SsrB regulates genes outside SPI-2 to affect *Salmonella* Typhimurium lifestyle

Thesis submitted to

Indian Institute of Science Education and Research Pune

In partial fulfilment of the requirements of

the BS-MS Dual Degree Programme

by

Snehal Girish Kadam

Student ID: 20131078

Under the guidance of

Prof. Linda Kenney

Mechanobiology Institute, National University of Singapore

Certificate

This is to certify that this dissertation entitled “SsrB regulates genes outside SPI-2 to affect *Salmonella* lifestyle” towards the partial fulfilment of the BS-MS dual degree programme at the Indian Institute of Science Education and Research, Pune represents study/work carried out by Snehal Girish Kadam at the Mechanobiology Institute, Singapore under the supervision of “Prof. Linda Kenney, Mechanobiology Institute during the academic year 2017-2018.

Prof. Linda Kenney

Mechanobiology Institute

National University of Singpaore

Snehal Girish Kadam

BS-MS program

IISER Pune

Declaration

I hereby declare that the matter embodied in the report entitled “SsrB regulates genes outside SPI-2 to affect *Salmonella* lifestyle” are the results of the work carried out by me at the Mechanobiology Institute, under the supervision of Prof. Linda Kenney and the same has not been submitted elsewhere for any other degree.

Prof. Linda Kenney

Mechanobiology Institute

National University of Singpaore

Snehal Girish Kadam

BS-MS program

IISER Pune

Contents

Abstract

Salmonella enterica serovar Typhimurium is a gram-negative intracellular pathogen known to cause gastro-intestinal infections. The Salmonella Pathogenicity Islands (SPI) are key to its pathogenesis. SsrB, encoded on SPI-2, acts as a regulatory switch between the intracellular disease-causing state and the extracellular biofilm forming state of *Salmonella* Typhimurium. Studying the SsrB regulon can provide insights into the role of a horizontally acquired regulator in modulating gene expression of the native chromosome and in turn regulation key aspects of pathogenesis. Transcriptome data from two day old *Salmonella* Typhimurium biofilms of an *ssrB* null strain identified a repertoire of genes that were differentially expressed. A subgroup which were downregulated included Hfq (an RNA chaperone), *dsrA* (a sRNA) and DksA (a stringent response regulator). To establish how SsrB regulates these genes, binding of SsrB to their regulatory regions was examined by electrophoretic mobility assays. SsrB was found to bind all three regulatory regions. DksA and *dsrA* are known to be important for the Acid Tolerance Response (ATR) in *Salmonella* Typhimurium. When we examined the ATR of the *ssrB* null strain, we found it to have a reduced survival in pH 3.3 as compared to the wild-type. In order to identify the mechanism of SsrB-mediated regulation of ATR, *dsrA* and *dksA* null strains were generated. However, due to high variations within experiments, further work is necessary to investigate this mechanism.

List of Figures

List of Tables

Acknowledgment

I would like to thank my supervisor, Prof. Linda Kenney for giving me the opportunity to carry out this work in her research group and for her support and guidance throughout the project. I am also grateful to Dr. Stuti Desai for guiding me through various steps in this study and in the lab. I thank Dr. Nishad Matange for his insights and advice through this project. I would also like to thank my lab members for their stimulating discussions and for providing a conducive work environment.

Introduction

Salmonellae are a leading cause for almost 25% of the cases of diarrhoeal diseases and 21 million typhoid-related cases reported in 2014 worldwide. *Salmonella enterica* serovar Typhimurium is a gram negative facultative pathogen known to infect rodents, chicken, livestock and humans (Rabsch et al., 2002). Its main mode of transmission is through contaminated food and water and leads to gastrointestinal diseases.

Salmonella pathogenesis is attributed to the presence of horizontally acquired pathogenicity islands in its genome (Hensel, 2004). These *Salmonella* Pathogenicity Islands (SPIs) encode numerous virulence genes that enable *Salmonella* to be a successful pathogen. Numerous studies on SPI-1 and SPI-2 have increased our understanding of these pathogenicity islands and the role they play in the lifestyle of the bacteria. Both encode Type Three Secretion Systems (T3SS), two component regulatory systems and effectors that are secreted into the host. SPI-1 is primarily required for the invasion of host intestinal epithelial cells (Mills et al., 2006) whereas SPI-2 enables the bacteria to replicate inside host cells (Cirillo et al., 1998; Hensel et al., 2000).

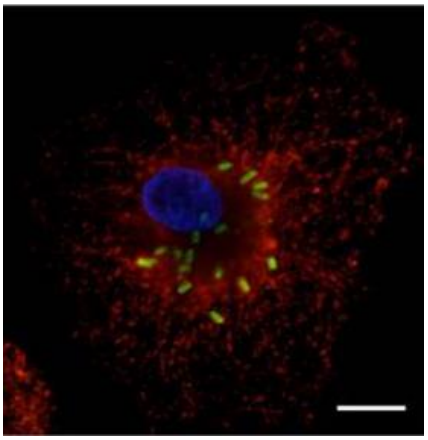
The Two Lifestyles of *Salmonella*

Like many pathogens, *Salmonella* too can survive within its host in two different forms – an infectious disease causing state or a carrier state (Figure 1). In its infectious state, the bacteria enter the gut and reside within intestinal epithelial cells or macrophages (Figure 1A). Once inside the host cells, the bacteria can go to deeper tissues and lead to inflammation. In systemic infections, bacteria are not restricted to localized tissues and can spread to other organs via the lymphoid tissues (Mastroeni and Grant, 2011). While the bacteria are present inside host cells, they are enclosed in a membrane bound vesicle called the *Salmonella* Containing Vacoule (SCV) (Dandekar et al., 2012). This unique vacuole enables the bacteria to modify various host cell properties and thereby, successfully proliferate inside the host.

However, they can also exist in a non-disease state in the form of biofilms on gallstones (Steenackers et al., 2012) (Figure 1B). This enables chronic carriage of the bacteria for longer periods of time, effectively turning the host into a carrier of these pathogens, which can later be transmitted to others or cause disease within the same host. Biofilm formation is not only advantageous from the perspective of transmission,

but has also been shown to contribute to an increased resistance to ciprofloxacin, a common antibiotic used to treat *Salmonella* infections (González et al., 2018).

A



B

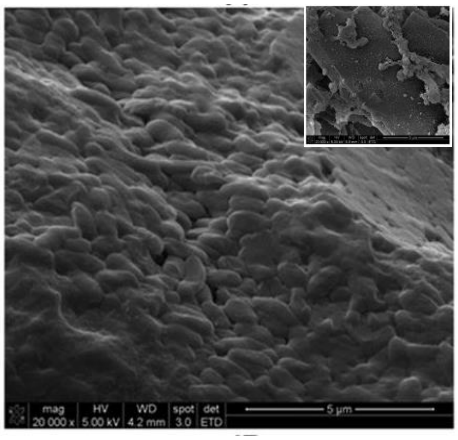


Figure 1. Dual lifestyles of *Salmonella*. A: Immunofluorescence microscopy of *Salmonella typhimuirum* (green) in human macrophage (Lathrop et al., 2015) depicting the intracellular lifestyle. B: Scanning Electron Micrograph of *Salmonella typhimuirum* biofilm on mouse gallstones 7 days post infection (inset: uninfected gallstone) (Gonzalez-Escobedo and Gunn, 2013).

SsrB acts as a lifestyle switch

SsrB, encoded on the SPI-2 pathogenicity island, is part of a two component regulatory system *ssrA/B*. SsrA is the membrane sensor kinase and SsrB is the response regulator. SsrB acts as a regulatory switch for these two different lifestyles for *Salmonella* Typhimurium (Desai et al., 2016). In its phosphorylated form, SsrB regulates the infectious lifestyle of *Salmonella.* Depending on environmental signals like acidic pH SsrA undergoes intramolecular phosphorylation and transfers the phosphate to SsrB. Phosphorylated SsrB transcriptionally activates SPI-2 genes, leading to the assembly of the type III secretion system and various effectors secreted into the host (Figure 2A). This enables the bacteria to survive and replicate intracellularly. In its unphosphorylated form, SsrB activates the expression of *csgD*, the master regulator of biofilm genes, by relieving H-NS (Histone-like Nucleoid Structuring protein) silencing (Figure 2B). This leads to a carrier state in the host by formation of biofilms.

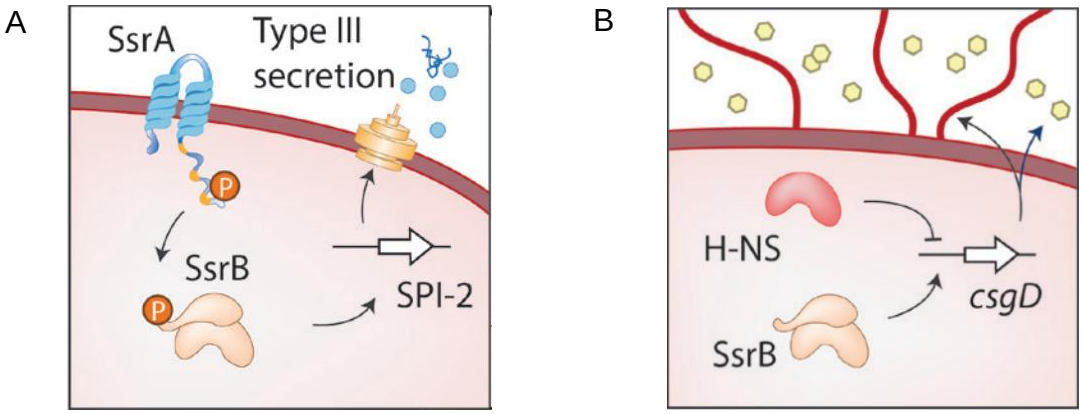


Figure 2. SsrB - a lifestyle switch for *Salmonella typhimurium* (Desai et al., 2016). A: Based on environmental conditions, SsrA, a membrane kinase, phosphorylates SsrB, which in turn activates SPI-2 gene expression. This enables the bacteria to survive and replicate within the host by secretion of effectors into the host via the type III secretion system. B: In its unphosphorylated form, SsrB activates expression of *csgD*, leading to biofilm formation.

This dual lifestyle revealed a unique role for SsrB. Traditionally SsrB has been thought to regulate genes within SPI-2, leading to virulence. However, this study showed the ability of SsrB, a horizontally acquired regulator, to activate the expression of a gene, *csgD*, lying outside its own pathogenicity island, influencing an important aspect of *Salmonella typhimuirum* infection (reviewed by Desai and Kenney, 2017).

This led to the hypothesis that the SsrB regulon could indeed include more such candidates lying outside its own pathogenicity island. In order to address this question, a whole genome transcriptome analysis of *ssrB* null biofilms was carried out (Stuti Desai, unpublished data). This revealed a repertoire of genes that were differentially regulated in the *ssrB* null strain as compared to the wildtype 14028s.

A key group of genes that were downregulated in this included sRNAs like *dsrA* and *rprA*, the stringent response regulator, *dksA* and an RNA chaperone *hfq*.

dsrA and *dksA* in Acid Tolerance Response:

Salmonella encounter numerous stressful environments within their hosts. One of the main defence mechanisms of the host it faces is the acidic environment in the gut or the acidification of the vacuole within host cells. The ability to survive within these acid conditions, termed the Acid Tolerance Response (ATR) (Foster and Hall, 1990), enables *Salmonella* to be a successful invader of the gut. The gut pH can reach values as low as 1.5 in the stomach and 4.0 in the small intestine (Watson et al., 1972). The

SCV luminal pH has also shown to reach pH < 4.5 upon acidification (Drecktrah et al., 2006; Rathman et al., 1996). ATR is defined as the resistance acquired to low pH after exposure to a mild acid pH (termed adaptation). The small RNA *dsrA* and the stringent

