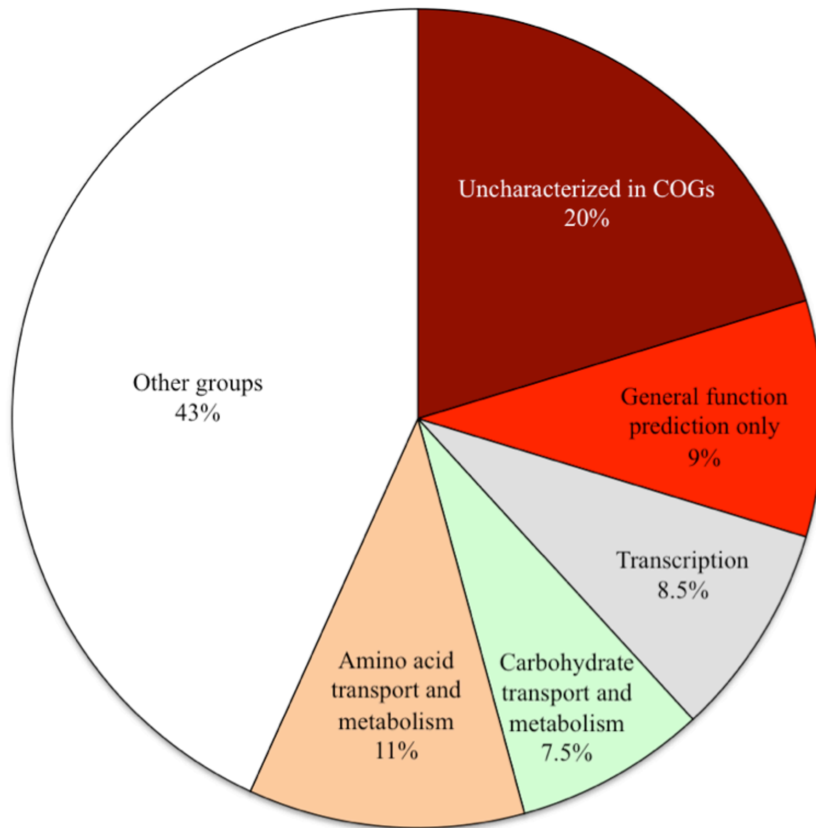

Figure 2. Major COGs classifications for primary screen candidates.

Figure 3. Representative motility plates with *ΔSTM1055*, *ΔSTM2151*, and *ΔSTM3792*

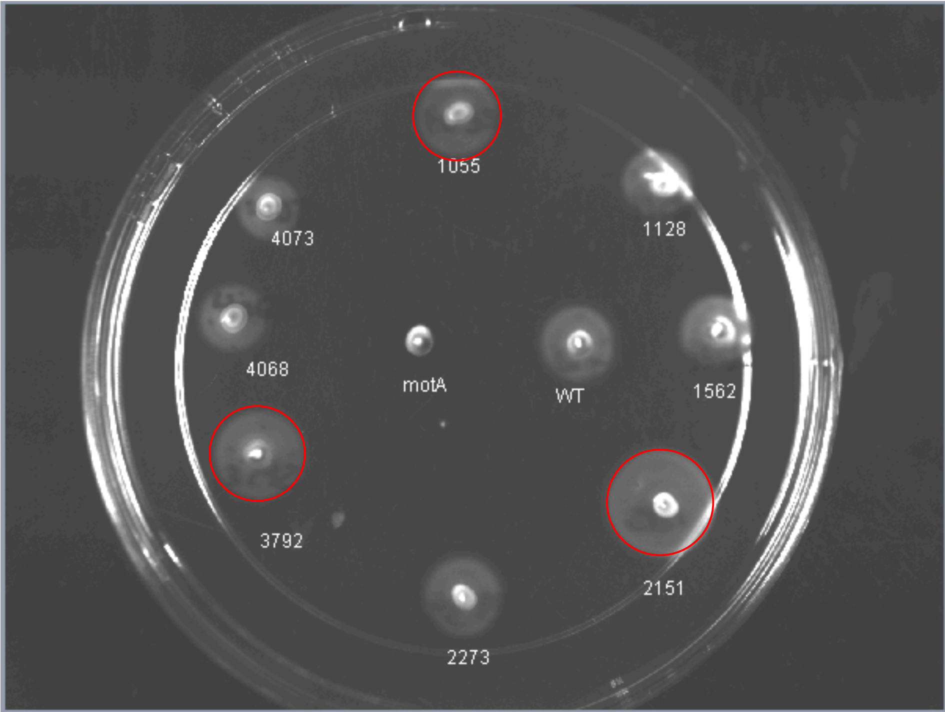

Figure 3a. The motility halos of *ΔSTM1055*, *ΔSTM2151*, and *ΔSTM3792* are larger than WT.