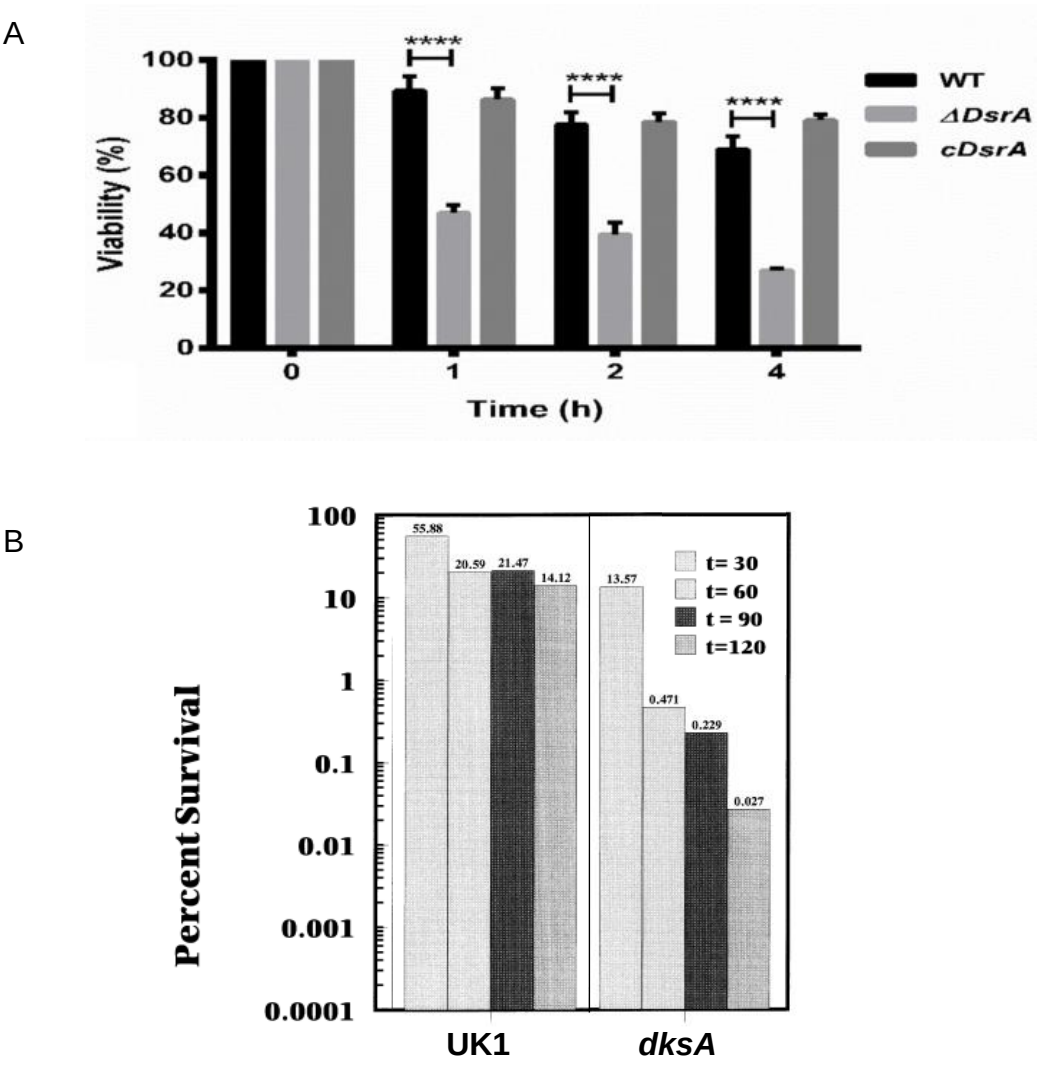


Figure 3. Acid Tolerance Assays for A. Δ*dsrA* (Ryan et al., 2016) and B. Δ*dksA* (Webb et al., 1999) show a reduced survival (post adaptation) for the mutants as compared to the wildtype strains upon acid challenge at pH 3.3 indicating an important role for these genes in acid survival.

response regulator DksA have been shown to be important for the ATR in *Salmonella typhimuirum* (Ryan et al., 2016; Webb et al., 1999). Both Δ*dsrA* and Δ*dksA* show reduced survival during acid challenge (Figure 3) in minimal E media of pH 3.1 (after adaptation at pH 4.4 for 1 hour in Figure 3A and for 30 minutes in Figure 3B).

dsrA: a regulator of *rpoS* and *hns*

sRNAs play an important role in modulating gene expression by interacting with mRNAs to either open up secondary structures, block various sites required for translation or target mRNAs for degradation . One such small RNA, *dsrA*, has been shown to regulate levels of *rpoS* and *hns* mRNAs in *E.coli* (Lease et al., 1998; Majdalani et al., 1998, 2001). It regulates *rpoS* by interacting with the mRNA to open up its secondary structure and enable translation. By binding immediately downstream of the start codon of the *hns* mRNA and recruiting the RNA degradation complex, *dsrA* represses H-NS expression. These two targets of *dsrA* are global regulators themselves, with RpoS being a stress sigma factor and H-NS being a global transcriptional silencer.

Hfq: a global regulator of sRNAs

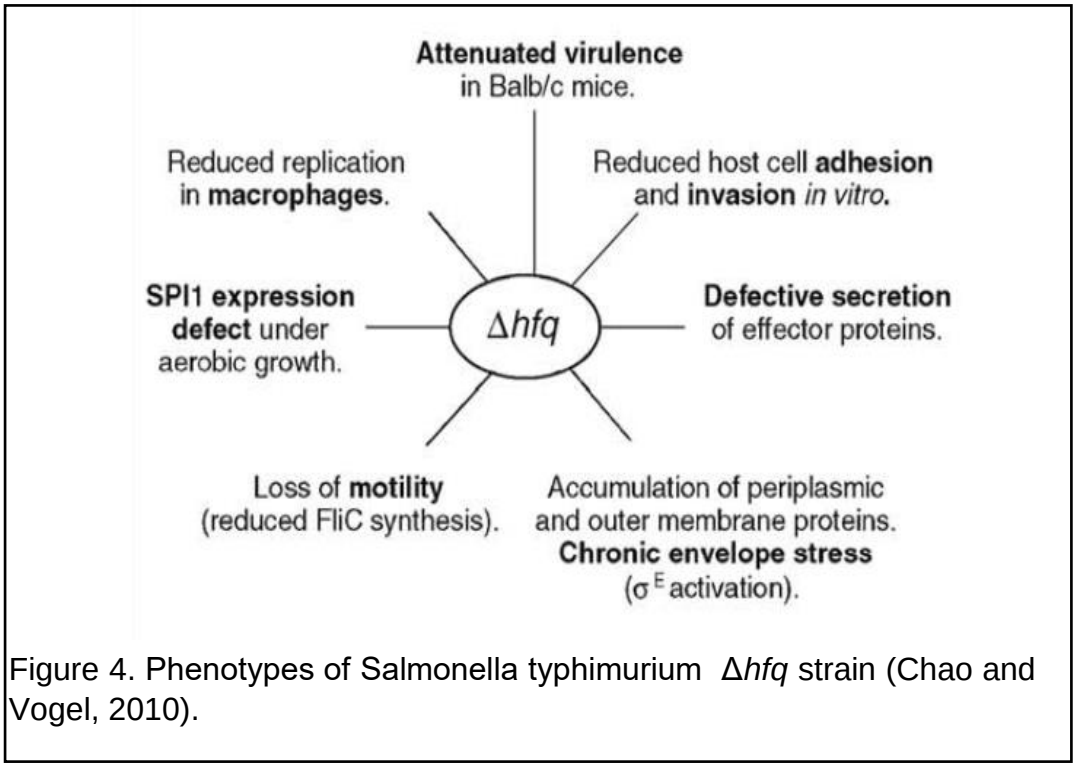


Figure 4. Phenotypes of Salmonella typhimurium Δ*hfq* strain (Chao and Vogel, 2010).

Hfq, a hexameric protein, plays a central role in numerous sRNA pathways. It has domains for binding sRNAs and mRNAs as well as a domain for interaction with other proteins involved in RNA decay (Brennan and Link, 2007). It functions as an RNA chaperone, assisting the binding of sRNAs to their target mRNAs facilitating post-transcriptional regulation. Hfq has emerged as a key player in virulence in bacterial pathogens (Chao and Vogel, 2010). In *Salmonella*, Hfq has been shown to be important for motility, invasion and intracellular growth (Sittka et al., 2007) (Figure 4).

Previous studies combining co-immunoprecipitation with RNA sequencing identified a dynamic change in Hfq-associated sRNAs in various growth conditions (Chao et al., 2012) and 63 of *Salmonella typhimuirum* sRNAs have been shown to be Hfq-dependent (Colgan et al., 2016). Hfq also plays an important role in biofilm formation via its regulation of *csgD* (Monteiro et al., 2012). Thus, Hfq is also a central regulatory player in *Salmonella*, leading to various physiological implications for the pathogen.

DksA: a stringent response regulator

DksA controls transcription by directly binding to RNA polymerase (Lennon et al., 2012), enabling it to affect the expression of a repertoire of genes. DksA regulates expression of a large number of genes including stress response genes leading to amino acid synthesis, repression of rRNA promoters (Paul et al., 2004), motility and *rpoS* regulation. It has also been shown to regulate genes required for virulence, not only in Salmonella pathogenesis (Azriel et al., 2016; Webb et al., 1999), but also various other bacterial pathogens (Jude et al., 2003; Mogull et al., 2001; Nakanishi et al., 2006; Yun et al., 2008). In *Salmonella*, it enhances survival under oxidative and nitrogen stress (Crawford et al., 2016; Henard and Vázquez-Torres, 2012), two key defence strategies adopted by host cells, thus contributing to *Salmonella* pathogenesis.

SsrB: A Master of Masters?

The 3 genes identified, *dksA, dsrA* and *hfq,* are central regulators and have many targets that play an important role in a repertoire of functions including virulence, biofilm formation, acid and nitrogen stress resistance, etc. Thus, we hypothesize a role of SsrB as a master regulator of these targets. It is also interesting that these 3 genes lie outside SPI-2, the pathogenicity island SsrB belongs to. Being a horizontally acquired regulator, we now see evidence of its adaptation to regulate genes within the native chromosome and thus play a truly central role in the lifestyle of the pathogen.

In this study, we addressed the question of SsrB directly binding these targets. We used the Acid Tolerance Response (ATR) assay as a phenotypic readout and showed that the *ssrB* null strain was defective in this response. We also construct *dksA* and *dsrA* null strains to further characterize this mechanism. However, due to high

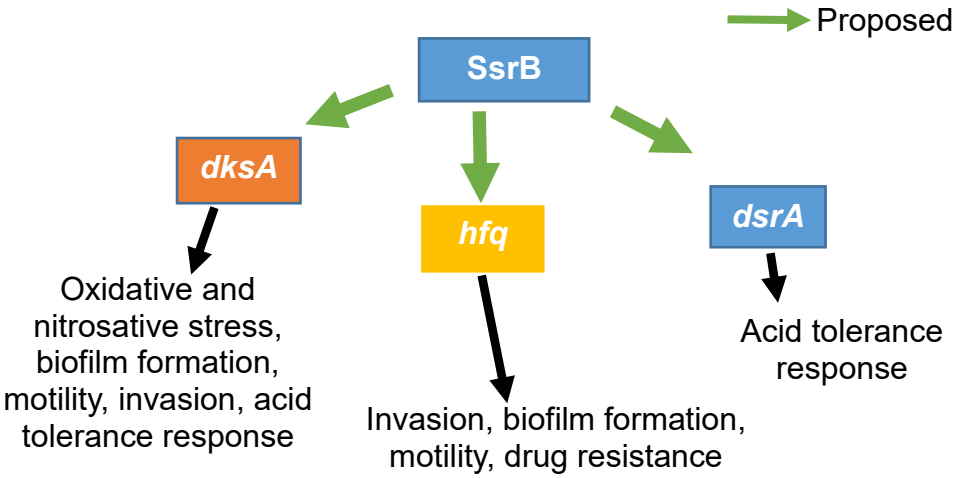


Figure 5. SsrB, a master regulator - Proposed genetic network for SsrB mediated regulation of *dksA*, *dsrA* and *hfq*.

variations within different experiments, further work is required to validate this regulatory circuit and its implications.

Materials and Methods

Bacterial strains and culture conditions: *Salmonella typhimurium* 14028s was used as the wild type strain. Strains used in this study are listed in Table 1. All bacterial cultures were grown in LB medium with 250 rpm shaking at 37°C in the presence of 100 µg/mL ampicillin (Amp^R), 25 µg/mL chloramphenicol (Cm^R) or 30 µg/mL kanamycin (Km^R) when necessary. For Acid Tolerance Assays (ATR), Vogel-Bonner Minimal E media supplemented with 0.5% glucose was used (Vogel and Bonner, 1956). For preparation of plates to isolate biofilms (*rdar* morphotype), LB-Agar medium containing 1% tryptone, 0.5% yeast extract and no salts was used. All media and buffer compositions used are described in Table 2.

Table 1. Bacterial Strains and Plasmids		
Strain/Plasmid	Description	Source

pKD4	Plasmid containing frt-flanked kanamycin cassette	(Datsenko and Wanner, 2000)
pKD3	Plasmid containing frt-flanked chloramphenicol cassette	
pKD46	Plasmid carrying λ red genes under arabinose inducible promoter and ampicillin resistance, temperature sensitive (grown at 30°C for plasmid maintenance)	
pCP20	Plasmid encoding FLP recombinase and and ampicillin resistance, temperature sensitive (grown at 30°C for plasmid maintenance)	
pET-Duet1	Vector carrying two T7 promoters for cloning of two target genes and ampicillin resistance	Addgene
pET-*dksA*	*dksA* cloned downstream of the T7 promoter in the pET-Duet1 vector backbone	This work
pET-*dsrA*	*dsrA* cloned downstream of the T7 promoter in the pET-Duet1 vector backbone	This work
Wild type	*Salmonella enterica* serovar Typhimurium 14028s	Lab Strain
ssrB::Km^R	*ssrB* replaced with Kanamycin in 14028s	Lab Strain
ssrB::Km^R pET-*dksA*	*ssrB*::Km^R harboring the *dksA* overexpression plasmid	This work
ssrB::Km^R pET-*dsrA*	*ssrB*::Km^R harboring the *dsrA* overexpression plasmid	This work

dksA::Cm^R	*dksA* replaced with Chloramphenicol in 14028s	This work
dksA::Cm^R pET-*dksA*	*dksA*::Cm^R harboring the *dksA* overexpression plasmid	This work
dsrA::Cm^R	*dsrA* replaced with Chloramphenicol in 14028s	This work
dsrA::Cm^R pET-dsrA	*dsrA*::Cm^R harboring the *dsrA* overexpression plasmid	This work
Δ*dsrA dksA*::Cm^R	*dksA* replaced with Chloramphenicol in Δ*dsrA*	This work

Table 2. Buffers and Media compositions

Buffer/Medium	Composition
Minimal E Media (50X) (autoclaved at 1X concentration)	670 mL milliQ H_2O 10 g $MgSO_4.7H_2O$ 100 g citric acid.H_2O 500 g K_2HPO_4 (anhydrous) 175 g $NaNH.HPO_4.4H_2O$
50X E Salts (for transducing broth)	100 mL milliQ H_2O 0.47 g $MgSO_4$ 10 g citric acid.H_2O 65.5 g K_2HPO_4. $3H_2O$ 17.5 g $NaNH_4HPO_4.H_2O$
Green Dye Stock (for Indicator plates in p22 transduction)	200 mL milliQ H_2O 1.3 g Aniline Blue 12.4 g Alizarin Yellow
Green Indicator Media	990 mL milliQ H_2O 8 g Bacto-Tryptone

	1 g Yeast Extract 5 g NaCl 15 g Agar 10 mL Green Dye Stock (After autoclaving, let media cool and add 16.8 mL of sterile 50% glucose and pour plates)

Table 3. Oligonucleotides used

Primer purpose	Sequence (5'-3')
dksA_ko_FP (forward, knockout)	CCTGATTTTCCCCCGAACATGGGGAT CGATAGTGCGTGTTGTGTAGGCTGGA GCTGCTTC
dksA_ko_RP (reverse, knockout)	GGGTAGAAACGAACGGGATTAACCC GCCATCTGTTTTTCGCATATGAATATC CTCCTTAG
dsrA_ko_FP (forward, knockout)	AAATATTTACTTGTCATGCAAAAAAAT TGCAGATAAGGTAGTGTAGGCTGGAG CTGCTTC
dsrA_ko_RP (reverse, knockout)	TGGCCGAAAAAAATCCCGGCCCTACG GGTCGGGATCAAACCATATGAATATC CTCCTTAG
dksA_flank_FP (forward, knockout confirmation)	CAT GGG GAT CGA TAG TGC G
dksA_flank_RP (reverse, knockout confirmation)	AAC GAA CGG GAT TAA CCC
dsrA_flank_FP (forward, knockout confirmation)	CCG AAC TAT TAG CGT GCT TAA
dsrA_flank_RP (reverse, knockout confirmation)	AAA TCC CGG CCC TAC
NcoI_dsrA (forward, dsrA cloning in pET-Duet vector)	TCA GTA CCA TGG AAT CTC ACA TCA GAT TTC CTG
dsrA_PstI (reverse, dsrA cloning in pET-Duet vector)	CTA ATA CTG CAG AAA AAA TCC CGG CCC TAC G

NcoI_dksA (forward, dksA cloning in pET-Duet vector)	TCA GTA CCA TGG AAG GAG AAG CAA CAT GCA AG
dksA_PstI (reverse, dksA cloning in pET-Duet vector)	GTT ACT CTG CAG TTA ACC CGC CAT CTG TTT TTC
dksA_gene_FP (forward, qRT-PCR)	CTT CAG CGT CTT CTC GAT C
dksA_gene_RP (reverse, qRT-PCR)	TGA CGC ACA TGC AGG AC
hfq_gene_FP (forward, qRT-PCR)	TGC AAG GTC AAA TCG AGT CC
hfq_gene_RP (reverse, qRT-PCR)	GGC ATT GTT GCT GTG ATG G
dsrA_gene_FP (forward, qRT-PCR)	AAT CCC GGC CCT ACG
dsrA_gene_RP (reverse, qRT-PCR)	AAT CTC ACA TCA GAT TTC CTG G
dksA_2_FPbio (forward, biotinylated, EMSA)	TGG AAT AAC AGC CTG ATT ATT AAG
dksA_2_RP (reverse, EMSA)	AAC ACG CAC TAT CGA TCC
hfq_FP_bio (forward, biotinylated, EMSA)	CGG GTA AAA CTT TAA CGG AGC
hfq_RP (reverse, EMSA)	TTC GCC CTC AAT GTA TGA CC
dsrA_FP_bio (forward, biotinylated, EMSA)	ACTATTAGCGTGCTTAATCATTC
dsrA_RP (reverse, EMSA)	CCA GGA AAT CTG ATG TGA G

Molecular Biology Techniques: All plasmid isolations, gel purifications, Polymerase Chain Reaction (PCR) purifications were carried out using reagents from Qiagen. Electroporation (Sambrook et al., 1989) was used for transformation of *Salmonella* strains. PCR for genetic knockouts, cloning and confirmation of constructs was carried out using standard protocols (Sambrook et al., 1989). All plasmids used are listed in Table 1. Various oligonucleotides used throughout the study are listed in Table 3. Gradient PCRs were used to find the optimal annealing temperatures of each primer pair.

Cloning of dksA and dsrA in overexpressing plasmids: The pET-Duet1 plasmid vector was used for cloning of the *dsrA* and *dksA* genes under the T7 promoter to generate two plasmids – pET-*dsrA* and pET-*dksA*. Primers with ~25 basepair homology to the respective gene and the restriction sites of NcoI (in the forward primer) and PstI (in the reverse primer) were designed. Using these primers, the respective gene was PCR

amplified and purified using gel elution (Qiagen Gel Extraction Kit). The pET-Duet-1 plasmid was isolated from DH5α cells. This plasmid and the two linear gene fragments of dksA and dsrA were digested in a 50 µL reaction with NcoI and PstI enzymes (Fermentas, Singapore) for 1 hour at 37°C. The dksA and dsrA reactions were purified using a PCR purification kit. The vector backbone was purified using gel elution. The backbone and each gene cassette was ligated using T4 DNA ligase (Thermo Scientific) and a ligation ratio of 1:10 in a 20 µL reaction. This was incubated at room temperature for 1 hour. The entire ligation reaction was the transformed into chemically competent DH5α cells.

Chemically Competent Cells and Transformation: An overnight culture of DH5α was grown from a single colony at 37°C. This was diluted 1:100 in 20 mL LB medium and allowed to grow at 37°C with shaking till OD (600nm) ~ 0.4 was reached. This culture was incubated on ice for 20 minutes. Cells were harvested by centrifugation at 4°C with 4000 rpm for 10 minutes. The cells were resuspended in 1 mL of 0.1M $CaCl_2$ and incubated on ice for 30 minutes. This was then centrifuged at 4°C with 4000 rpm for 10 minutes and the pellet was resuspended in a mixture of 175 µL 0.1M $CaCl_2$ and 75 µL of 50% glycerol. This was then split into 50 µL aliquots of chemically competent cells. Each ligation reaction was mixed with one aliquot of these cells and incubated on ice for 30 minutes. A no-DNA aliquot was also used as a control. These were then heat shocked at 42°C for 45 seconds and then incubated on ice for 5 minutes. 200 µL LB media was added and the samples were incubated at 37°C for 1 hour. The entire volume was then plated on ampicillin plates for selection.

Electroporation: The strain to be transformed was inoculated from a single colony in LB medium (with the respective antibiotic where appropriate) and incubated overnight at 37°C with shaking. This culture was then diluted 1:100 in 10mL LB medium and grown at 37°C with shaking to an OD (600nm) of ~ 0.6. The culture was kept on ice for 10 minutes. Cells were then pelleted by centrifugation at 5000 rpm for 6 minutes at 4°C.The cells were washed with 10mL ice-cold sterile milliQ water and centrifugation and washing was repeated thrice. After 3 washes, the cells were concentrated by resuspension in 200 µL ice-cold sterile milliQ water. Electroporation was carried out using 100 µL of cells and 150-200 ng of DNA. The cells were recovered in SOC medium for 30-45 minutes at 37°C with shaking and plated on chloramphenicol

selection media plates. For the pKD46 and pCP20 plasmids, a temperature of 30°C was used in all steps of electroporation.

Strain Construction: The *dsrA*::Cm^R and *dksA*::Cm^R strains were generated by replacing the respective genes with a chloramphenicol cassette flanked by FRT sites as described previously (Datsenko and Wanner, 2000). Briefly, primers were designed with ~40 bp homology to the gene of interest and 20 bp homology to the cassette for replacement. The chloramphenicol cassette was PCR amplified from the pKD3 plasmid and gel purified. A strain harboring the lambda red plasmid (pKD46) was grown overnight from a single colony in LB medium with ampicillin selection at 30°C with shaking. This overnight culture was diluted (1:100) in 10 mL LB media with 0.2% arabinose and ampicillin selection and grown till OD (600nm) ~ 0.6 at 30°C with shaking. The cells were washed thrice with ice-cold sterile milliQ water and resuspended as described above. Electroporation was carried out using 100 µL of cells and 800 ng of the chloramphenicol cassette PCR product. The cells were recovered in SOC medium for 5 hours at 30°C with shaking and plated on chloramphenicol selection media plates. The replacements were confirmed using colony PCR with primers flanking the respective gene region.

Eliminating Antibiotic Resistance Gene: In order to generate the Δ*dsrA dksA*::Cm^R double mutant, elimination of the chloramphenicol cassette from one of the single mutants was necessary. The *dsrA*::Cm^R mutant was transformed with pCP20, a plasmid expressing the FLP recombinase as well as the ampicillin resistance gene in a temperature sensitive manner. Transformants were selected on ampicillin plates after incubation at 30°C. These were streaked for single colonies in LB plates (no antibiotic selection) and incubated at 43°C overnight for the induction of the FLP system. Few colonies from this were tested for loss of antibiotic resistance by simultaneous streaking on chloramphenicol, ampicillin and LB plates. Only the clones that grew on LB plates, but not on chloramphenicol and ampicillin plates were selected to obtain the Δ*dsrA* strain. Using PCR, the loss of the chloramphenicol cassette was confirmed. The P22 lysate of the *dksA*::Cm^R single mutant was used to replace *dksA* in the Δ*dsrA* strain (after FLP recombination). This was confirmed using PCR with primers flanking the *dksA* region.

P22 Phage Transduction: In order to move all mutations and replacements to the wild type (14028s) background, P22 phage was used for transduction. The host strain (containing the mutation to be transferred) was grown overnight in LB at 37°C with shaking. Transducing broth was prepared using 4 mL LB, 100 µL of 50X E salts (described in Table 2) and 20 µL of 50% glucose. To this broth, 100 µL of the p22 phage and 1 mL of the overnight culture was added and incubated overnight at 37°C with shaking. The next day, 100 µL chloroform was added to overnight culture and was centrifuged at 10000 rpm for 20 minutes at 4°C. The supernatant was transferred to a sterile falcon and the chloroform addition and centrifugation was repeated thrice. After the final step, 100 µL chloroform was added prior to storage of this lysate at 4°C. The recipient cells (14028s) were grown overnight in LB. 100 µL of this overnight culture was incubated with 100 µL of the appropriate host strain lysate at 37°C for 60 minutes with shaking. A no-lysate control of recipient cells was also used. These were plated directly on selective media plates. Green indicator plates were prepared as described in Table 2. Transductants were picked early and streaked for single colonies on green indicator plates containing the appropriate antibiotic selection. This was incubated at 37°C overnight (or 10 hours). Light/pale yellow coloured colonies from this green plate were streaked onto a second green indicator plate with the same antibiotic selection. Single pale yellow colonies from this were streaked back onto LB plates supplemented with the appropriate antibiotic and grown overnight at 37°C. A single colony from this plate was used to confirm the knockouts generated.

RNA isolation and qRT-PCR: RNA was isolated from two day old biofilms of 14028s and *ssrB*::Km^R strains using the GeneAll RiboEx RNA kit. Isolated RNA was run on a bleach gel (Aranda et al., 2012) to check the RNA integrity. A two-step DNAse I treatment to digest genomic DNA was carried out for this RNA using Ambion Turbo DNAse enzyme as described in the kit protocol. From this DNAse digested RNA, cDNA preps were made using iScript Reverse Transcription Supermix from BioRad. A minus RT reaction (no reverse transcriptase added) was used as a control. A saturated PCR for 16S rRNA gene (housekeeping gene) was used to check genomic DNA contamination in the minus RT control. The cDNA prep was then used for qRT-PCR analysis of various target genes using the SsoFast Evagreen Supermix from BioRad. 16S rRNA was used as the reference gene.

Electrophoretic mobility assays (EMSA): Primers were designed for the regulatory regions to be tested. These primers were biotin-tagged at the 5'-end of the forward primer. The fragment of interest was PCR amplified and gel purified. This DNA fragment was incubated with purified full-length SsrB for 20 minutes at room temperature in a 20uL reaction using the components of the LightShift Chemiluminescent EMSA Kit (Thermo Scientific). This was followed by a 10 minute incubation on ice. A no-protein added sample was used as a control. All reactions were run at 100V in a 4% poly-acrylamide gel (acrylamide:bis ratio of 37.5:1) for 50 minutes. This was then transferred to a nylon membrane and processed for biotin label detection (LightShift Chemiluminescent EMSA Kit). The membrane was imaged using ChemiDoc Touch Imaging system (Bio-rad). For the competition assay, a non-biotinylated DNA fragment of the same sequence was used. A 200-fold higher amount of this fragment was added into the reaction mix simultaneously with the biotinylated DNA. A DNA-binding SsrB mutant, K179A, was also used as a control.

Acid Tolerance Assays (ATR): ATR assay was performed as previously described (Riesenberg-Wilmes et al., 1996). An overnight culture of the strains to be tested was inoculated from a single colony of each strain into 1X Minimal E glucose media (pH = 7.6 ± 0.1). The next day, this culture was diluted 1:100 in 1X Minimal E glucose media (pH = 7.6 ± 0.1). The cultures were allowed to grow to an O.D. (600nm) of ~ 0.2. The pH of the culture was then adjusted to 5.8 ± 0.1 using HCl. These cultures were then allowed to reach an O.D. of ~ 0.4. This was considered at time point zero (t = 0 hour). The pH of the culture was adjusted to 3.3 ± 0.1 using HCl. Colony Forming Unit (CFU) counts were determined by making the appropriate dilutions and plating on LB agar plates (supplemented with 100µg/mL ampicillin for the complemented strains) for 1 and 2 hours post acid challenge.