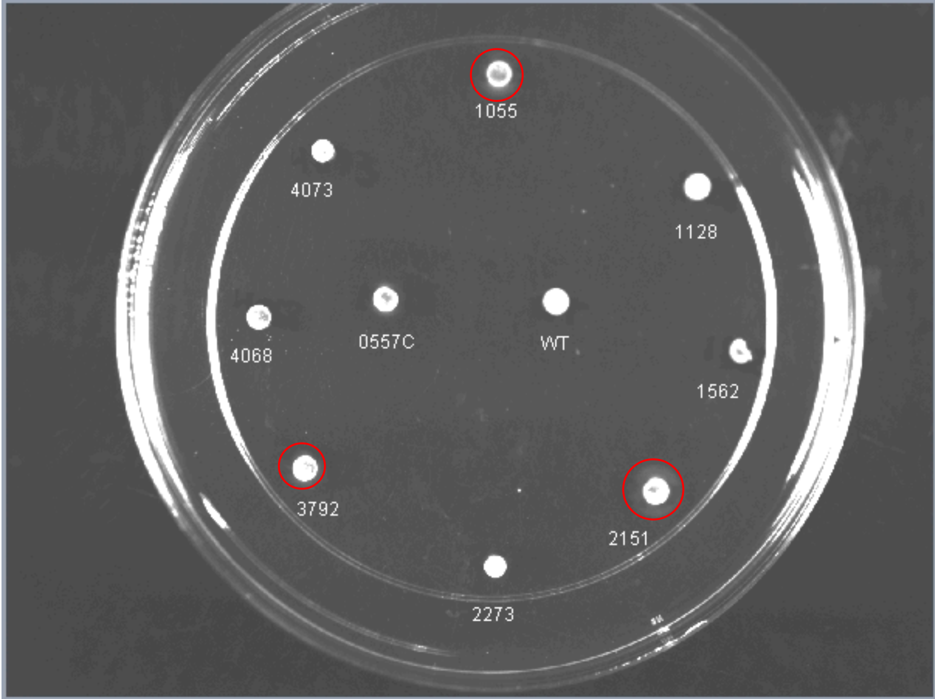

Figure 3b. *ΔSTM1055*, *ΔSTM2151*, and *ΔSTM3792* are producing motility halos in SPI-2-inducing media, while WT is non-motile.

Figure 4. Representative motility plates with *ΔSTM1697*

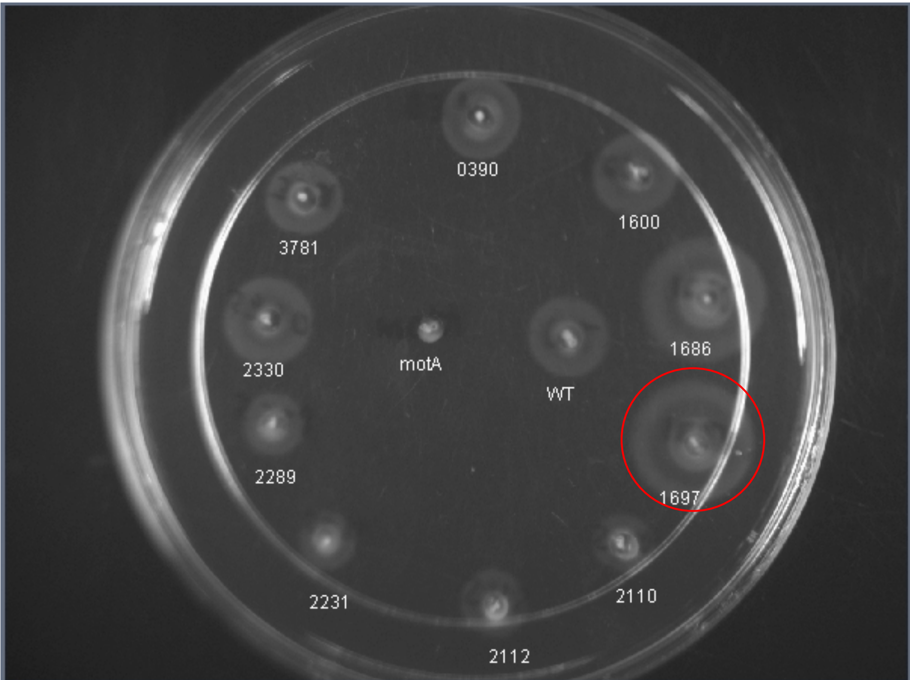

Figure 4a. The motility halo of *ΔSTM1697* is larger than WT.