Results and Discussion

Loss of ssrB affects expression of a repertoire of genes: The RNA-sequencing data (Stuti Desai, unpublished data) of two day old *Salmonella typhimurium* biofilms of a *ssrB*::Km^R strain revealed a downregulation in expression of *hfq*, *dksA*, and *dsrA*. While *hfq* and *dksA* showed an approximate 2 fold decrease in expression in the *ssrB*::Km^R strain, *dsrA* showed a 25 fold decrease.

Table 4. Fold Change in expression of various genes identified using RNA-sequencing of two day old *Salmonella* biofilms

Gene	Fold Change in *ssrB*::Km^R vs WT (RNA-seq data)
hfq	2.0 ↓
dsrA	25 ↓
dksA	2.6 ↓

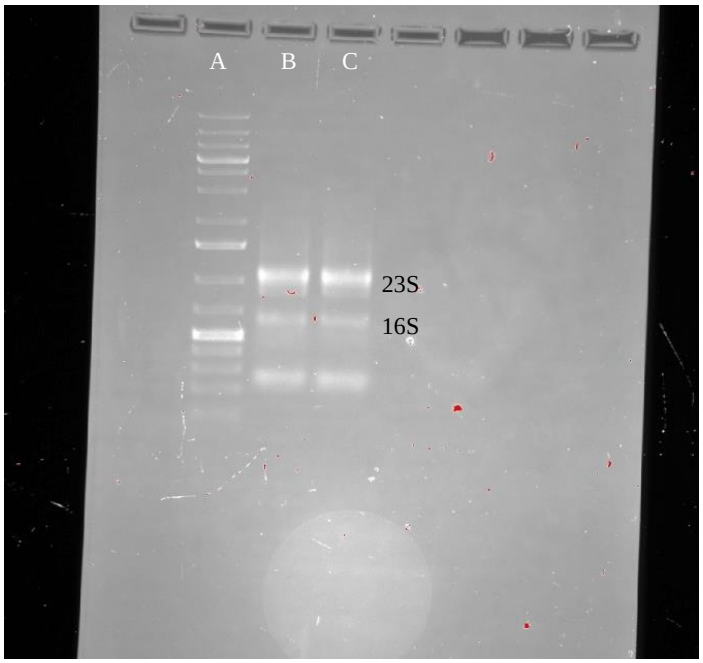


Figure 6. Bleach agarose gel showing the RNA isolated from WT (lane B) and *ssrB*::KmR (lane C). Lane A: 2 log DNA ladder

qRT-PCR validation for RNA-sequencing data: RNA isolated from 2 day old biofilms of WT and *ssrB::*Km^R strain grown of LB (no salt) plates at 30°C was run on a bleach

gel (Aranda et al., 2012) to check the RNA quality (Figure 6). The bleach gel showed the presence of both 23S and 16S rRNA bands and no smear, indicating that the RNA was not degraded.

To check genomic DNA contamination in the RNA after DNase I treatment, a saturated PCR for the *rrsA* gene (16S rRNA, reference gene) was run for the minus RT cDNA prep, which had no reverse transcriptase added. Initial results indicated the presence of genomic DNA (Figure 7). Briefly, the initial protocol followed used 10 µg of RNA for DNase treatment. The enzyme was added in 2 steps, with each step consisting of a 30 minute incubation at 37°C. The enzyme was inactivated by incubation at room temperature for 5 minutes after addition of the DNase inactivation reagent. The sample was then centrifuged to separate the DNase digested RNA.

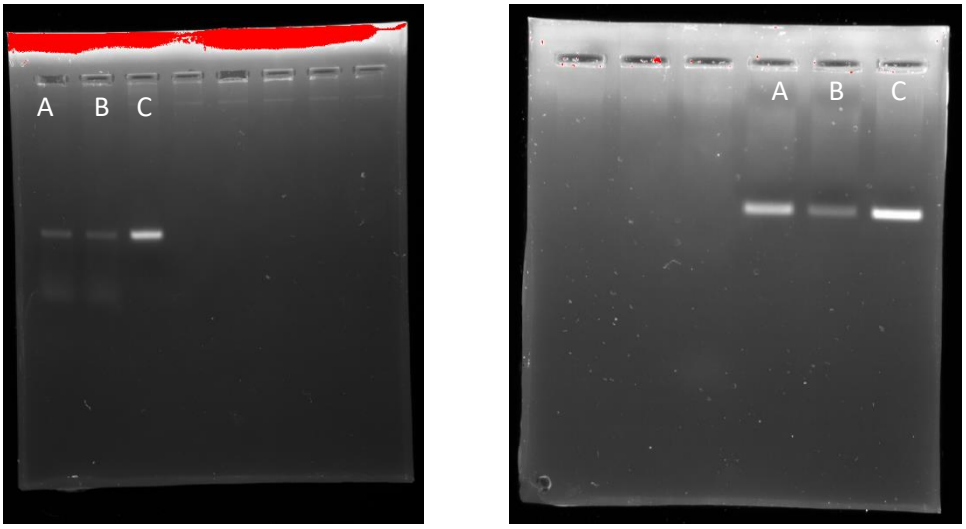


Figure 7. The presence of bands in minus RT control of WT (lane A) and *ssrB*::Km^R (lane B) indicates that the cDNA preps have genomic DNA contamination. Lane C shows the PCR product using an equivalent amount of genomic DNA as template.

To prevent any genomic DNA contamination in further cDNA preps, some aspects in the protocol were modified. Firstly, a lower amount of RNA was used in the DNase treatment (8µg). Prior to the addition of DNase inactivation reagent after DNase I digestion, the reaction volume was transferred to a fresh autoclaved tube. The absence of any band after PCR amplification of *rrsA* gene in minus RT control preps indicated that the cDNA preps were free of any genomic DNA contamination after these modifications (Figure 8).

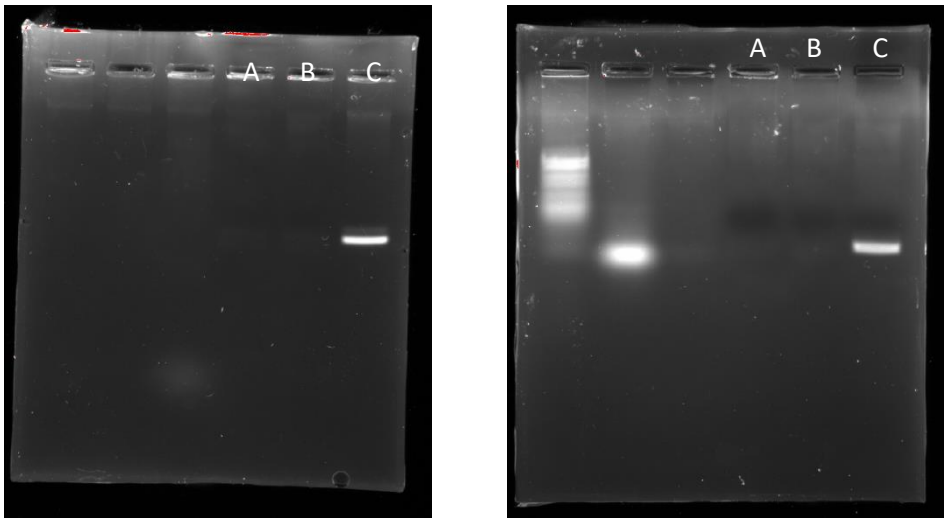


Figure 8. The absence of bands in minus RT control cDNA of WT (lane A) and *ssrB*::Km^R (lane B) indicates that the cDNA preps are free of any genomic DNA contamination. Lane C shows the PCR product using the equimolar amount of genomic DNA as template.

Using qRT-PCR, *dksA* expression in *ssrB*::Km^R was compared to that in WT (n =6 in triplicates). Similarly, *hfq* (n = 3) and *dsrA* (n =2) levels were also compared. Expression ratios were calculated using the $\Delta\Delta C_T$ analysis method (Livak and Schmittgen, 2001). All sets that had C_T values greater than 31 were not included in the calculations. An average expression ratio (mean ± standard deviation) of 0.3 ± 0.85 (in *ssrB*::Km^R compared to 14028s) was obtained for *dksA* (using sets n = 1 and n = 6), 0.36 ± 0.87 for *hfq* (sets n =1, n =2, n = 3) and 0.25 ± 0.79 for *dsrA* (n=1). However, given the large errors, due to differences in C_T values between triplicates, a better comparison is required for further validation of the trends observed in the RNA-sequencing data.

Table 5. C_T values for qRT-PCR

gene	WT (14028s)			*SsrB*::Km^R		
n = 1						
rrsA	9.8	9.75	9.72	8.63	8.62	8.83
dksA	26.15	25.88	26.19	27.31	27	27.37
hfq	23.88	23.78	23.87	24.08	25.14	24.55
dsrA	26.2	25.67	26.22	26.93	26.93	26.99
n = 2						
rrsA	12.06	13.33	11.96	12.96	13.27	12.9
dksA	29.75	29.08	29.21	31.15	30.9	31.54
hfq	27.68	27.33	27.26	28.62	29.11	28.64
n = 3						
rrsA	11.17	12.68	10.99	9.75	10.10	11.02
dksA	26.89	26.64	26.89	27.21	27.07	27.30
hfq	23.77	23.95	23.98	24.84	24.94	24.32
n = 4						
rrsA	15	15.38	15.46	12.77	11.53	12.16
dksA	31.32	31.69	31.37	30.95	30.85	30.54
hfq	30.59	30.13	30.63	29.55	29.53	29.42
dsrA	31.98	32.02	31.96	31.07	31.69	31.81
n = 5						
rrsA	12.76	12.52	12.44	12.96	13.21	13.57
dksA	28.78	28.91	28.87	31.77	31.58	31.54
n = 6						

rrsA	8.64	7.96	7.22	8.92	10.30	7.52
dksA	25.31	24.29	24.39	27.84	27.16	25.91

SsrB binding sites in regulatory regions of dksA, hfq and dsrA: SsrB activates transcription of SPI-2 genes. The regulatory regions of these genes have been found to be AT-rich. However, a single consensus sequence has not been identified. Studies indicate the presence of either a TTAAT motif (Feng et al., 2004) or an AT-rich 18 base pair palindrome sequence (Tomljenovic-Berube et al., 2010). However reports indicate that SsrB can tolerate degeneracy within these identified binding sites. Thus, a non-stringent search for these two sites was done using the DNA Pattern Find bioinformatics tool (http://www.bioinformatics.org/sms2/dna_pattern.html) for the regulatory regions upstream of *dksA*, *hfq* and *dsrA*. Such sites were found upstream of all these three genes.

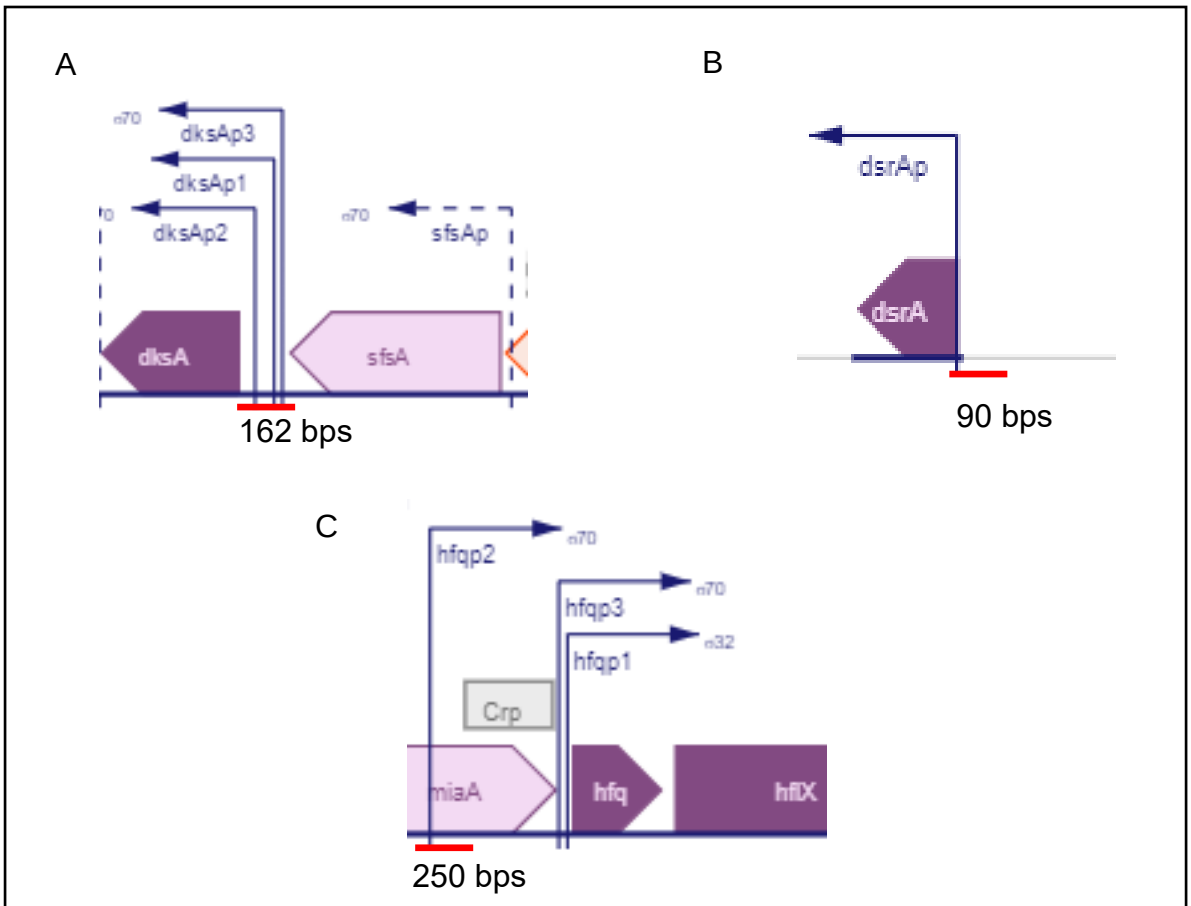
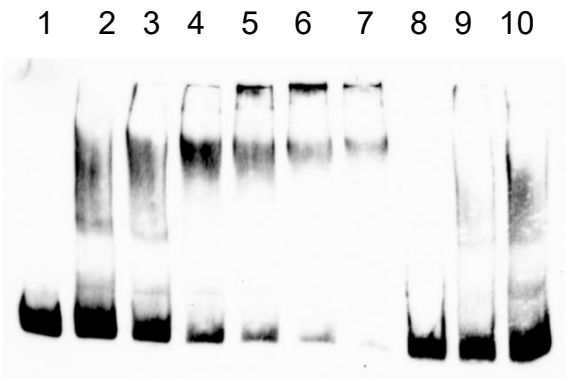


Figure 9. SsrB binding sites identified in regulatory regions of target genes. The red lines show the region chosen and the length of the fragment. A. *dksA*, AT-rich 18 bps sequence. B. *dsrA*, TTAAT sequence. C. *hfq*, AT-rich 18 bps sequence

SsrB *binds to the dksA regulatory region:* To examine whether SsrB directly regulates *dksA*, an electrophoretic mobility shift assay (EMSA) was used. The full length SsrB



Lane 1: dksA (0.95 fmoles)

Lane 2: dksA (0.95 fmoles) + 0.5 µM SsrB

Lane 3: dksA (0.95 fmoles) + 1 µM SsrB

Lane 4: dksA (0.95 fmoles) + 1.5 µM SsrB

Lane 5: dksA (0.95 fmoles) + 2 µM SsrB

Lane 6: dksA (0.95 fmoles) + 2.5 µM SsrB

Lane 7: dksA (0.95 fmoles) + 3 µM SsrB

Lane 8: dksA (0.95 fmoles) + 1 µM K179A

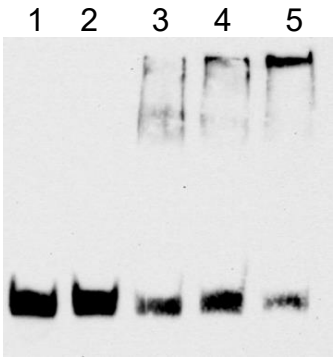
Lane 9: dksA (0.95 fmoles) + 1 µM SsrB + 200x comp DNA (added at same time)

Lane 10: dksA (0.95 fmoles) + 1 µM SsrB + 200x comp DNA (20 min prior incubation with unlabelled DNA)

Figure 10. SsrB binds to the *dksA* regulatory region. Using a DNA binding mutant (K179A) and competitor DNA as controls, we showed that SsrB does specifically interact with the *dksA* regulatory region at concentrations as low as 0.5 µM.

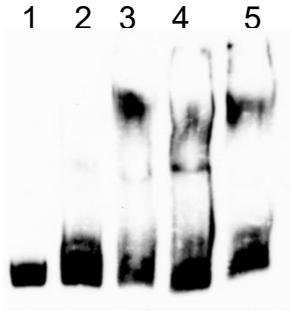
protein was incubated with a 162 base pair DNA fragment of the region immediately upstream of the *dksA* coding sequence. This region contained the plausible SsrB binding site. Initial experiments indicated a presence of binding. The presence of SsrB resulted in a shift in the DNA fragment band (Figure 10). This shift, resulting from the DNA-protein complex moving slower in the acrylamide gel as compared to the free DNA, indicates that SsrB does indeed interact with the *dksA* regulatory region. A DNA binding mutant K179A was used as a control (Figure 10, Lane 8). In the presence of this protein no shift was observed. A competition assay using unlabelled *dksA* was also carried out, where SsrB was incubated with both labelled as well as unlabelled DNA (Figure 10, Lane 9). The unlabelled DNA was added in excess (200 times the labelled DNA) to enable easy visualization of the loss of signal of the SsrB-labelled *dksA* complex. A similar competition reaction was set up with the unlabelled DNA incubated 20 minutes prior to the labelled DNA (Lane 10). However, the competition assay with simultaneous incubation provided easier visualization of the signal loss.

SsrB *binds to the dsrA and hfq regulatory regions:* EMSA assays were also used to check binding of SsrB to the regulatory regions of *dsrA* (Figure 11) and *hfq* (Figure 12). For both genes, a shift in the band was observed, when incubated with a range of SsrB concentrations, indicating a direct interaction with SsrB. However, the band quality was not sharp for all protein-DNA complexes observed for *dksA*, *dsrA* and *hfq*. Hence, there was a need to test different binding and gel-running conditions to improve the quality of the EMSA assays.



Lane 1: dsrA (1.8 fmoles)
Lane 2: dsrA (1.8 fmoles) + 0.5 μM SsrB
Lane 3: dsrA (1.8 fmoles) +1 μM SsrB
Lane 4: dsrA (1.8 fmoles) + 1.5 μM SsrB
Lane 5: dsrA (1.8 fmoles) + 2 μM SsrB

Figure 11. SsrB interacts with *dsrA* regulatory region, detectable at concentrations ≥ 1 μM. The shift in band indicates a direct binding. However, further experiments are needed to fully characterize this binding.



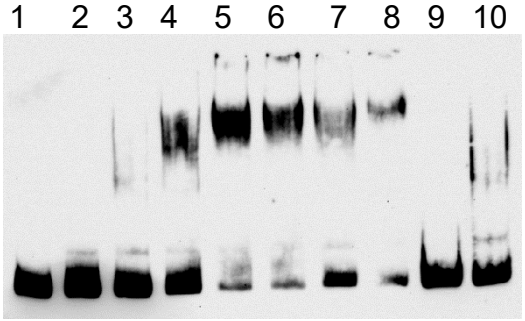
Lane 1: hfq (1.2 fmoles)
Lane 2: hfq (1.2 fmoles) + 0.5 μM SsrB
Lane 3: hfq (1.2 fmoles) +1 μM SsrB
Lane 4: hfq (1.2 fmoles) + 1.5 μM SsrB
Lane 5: hfq (1.2 fmoles) + 2 μM SsrB

Figure 12. SsrB interacts with *hfq* regulatory region. The shift in band, observed at SsrB ≥ 1 μM, indicates a direct binding. However, further experiments are needed to improve the quality of the assay.

Optimization of the EMSA assay to improve band quality: Though EMSA assays indicated towards a regulation of the target genes by SsrB via direct binding, the bands in the assays appeared smeared. In order to improve the quality of the assays, various different binding and running conditions were tried. The SsrB protein was stored in different aliquots at -20°C. The absorbance at 280 nm was measured only once, when

the protein prep was diluted from 21µM to 5 µM. After this dilution, the diluted prep was aliquoted into smaller volumes to use in each EMSA assay. However, the absorbance was not measured each time. After various troubleshooting steps did not result in any improvements in band quality, a spectrophotometric reading of the protein prep (both diluted and stock) revealed a relatively flat line rather than a peak at 280 nm. This led to the conclusion that the protein prep was degraded.

(a) Lower volumes loaded: A large loading volume can result in differences in the sample movement through the gel. A volume of 20 µL used in the above EMSA reaction, fills the entire volume of the wells in the gel. This can result in the



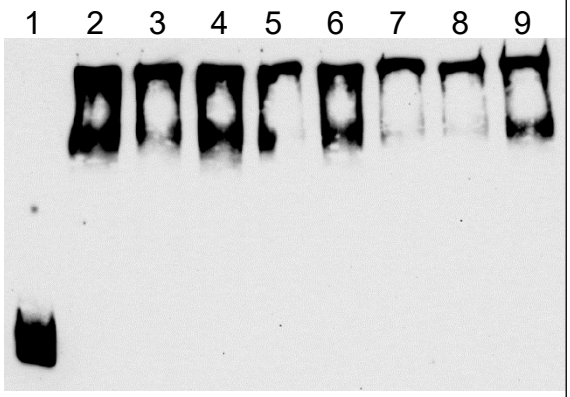
Lane 1: dksA (0.95 fmoles)
Lane 2: dksA (0.95 fmoles) + 0.25 µM SsrB
Lane 3: dksA (0.95 fmoles) + 0.5 µM SsrB
Lane 4: dksA (0.95 fmoles) + 1 µM SsrB
Lane 5: dksA (0.95 fmoles) + 1.5 µM SsrB
Lane 6: dksA (0.95 fmoles) + 2 µM SsrB
Lane 7: dksA (0.95 fmoles) + 2.5 µM SsrB
Lane 8: dksA (0.95 fmoles) + 3 µM SsrB
Lane 9: dksA (0.95 fmoles) + 1 µM K179A
Lane 10: dksA (0.95 fmoles) + 1 µM SsrB + 200x comp DNA (added at same time)

Figure 13. Loading only a fraction of the sample volume results in loss of detectable signal at lower SsrB concentrations and does not improve band quality.

sample effectively moving in different layers, with the lowest most layer entering the gel first and moving faster. Comparatively, the top of the sample would take enter at a later time point and thus be slower as compared to the sample front. This could thus result in a smear. In order to test this, the EMSA assay was repeated for the *dksA* fragment and only 10 µL of the reaction was loaded onto the gel. Though the band was slightly less smeared, the lower loading volumes resulted in loss of signal that was initially detected when the entire reaction volume was loaded. For example, the band shift observed for 0.5 µM SsrB was undetectable when the loading volume was reduced from 20 to 10 µL (compare Lane 3 (Figure 13) and Lane 2 (Figure 10)). This indicated that reducing the volume led to a loss of signal which could be misinterpreted as absence of

binding at that particular SsrB concentration. Thus, failure to load the entire sample did not improve the EMSA assay.

(b) Increased gel percentage and varying glycerol concentration: Increasing gel percentage has been shown to result in crisper bands. Thus, a 5% polyacrylamide gel was used instead of 4%. The binding reactions contain glycerol (3.75%). Glycerol amounts in the sample can play very crucial roles in the binding as well running of the samples. Glycerol helps stabilize protein-DNA complexes formed and hence is important to see the complexes. It also helps the samples sink into the wells during loading. However, higher amounts of glycerol can result in a high viscosity of the sample. This leads to defects in running through the polyacrylamide gel. Thus, varying glycerol concentrations may improve the EMSA assay quality. In this assay, we also varied glycerol concentration of 2.5, 3.75 and 5%. The SsrB concentrations used were 1 and 1.5 µM, since we had previously observed complex formation for these concentrations using a 4% gel and 3.75% glycerol. This enabled the two samples to serve as a control for change in gel percentage. The remaining samples would indicate if changes in glycerol affected the assay quality. However, the 5% gel resulted in none of the protein-DNA complexes travelling down from the wells (Figure 14). All protein-DNA complexes remained within



Lane 1: dksA (0.95 fmoles)

Lane 2: dksA + 1 µM SsrB (3.75% glycerol)

Lane 3: dksA + 1.5 µM SsrB (3.75% glycerol)

Lane 4: dksA + 1 µM SsrB (2.5% glycerol)

Lane 5: dksA + 1.5 µM SsrB (2.5% glycerol)

Lane 6: dksA + 1 µM SsrB (5% glycerol)

Lane 7: dksA + 1.5 µM SsrB (5% glycerol)

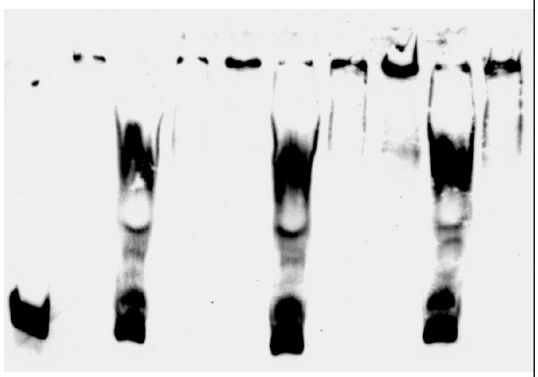
Lane 8: dksA + 1.5 µM SsrB (no glycerol in binding reaction) + 10% glycerol while loading

Lane 9: dksA + 1 µM SsrB (3.75% glycerol) + 10% glycerol while loading

Figure 14. Use of a 5% gel results in the samples not running into the gel. The higher pore size leads to the sample getting stuck in the wells. Thus a 5% gel is not useful to study the interactions between SsrB and *dksA*.

the wells whereas the free DNA moved into the gel. This could likely be due to the smaller pore sizes of the gel. We can exclude the fact that this observation resulted from the glycerol variation, since the 1 and 1.5 µM SsrB concentration had resulted in the complex moving into the 4% gel. Thus, a 5% gel was not optimal for the EMSA assay.