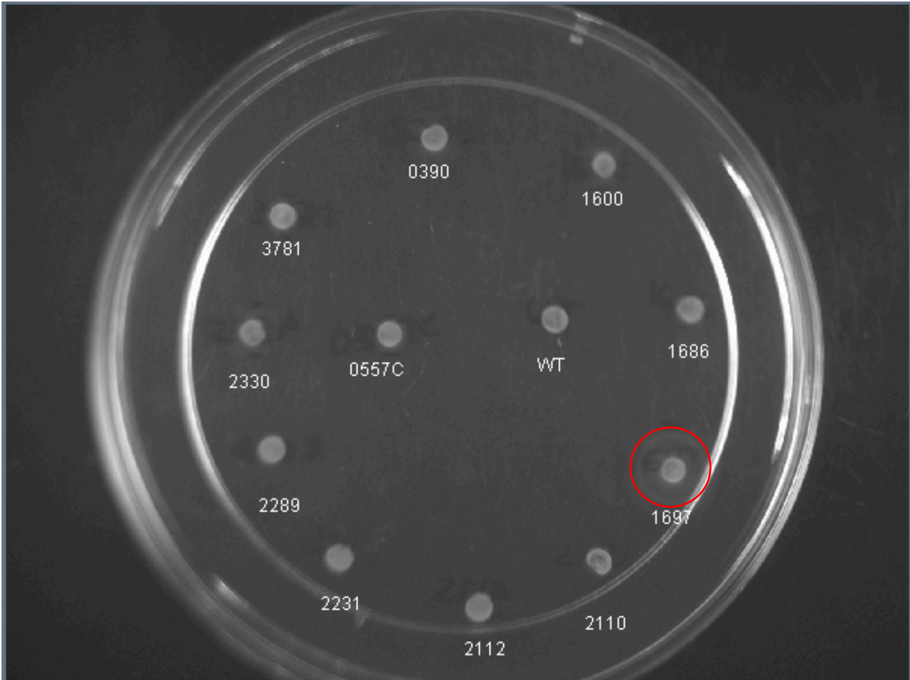

Figure 4b. *ΔSTM1697* is producing a large motility halo in SPI-2 inducing media, while WT is non-motile.

Figure 5. Western blot analysis identifies mutants expressing flagellins under LB conditions

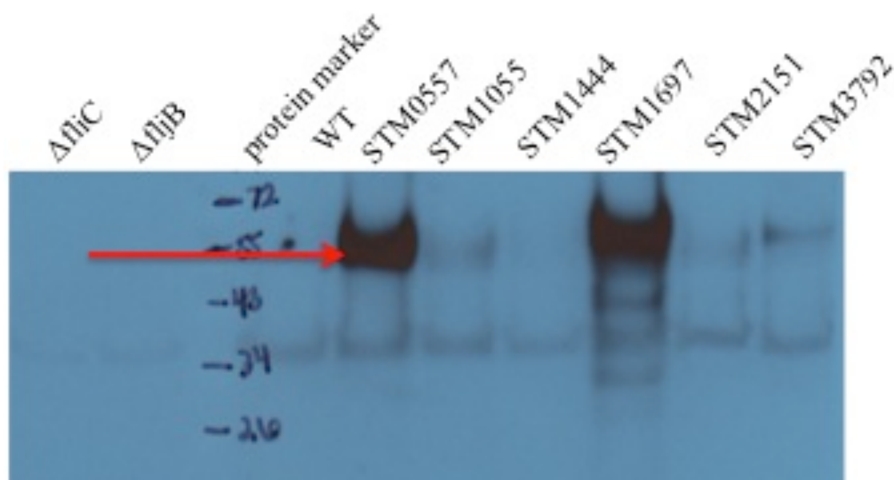

Figure 5a. Samples prepared in LB and probed with anti-FljB flagellin antibodies. Flagellin expression is shown around 55kDa. Strong expression is seen in STM0557 and STM1697. Weak expression is seen in *ΔSTM1055*, *ΔSTM2151*, and *ΔSTM3792*. *ΔfljB* was included as antibody control.

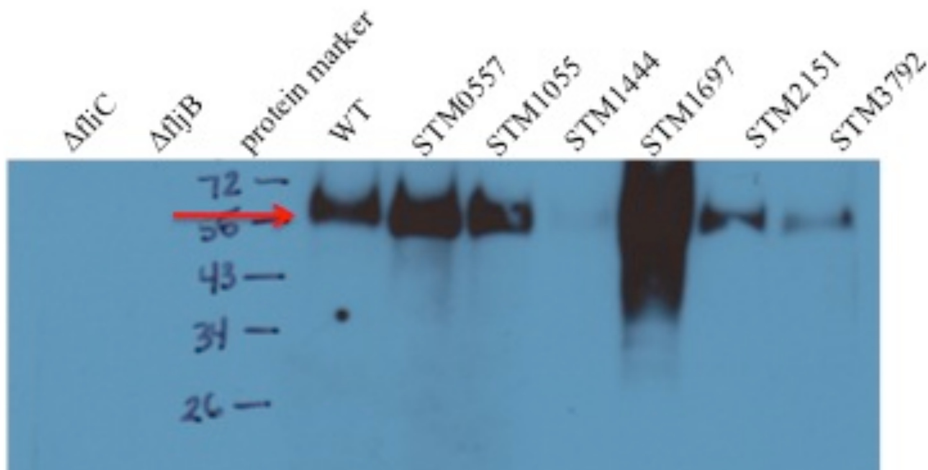

Figure 5b. Samples prepared in LB and probed with anti-FliC flagellin antibodies. Flagellin expression is shown around 55kDa. Strong expression is seen in WT, *ΔSTM0557*, *ΔSTM1055*, *ΔSTM1697*, *ΔSTM2151*, and *ΔSTM3792*. A weak expression is seen in *ΔSTM1444*. *ΔfliC* was included as antibody control.

Figure 6. Western blot analysis identifies mutants expressing flagellins under SPI-2 inducing conditions

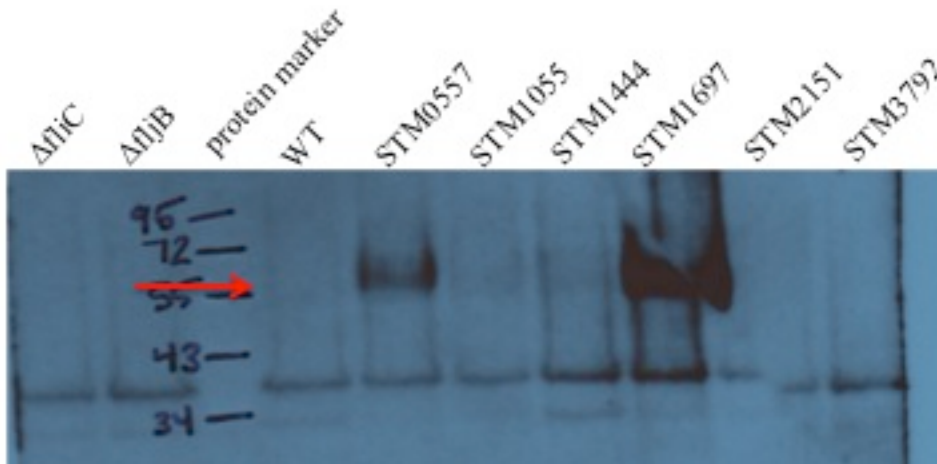

Figure 6a. Samples prepared in SPI-2 and probed with anti-FljB flagellin antibodies. Flagellin expression is shown around 55kDa. Strong expression is seen in *ΔSTM0557* and *ΔSTM1697*. Weak expression is seen in *ΔSTM1055* and *ΔSTM1444*. No expression is shown in WT. *ΔfljB* was included as antibody control.