(c) Effects of loading dye and voltage: Various loading dyes can interact with proteins and thus interfere with DNA-protein complexes. This could also result in diffused bands in a gel. Hence, some protocols suggest not adding loading dyes to the samples, but loading it into an empty well to serve the purpose of tracking the samples. To check the effect of the loading dye, an assay with *dksA* and SsrB. A 4% gel and 1.5 µM SsrB was used. Varying glycerol concentrations were also tried (2.5, 3.75 and 5%). The gel was run using higher volumes of buffer (twice the volume as before, filling the gel chamber upto the bottom well boundary). This was to prevent any heating of the gel, which could also result



Lane 1: dksA (0.95 fmoles)

Lane 2: dksA + 1.5 µM SsrB (2.5% glycerol) + no loading dye

Lane 3: dksA + 1.5 µM SsrB (2.5% glycerol) + kit loading buffer

Lane 4: dksA + 1.5 µM SsrB (2.5% glycerol) + 6X loading dye

Lane 5: dksA + 1.5 µM SsrB (3.75% glycerol) + no loading dye

Lane 6: dksA + 1 µM SsrB (3.75% glycerol) + kit loading buffer

Lane 7: dksA + 1.5 µM SsrB (3.75% glycerol) 6X loading dye

Lane 8: dksA + 1.5 µM SsrB (5% glycerol) + no loading dye

Lane 9: dksA + 1.5 µM SsrB (5% glycerol) + kit loading buffer

Lane 10: dksA + 1.5 µM SsrB (5% glycerol) 6X loading dye

Figure 15. Only use of the kit-provided loading buffer enabled the samples to migrate into the gel. The absence of loading dye or use of the usual 6X DNA loading dye resulted in the samples getting stuck in the well.

in diffused bands. A higher voltage was also used (200V). Three different loading dye were used – a no loading dye control, 5X loading buffer (provided with the Thermo Scientific EMSA kit) and a 6X loading dye containing bromophenol blue (used for DNA agarose gels). The exact components of the 5X loading buffer are unknown. We observed that only the samples containing the loading buffer provided with the EMSA kit moved into the gel (Figure 15). All other samples remained within the wells or were not clearly detected. This indicates that the loading buffer has some specific component apart from the tracking dye which enables the samples to sink into the wells and migrate into the gel. Thus, addition of the loading buffer was necessary in our system. The change in voltage and addition of running buffer did not improve the band quality.

(d) Similarly, we also checked for other aspects of the assay that could be modulated. Various conditions like changing the bis:acrylamide ratio to 29:1, eliminating the 10 minute incubation on ice in the binding conditions, controlling running temperature by running the gels at 4°C were tried. However, none of these resulted in an improved band quality as compared to initial assays. Finally, a spectrophometeric reading of the protein aliquot as well as the master stock indicated possible degradation and formation of aggregates. This could explain the diffused bands as well as the samples stuck in the well due to large aggregate formation.

SsrB::Km^R *strain shows decreased cell viability after acid challenge*: Initial acid tolerance assays were carried out using the fresh medium ATR assay (Bearson et al., 1998). The protocol involved replacing the spent media with minimal media whose pH was pre-adjusted to the desired value (5.8 for adaptation and 3.3 for acid challenge). However, the relatively low optical density at 600 nm (OD600 = 0.2) required prior to adaptation made it difficult to visualize the bacterial cell pellet during resuspension in new pH adjusted media. This resulted in loss of cells and a significant difference in the cell density for WT and *SsrB::*Km^R. Hence, the protocol was changed to follow the standard ATR assay (Foster and Hall, 1990) which involved addition of HCl to adjust pH directly to the bacterial culture.

This ATR assay revealed a decrease in percentage of viable cells after exposure to pH 3.3 for the *SsrB::*Km^R strain (Figure 16). In comparison, the wildtype strain was able to mount an ATR. Compared to previously reported values for the wild-type strains (see Figure 3), our experiments yielded a lower percent survival. The *dsrA* study (Ryan et al., 2016) quantified 80% survival for WT (SB300 strain) and the *dksA* study (Webb et al., 1999) showed 55% survival for WT (UK1 strain) at 1 hour post acid challenge. Prior to acid challenge at pH 3.3, both strains were grown in pH 5.8 for adaptation. Previous work has shown that adapted cells survive better in an acid challenge as compared to unadapted cells (Riesenberg-Wilmes et al., 1996). Despite an adaptation at pH 5.8, the *SsrB::*Km^R strain was defective in survival in an acidic

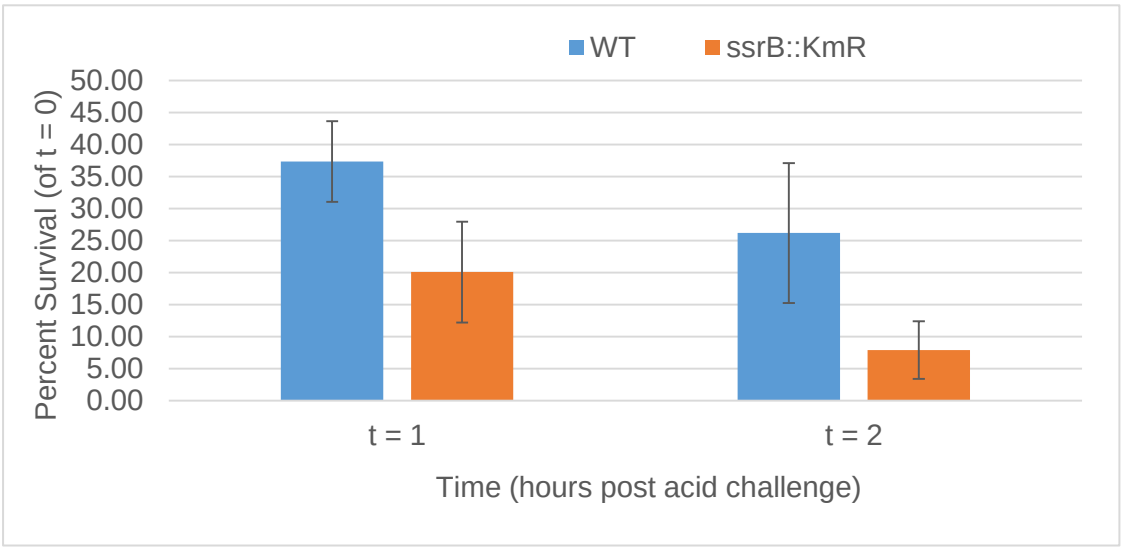


Figure 16. Acid Tolerance Assay (ATR) revealed a reduced survival of the *ssrB* null strain at 1 and 2 hours post acid challenge. In absence of *ssrB*, cells are have reduced survival in acidic conditions (pH = 3.3) as compared to the WT. (N = 5, duplicates, $p < 0.0001$).

environment. However, the assays show a high variation within each time point. *The* experiment was carried out for n =5 (in duplicates, with two dilutions). The dilutions and duplicates did not match (supplementary information). Further controls and experiments are needed to reduce the source of these errors.

Previous work had established the roles of *dksA* and *dsrA* in acid tolerance response in *Salmonella* Typhimurium (Figure 3). RNA-sequencing data (Stuti Desai unplublished) as well as EMSA assays (Figures 10 and 11) indicated that SsrB directly regulated both *dksA* and *dsrA*. We thus hypothesized that the observed reduction in acid survival of the *ssrB*::Km^R strain could be via the regulation of *dksA* or *dsrA* by

SsrB. In order to test this hypothesis, it was necessary to test the acid tolerance responses of the *dksA/dsrA* null strains as well as complementing the *ssrB*::Km^R strain with *dksA/dsrA* overexpression plasmids to test for a rescue of the ATR-deficient phenotype.

dksA and dsrA null strains generated using lambda red system: Using primers with overhangs homologous to the respective genes, the chloramphenicol cassette was PCR amplified from the pKD3 plasmid. This generated a linear DNA fragment containing the chloramphenicol cassette flanked by FRT sites and a region homologous to the *dksA/dsrA* gene on each end of the fragment. This was then transformed into a lambda red background strain (14028s pKD46) using electroporation. Transformants were screened using colony PCR. The screening was done using primers flanking the respective gene locations. After confirmation of transformants, these were moved from the lambda red strain to a clean wild-type background (14028s) using P22 phage transduction. A colony PCR was done after transduction to confirm the replacement of the respective genes in this wild-type background (Figure 17). The PCR indicated that the original gene (*dksA* ~ 0.5 kb and *dsrA* ~ 0.16 kb) had been replaced with the chloramphenicol cassette (~1.1 -1.2 kb).

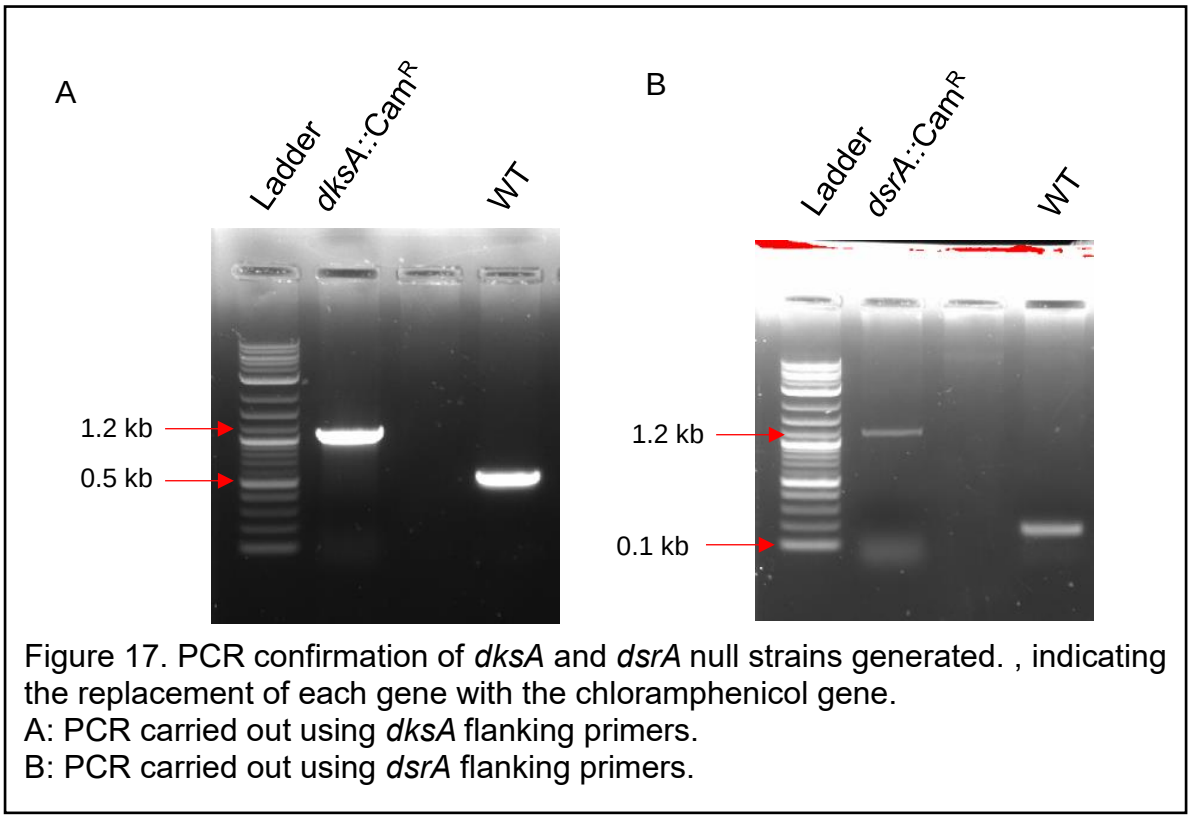


Figure 17. PCR confirmation of *dksA* and *dsrA* null strains generated. , indicating the replacement of each gene with the chloramphenicol gene.
A: PCR carried out using *dksA* flanking primers.
B: PCR carried out using *dsrA* flanking primers.

Sizes of respective bands corresponded to the original gene length + the extra length (outward) covered by the flanking primers.

ΔdsrA dksA::Cm^R strain generated by using the FLP system: The ssrB::Km^R strain showed a downregulation of both *dksA* and *dsrA*. Using a *dsrA dksA* double mutant would thus be an informative control in testing the acid tolerance response. In order to generate this double mutant strain, the *dsrA*::Cm^R strain was transformed with pCP20, a plasmid containing the FLP recombinase under thermal induction. After selection of ampicillin positive transformants, the colonies were grown non-selectively at 43°C to induce FLP mediated recombination. These colonies were then tested for loss of chloramphenicol and ampicillin resistance, resulting in the generation of the Δ*dsrA* strain. The dksA::Cm^R P22 lysate was then grown on Δ*dsrA* and chloramphenicol positive colonies were screened for the *dsrA* and *dksA* gene locations using colony PCR (Figure 18). This indicated that the ~1.2 kb chloramphenicol cassette had been

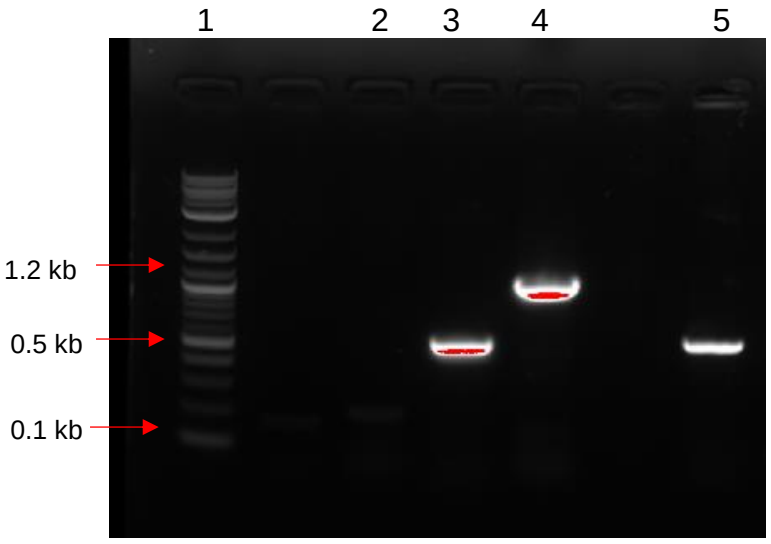


Figure 18. PCR confirmation of the *dsrA dksA* double mutant generated. The PCR indicated the chloramphenicol cassette at the *dsrA* location had been lost (Lane 2) and the *dksA* gene (Lane 3) had been replaced with a chloramphenicol cassette (Lane 4).

lost from the *dsrA* location and replaced with the FRT scar site (Lane 2). Using the *dksA* flanking primers, we observed that the previously ~0.5kb band (corresponding to the *dksA* gene in Δ*dsrA*, Lane 3) had been replaced with the ~1.1-1.2kb chloramphenicol cassette (Lane 4) after transduction. Thus, the strain obtained had

lost *dsrA* and had the *dksA* gene replaced with chloramphenicol indicating successful generation of the Δ*dsrA dksA*::Cm^R strain.