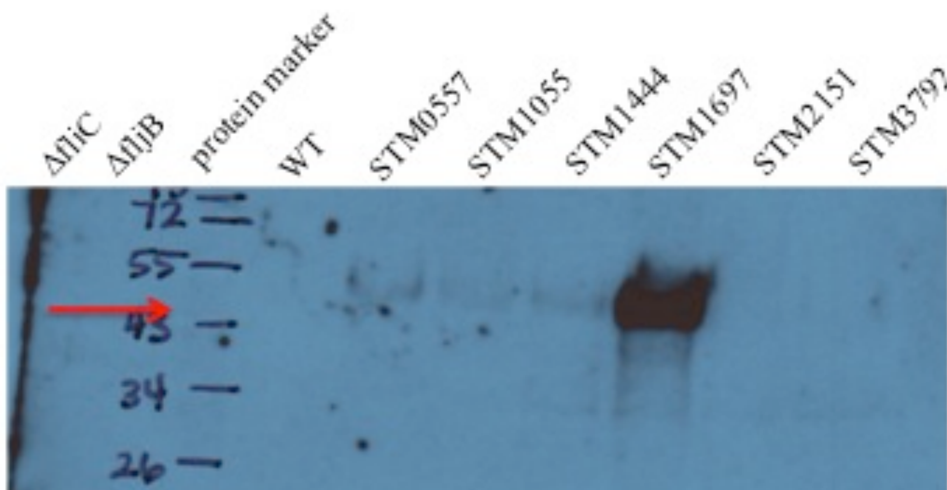

Figure 6b. Samples prepared in SPI-2 and probed with anti-FliC flagellin antibodies. Flagellin expression is shown around 55kDa. Strong expression is seen in STM1697. Weak expression is seen in *ΔSTM0557*, *ΔSTM1055*, and *ΔSTM1444*. No expression is shown in WT. Δ*fliC* was included as antibody control.

Figure 7. Western blot verifies SPI-2 activation for most mutants

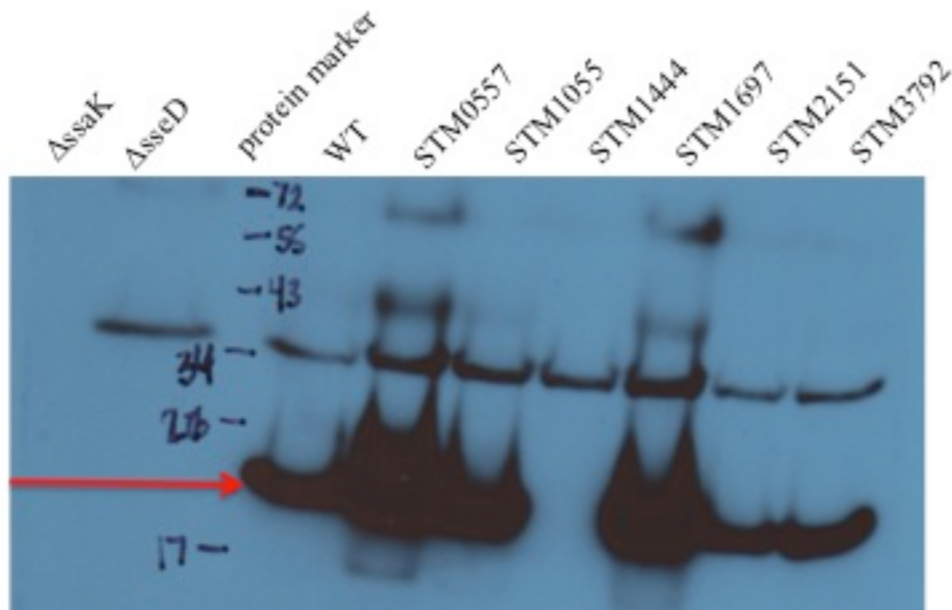

Figure 7. Supernatant of samples prepared in SPI-2 media and probed with anti-*sseD* antibodies. SseD expression is shown around 22 kDa. Significant SseD expression, confirming SPI-2 activation, is found in all samples except antibody control, *ΔsseD*, as well as *ΔssaK* (cannot make SPI-2 machinery) and *ΔSTM1444* (cannot activate SPI-2).

TABLES

Table 1. Candidate mutants tested by secondary screening

Strain	Gene	LB %WT[a]	LB SD %WT[b]	LB T-Test[c]	SPI-2 %WT	SPI-2 SD %WT	SPI-2 T-Test
STM1444[d,e]	*slyA*	111.74	18.89	0.683	262.90	33.67	0.018
STM1697		142.48	8.52	0.050	231.00	121.71	0.208
STM0390	*aroM*	96.88	8.67	0.779	222.68	86.45	0.150
STM2151	*stcB*	114.93	27.61	0.597	208.94	30.15	0.015
STM1055		124.98	14.95	0.228	180.33	12.21	0.013
STM1600		99.42	14.51	0.967	176.90	37.04	0.092
STM3792		116.89	14.14	0.347	170.30	29.35	0.067
STM2330	*lrhA*	106.79	4.71	0.122	168.25	28.44	0.060
STM2907	*pphB*	142.81	13.45	0.081	157.85	25.85	0.048
STM3806		97.69	15.37	0.922	155.13	89.36	0.402
STM0549	*fimZ*	103.02	11.91	0.859	148.44	13.73	0.035
STM1660	*fnr*	114.36	15.04	0.552	142.95	50.84	0.303
STM1686	*pspE*	137.76	4.57	0.024	139.54	28.51	0.190
csrB		105.35	20.03	0.842	133.00	45.77	0.387
STM0557		147.94	12.46	0.017	124.38	21.80	0.252
STM1947	*uvrY*	86.70	8.34	0.544	122.07	40.88	0.468
STM2110	*wcaF*	78.29	2.98	0.031	121.11	29.12	0.360
STM1260		90.93	5.40	0.540	121.10	6.33	0.048
STM3781		84.23	3.83	0.053	118.89	26.20	0.369
STM2273		101.42	5.99	0.902	118.78	30.67	0.447
STM4068		106.66	10.34	0.678	117.33	31.77	0.484
STM1391	*ssrB*	85.37	10.04	0.479	106.17	5.83	0.480
STM2112	*wcaD*	85.08	3.92	0.074	105.05	1.03	0.372
STM3124		84.31	11.04	0.516	104.80	4.09	0.281
STM1547		75.93	14.34	0.396	103.78	3.75	0.563
STM2796	*yqaE*	95.32	10.38	0.826	103.44	4.58	0.428
STM4073	*ydeW*	102.58	10.26	0.836	103.36	15.36	0.795
STM3648	*yiaG*	87.17	8.74	0.562	103.22	9.58	0.679
STM1128		102.03	11.76	0.879	103.06	8.56	0.788
STM0548	*fimF*	98.73	5.23	0.931	102.28	7.02	0.708
STM2289		79.45	2.37	0.057	101.91	2.04	0.739
STM1816	*minE*	93.92	2.11	0.717	101.90	4.50	0.447
ryeB		109.38	26.77	0.761	101.22	3.47	0.852
STM0764		85.97	10.47	0.548	101.10	4.43	0.790
STM3860		104.32	4.77	0.719	101.02	1.07	0.787

STM4506		84.27	5.96	0.256	101.00	2.65	0.862
STM0304	*sinR*	98.67	3.49	0.925	100.91	5.09	0.322
STM1159	*yceO*	96.72	11.07	0.881	100.77	5.73	0.876
STM1562	*hdeB*	105.03	21.47	0.519	100.11	5.23	0.987
STM4318		93.14	9.30	0.628	100.05	4.00	0.981
STM4307		88.88	14.83	0.645	99.89	4.80	0.974
STM4547	*yjjQ*	83.52	3.89	0.179	98.51	2.15	0.621
STM2231		87.12	7.15	0.079	98.25	6.77	0.814
STM2395	*pgtE*	93.89	13.36	0.803	97.95	3.56	0.755
STM0654	*ybeQ*	87.03	4.54	0.300	97.90	2.71	0.710
STM0301	*safC*	91.79	4.84	0.597	97.62	3.37	0.111
STM2509	*nupC*	83.74	6.40	0.241	96.56	9.64	0.721
STM2912		95.44	5.41	0.754	95.79	0.71	0.305
STM3191		84.96	8.66	0.505	95.64	5.59	0.406
STM4505		87.31	4.28	0.302	95.56	5.09	0.542
STM3771		84.27	13.30	0.530	95.36	1.14	0.056
STM4308		92.98	8.01	0.596	95.00	4.41	0.498
STM2614		85.62	9.37	0.529	94.68	9.16	0.472
STM3770		88.75	6.95	0.402	94.68	6.59	0.544
STM4395	*yifZ*	83.60	4.35	0.063	93.45	7.08	0.441
STM2613		86.11	6.05	0.306	92.07	7.22	0.411

[a]Motility halo size is reported as a percentage of the WT sample incubated on the same motility plate as the mutant.
[b]Standard deviation across motility halo diameters measured in triplicate
[c]Student's T-Test was used to identify motility halos of statistically significant diameter compared to WT. $p<0.05$
[d]Strains identified in bold show a statistically significant motility halo in either LB or SPI-2 conditions
[e]Strains highlighted in yellow were selected for Western blot analysis. Strains are listed in order of largest %WT motility halo in SPI-2-inducing conditions to smallest.