Cloning of dksA and dsrA genes in an overexpression system: Using a genetics approach, we wanted to test if the reduced acid survival phenotype of *ssrB*::Km^R could be rescued by overexpression of the *dksA* and *dsrA* in trans on respective pET-*dksA* and pET-*dsrA* plasmids. To construct this overexpression system for *dksA* and *dsrA*, a pET-Duet-1 plasmid (Figure 19A) was used. The respective gene was cloned using restriction digestion and ligation downstream of the T7 promoter to generate the pET-

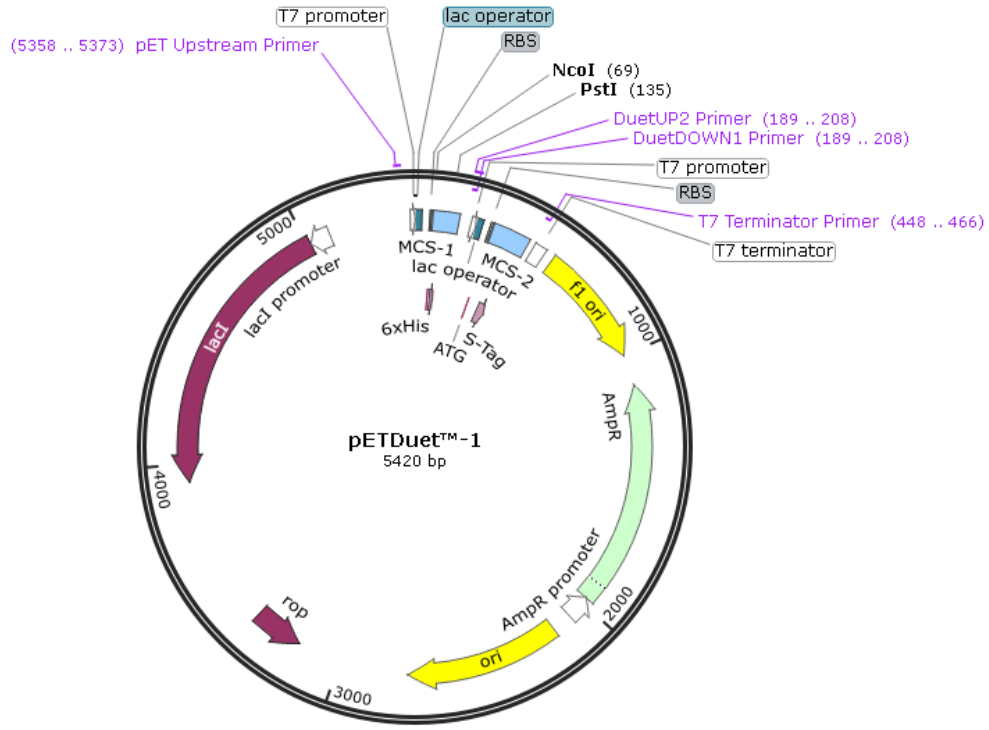


Figure 19A. pET-Duet-1 plasmid map.

dksA (Figure 19B) and pET-*dsrA* plasmids (Figure 19C). The clones were confirmed by a PCR reaction using gene specific primers. The clones showed the expected gene lengths for both *dksA* and *dsrA* (Figure 20) after PCR. The pET-Duet-1 vector PCR showed no band when it was used as the template for both gene primer pairs,

indicating that indeed the bands obtained for the pET-*dksA* and pET-*dsrA* were the expected gene bands.

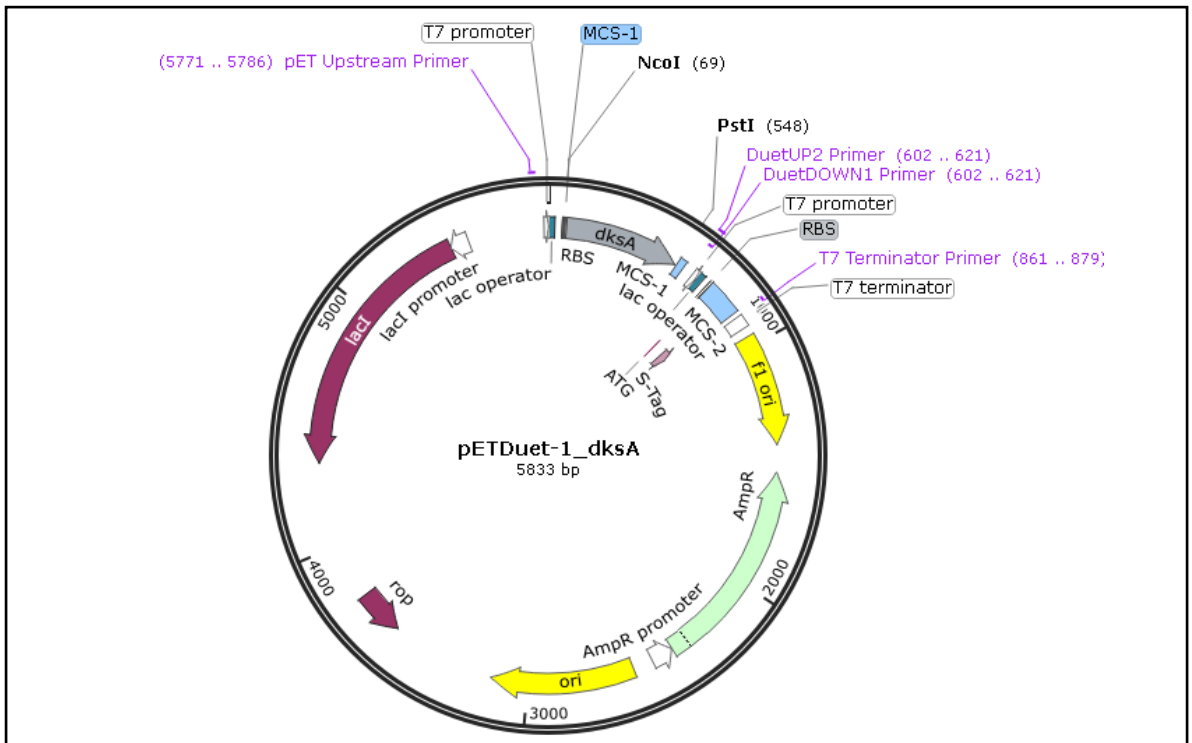


Figure 19B. pET-*dksA* plasmid map.

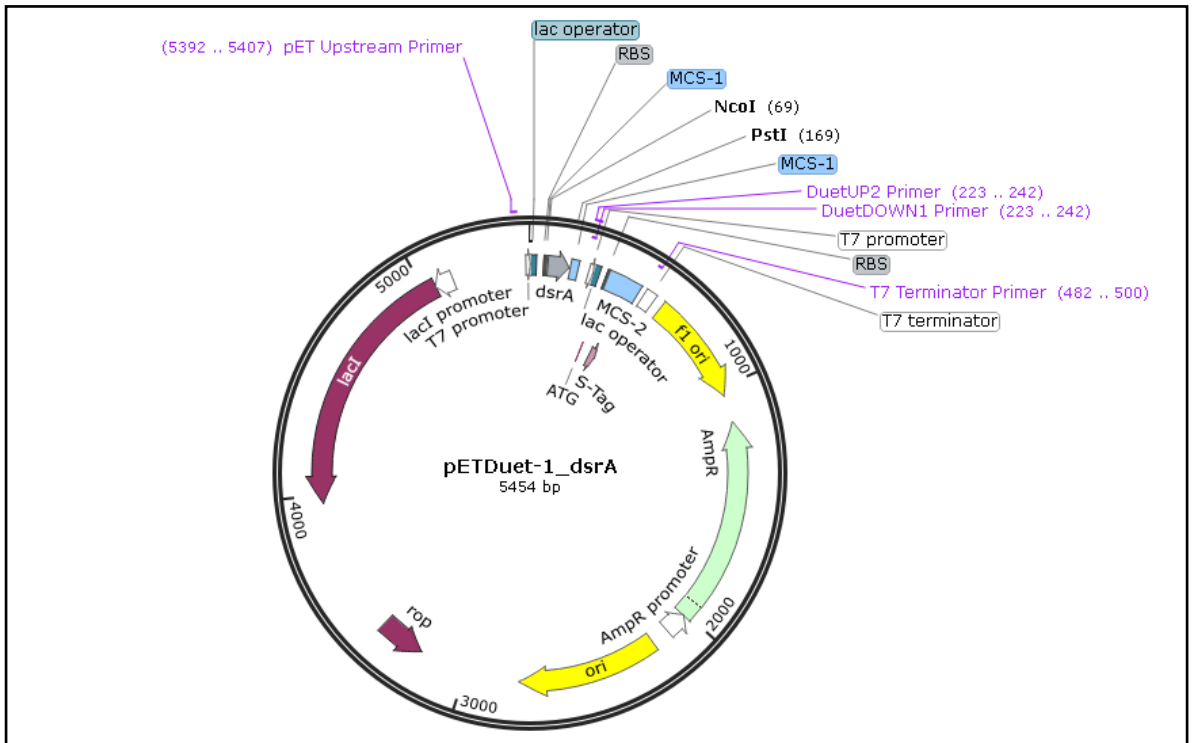
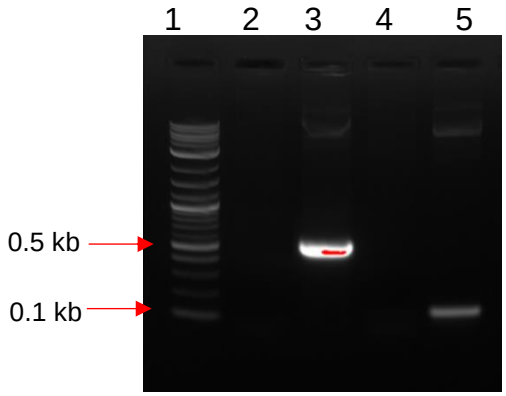


Figure 19C. pET-*dsrA* plasmid map.



Lane 2: pET-Duet-1 + *dksA* primers

Lane 3: pET-*dksA* + *dksA* primers

Lane 4: pET-Duet-1 + *dsrA* primers

Figure 20. PCR confirmation of the *dsrA* and *dksA* expressing clones obtained.

Since these plasmids were to be used for expression of *dsrA* and *dksA* in various strains, it was important to check the sequence of the inserts as well as their alignments. Both forward and reverse primers were used for DNA sequencing (1st BASE DNA Sequencing Services), enabling two reads for each plasmid. The two reads obtained were combined to reconstruct the full sequence and this was aligned with the original gene sequence. A 100% sequence alignment was obtained for the

Score	Expect	Identities	Gaps	Strand
867 bits(469)	0.0	469/469(100%)	0/469(0%)	Plus/Plus

```
Query  7    AAGGAGAAGCAACATGCAAGAAGGGCAAAACCGTAAAACATCGTCCCTGAGTATTCTCGC  66
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1    AAGGAGAAGCAACATGCAAGAAGGGCAAAACCGTAAAACATCGTCCCTGAGTATTCTCGC  60

Query  67   CATCGCTGGGGTGGAGCCGTACCAGGAGAAACCGGGCGAAGAGTATATGAACGAAGCCCA  126
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  61   CATCGCTGGGGTGGAGCCGTACCAGGAGAAACCGGGCGAAGAGTATATGAACGAAGCCCA  120

Query  127  GCTATCGCACTTCAAGCGTATTCTTGAAGCATGGCGTAATCAACTCCGGGATGAAGTCGA  186
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  121  GCTATCGCACTTCAAGCGTATTCTTGAAGCATGGCGTAATCAACTCCGGGATGAAGTCGA  180

Query  187  TCGCACTGTGACGCACATGCAGGACGAAGCCGCCAACTTCCCCGATCCGGTCGATCGCGC  246
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  181  TCGCACTGTGACGCACATGCAGGACGAAGCCGCCAACTTCCCCGATCCGGTCGATCGCGC  240

Query  247  CGCGCAGGAAGAGGAGTTTAGCCTGGAGTTACGTAATCGTGACCGTGAGCGCAAACTGAT  306
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  241  CGCGCAGGAAGAGGAGTTTAGCCTGGAGTTACGTAATCGTGACCGTGAGCGCAAACTGAT  300

Query  307  CAAAAAGATCGAGAAGACGCTGAAGAAAGTGGAAGATGAAGACTTCGGTTATTGCGAGTC  366
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  301  CAAAAAGATCGAGAAGACGCTGAAGAAAGTGGAAGATGAAGACTTCGGTTATTGCGAGTC  360

Query  367  CTGCGGGGTGGAGATTGGTATCCGCCGCCTGGAAGCGCGTCCAACAGCCGATCTGTGCAT  426
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  361  CTGCGGGGTGGAGATTGGTATCCGCCGCCTGGAAGCGCGTCCAACAGCCGATCTGTGCAT  420

Query  427  CGACTGCAAAACGCTGGCTGAAATTCGCGAAAAACAGATGGCGGGTTAA  475
            ||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  421  CGACTGCAAAACGCTGGCTGAAATTCGCGAAAAACAGATGGCGGGTTAA  469
```

Figure 21A. Sequence alignment of pET-*dksA* with the dksA coding sequence showing a 100% alignment (red circle). Query – Reconstructed sequence from the forward and reverse reads of the sequencing results. Subjct – coding sequence of dksA

Score	Expect	Identities	Gaps	Strand
163 bits(180)	4e-46	90/90(100%)	0/90(0%)	Plus/Plus

```
Query  7   AATCTCACATCAGATTTCCTGGTGTAACGAATTTTCAAGTGCTTCTTGCATAAGCAAGTT  66
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1   AATCTCACATCAGATTTCCTGGTGTAACGAATTTTCAAGTGCTTCTTGCATAAGCAAGTT  60

Query  67  TGATCCCGACCCGTAGGGCCGGGATTTTTT  96
           |||||||||||||||||||||||||||||
Sbjct  61  TGATCCCGACCCGTAGGGCCGGGATTTTTT  90
```

Figure 21B. Sequence alignments of pET-*dsrA* with the *dsrA* coding sequence showing a 100% alignment (red circle). Query – Reconstructed sequence from the forward and reverse reads of the sequencing results. Subjct – coding sequence of *dsrA*

sequencing reads of the plasmid and the gene coding sequences for *dksA* (Figure 21A) and *dsrA* (Figure 21B). Thus, the clones obtained were in the correct alignment and had the accurate sequence of the *dksA/dsrA* gene. These were then transformed into the various strains constructed.

The dsrA null strain did not show an acid tolerance phenotype in our system: To test the various strains constructed, we began initial control experiments using only the WT (14028s), *dsrA*::Cm^R and *dsrA*::Cm^R pET-*dsrA* strains. The ATR assay was

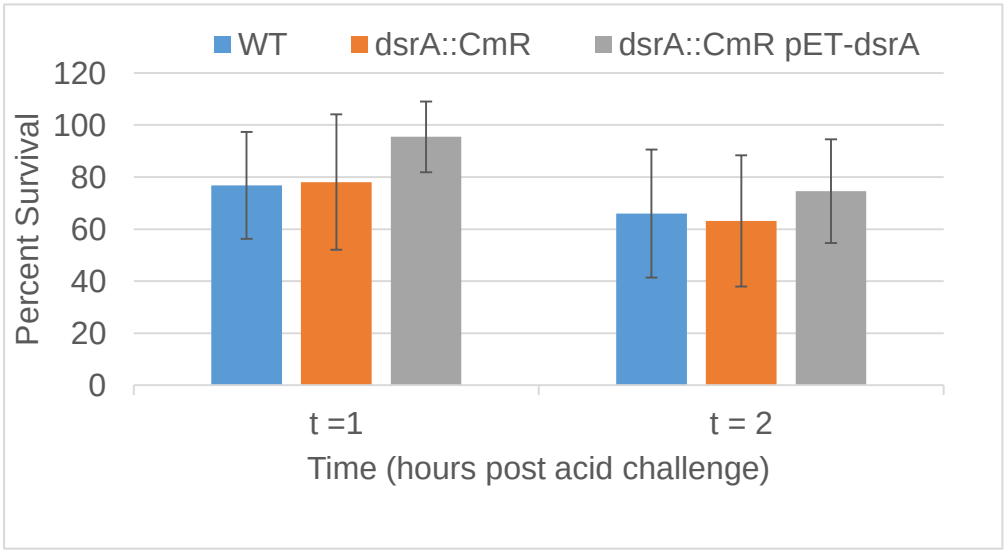


Figure 22. We were unable to replicate the ATR phenomenon in the *dsrA* null as previously described (Webb et al., 1999). (N=4 in duplicates)

performed as described before. There was reasonable agreement between the duplicates and dilutions (supplementary information). However, the *dsrA*::Cm^R strain did not show any differences in survival as compared to the wildtype (Figure 21). A

difference was observed in one data set (out of 5) (however, the initial OD of that experiment prior to adaptation had reached 0.27). Thus, we were unable to replicate the previous report (Ryan et al., 2016) that showed a role for *dsrA* in acid tolerance . This study used a different protocol to show the acid tolerance response, wherein they adapt the cells to a pH of 4.4 at OD 0.4 for 1 hour. Another observation was that the wild-type survival was different as compared to previous experiments (Figure 20 vs Figure 15). The percent survival obtained in this experiment (80%) at t = 1 hour was similar to previously reported values in the literature (Figure 3). Due these discrepancies within experiments, further experiments are required to replicate previous reports to test if *dsrA* played a role in the acid tolerance response of *Salmonella* Typhimurium.

The dksA null strain shows a growth defect at pH 7.6 in minimal media: To characterize the ATR of the *dksA* null strain, single colonies of WT (14028s), *dksA*::CmR and *dksA*::Cm^R pET-*dksA* strains were inoculated into minimal E media (pH = 7.6) and grown overnight (as described for standard ATR assay). However, the *dksA*::Cm^R did not grown into a saturated culture despite 16 hours at 37°C. To faciliate growth of the strain, initially the strain was grown in rich media (LB) from a single colony. After overnight growth, the cells were collected and the media was removed. This was resuspended in equal volume of the minimal E media. This resuspension was used for a 1:100 inoculation into 1 mL minimal E media. This was allowed to grow overnight at 37°C with shaking. Despite the initial growth in rich media, the *dksA* null strain was still unable to reach saturation. The growth was then checked by inoculation of *dksA*::Cm^R in minimal E media supplemented with casamino acids (0.05%) as previously described (Webb et al., 1999). The null strain was still unable to reach saturation after 16 hours.

The *dksA* null strain showed a growth defect in minimal E media and thus, we were unable to proceed and characterize its acid tolerance response. Growth measurements of this strain in minimal E media using a range of casamino acids concentrations are necessary in the future prior to studying its ATR.

Conclusions and Future Work

In this study, we characterized three plausible gene targets, *dksA, dsrA* and *hfq*, of the SsrB regulon using RNA-sequencing data of 2 day old *Salmonella* Typhimurium biofilms (Stuti Desai, unpublished data). The direct binding of SsrB with the regulatory regions of these genes was tested using electrophoretic mobility shift assays. These assays did indicate a direct interaction of *dksA, dsrA* and *hfq* regulatory regions with SsrB. However, the quality of the assay could not be improved, likely due to degradation of the protein aliquot, due to a misinterpretation of the spectrophotometric peak. Further experiments are needed to better characterize this interaction, including improving the quality of the bands obtained, as well as finding minimum concentrations of binding. The use of the C-terminal of SsrB (the DNA binding domain, SsrBc), could also help improve the assay quality since this form of the protein is more stable than the full length SsrB.

Two genes, *dksA* and *dsrA*, have been previously shown to play a role in acid tolerance. The *ssrB* null strain, which showed lower expression levels of these two genes in biofilm inducing conditions in RNA-sequencing analysis (Stuti Desai, unpublished data), was tested for its acid survival. A reduction in the percent survival in pH 3.3 was obtained for the *ssrB* null strain as compared to the wild-type. However, the assay showed high variation (within duplicates and within dilutions plated). In order to shed light on the mechanism of this regulation, a genetics approach was used. Using lambda red recombination and P22 transductions, *dsrA* and *dksA* null strains, as well as a *dsrA dksA* double mutant were generated. The two genes were also cloned into the pET-Duet-1 plasmid for use in complementation studies. However, we were unable to characterize the ATR in the *dsrA* null strain. The *dksA* null strain showed a growth defect in minimal E media at pH 7.6 itself and hence its ATR was also not characterized. The wild-type ATR showed a high variation within experiments and hence, future work must work towards accurately characterizing this phenotype.

Despite this, it was evident that the *ssrB*::Km^R strain was defective in its ATR. This study indicates a novel role for SsrB in the acid tolerance response. Using existing transcriptome data in the lab, we were able to identify three new targets of SsrB, *dsrA, dksA* and *hfq*, thus expanding the known SsrB regulon. The three genes identified lie outside SPI-2, the pathogenicity island harboring *ssrB* gene. This shows that a

horizontally acquired response regulator can also modulate genes on the native chromosome. These three genes are regulators of aspects like virulence, motility, stress responses, biofilm formation, etc. in *Salmonella* lifestyle and pathogenesis and have many downstream targets. This leads to the proposal of SsrB being a master regulator of regulators which needs to be further investigated in future to characterize what role this could play in the pathogenesis of *Salmonella*.

References

Aranda, P.S., LaJoie, D.M., and Jorcyk, C.L. (2012). Bleach gel: a simple agarose gel for analyzing RNA quality. Electrophoresis *33*, 366–369.

Azriel, S., Goren, A., Rahav, G., and Gal-mor, O. (2016). The Stringent Response Regulator DksA Is Required for Salmonella enterica Serovar Typhimurium Growth in Minimal Medium , Motility , Biofilm Formation , and Intestinal Colonization. *84*, 375–384.

Bearson, B.L., Wilson, L., and Foster, J.W. (1998). A low pH-inducible, PhoPQ-dependent acid tolerance response protects Salmonella typhimurium against inorganic acid stress. J. Bacteriol. *180*, 2409–2417.

Brennan, R.G., and Link, T.M. (2007). Hfq structure, function and ligand binding. Curr. Opin. Microbiol. *10*, 125–133.

Chao, Y., and Vogel, J. (2010). The role of Hfq in bacterial pathogens. Curr. Opin. Microbiol. *13*, 24–33.

Chao, Y., Papenfort, K., Reinhardt, R., Sharma, C.M., and Vogel, J. (2012). An atlas of Hfq-bound transcripts reveals 3′ UTRs as a genomic reservoir of regulatory small RNAs. EMBO J. *31*, 4005–4019.

Cirillo, D.M., Valdivia, R.H., Monack, D.M., and Falkow, S. (1998). Macrophage-dependent induction of the Salmonella pathogenicity island 2 type III secretion system and its role in intracellular survival. Mol. Microbiol. *30*, 175–188.

Colgan, A.M., Kroeger, C., Diard, M., Hardt, W.D., Puente, J.L., Sivasankaran, S.K., Hokamp, K., and Hinton, J.C.D. (2016). The Impact of 18 Ancestral and Horizontally-Acquired Regulatory Proteins upon the Transcriptome and sRNA Landscape of Salmonella enterica serovar Typhimurium. PLoS Genet. *12*, 1–42.

Crawford, M.A., Henard, C.A., Tapscott, T., Porwollik, S., McClelland, M., and Vázquez-Torres, A. (2016). DksA-Dependent Transcriptional Regulation in Salmonella Experiencing Nitrosative Stress. Front. Microbiol. *7*, 444.

Dandekar, T., Fieselmann, A., Popp, J., and Hensel, M. (2012). Salmonella enterica: A surprisingly well-adapted intracellular lifestyle. Front. Microbiol. *3*, 1–11.

Datsenko, K.A., and Wanner, B.L. (2000). One-step inactivation of chromosomal

genes in Escherichia coli K-12 using PCR products. Proc. Natl. Acad. Sci. U. S. A. *97*, 6640–6645.

Desai, S.K., and Kenney, L.J. (2017). To ~P or Not to ~P? Non-canonical activation by two-component response regulators. Mol. Microbiol. *103*, 203–213.

Desai, S.K., Winardhi, R.S., Periasamy, S., Dykas, M.M., Jie, Y., and Kenney, L.J. (2016). The horizontally-acquired response regulator SsrB drives a Salmonella lifestyle switch by relieving biofilm silencing. Elife *5*, 1–23.

Drecktrah, D., Knodler, L.A., Ireland, R., and Steele-Mortimer, O. (2006). The mechanism of Salmonella entry determines the vacuolar environment and intracellular gene expression. Traffic *7*, 39–51.

Feng, X., Walthers, D., Oropeza, R., and Kenney, L.J. (2004). The response regulator SsrB activates transcription and binds to a region overlapping OmpR binding sites at Salmonella pathogenicity island 2. Mol. Microbiol. *54*, 823–835.

Foster, J.W., and Hall, H.K. (1990). Adaptive acidification tolerance response of Salmonella typhimurium. J. Bacteriol. *172*, 771–778.

González, J.F., Alberts, H., Lee, J., Doolittle, L., and Gunn, J.S. (2018). Biofilm Formation Protects Salmonella from the Antibiotic Ciprofloxacin In Vitro and In Vivo in the Mouse Model of chronic Carriage. Sci. Rep. *8*, 222.

Gonzalez-Escobedo, G., and Gunn, J.S. (2013). Identification of Salmonella enterica Serovar Typhimurium Genes Regulated during Biofilm Formation on Cholesterol Gallstone Surfaces. Infect. Immun. *81*, 3770–3780.

Henard, C.A., and Vázquez-Torres, A. (2012). Dksa-dependent resistance of Salmonella enterica Serovar typhimurium against the antimicrobial activity of inducible nitric oxide synthase. Infect. Immun. *80*, 1373–1380.

Hensel, M. (2004). Evolution of pathogenicity islands of Salmonella enterica. Int. J. Med. Microbiol. *294*, 95–102.

Hensel, M., Bakteriologie, L., and Pettenkofer-institut, M. Von (2000). Salmonella pathogenicity island 2. Mol. Microbiol. *36*, 1015–1023.

Jude, F., Köhler, T., Branny, P., Perron, K., Mayer, M.P., Comte, R., and Van

Delden, C. (2003). Posttranscriptional control of quorum-sensing-dependent virulence genes by DksA in Pseudomonas aeruginosa. J. Bacteriol. *185*, 3558–3566.

Lathrop, S.K., Binder, K.A., Starr, T., Cooper, K.G., Chong, A., Carmody, A.B., and Steele-Mortimer, O. (2015). Replication of Salmonella enterica Serovar Typhimurium in Human Monocyte-Derived Macrophages. Infect. Immun. *83*, 2661–2671.

Lease, R.A., Cusick, M.E., and Belfort, M. (1998). Riboregulation in Escherichia coli: DsrA RNA acts by RNA:RNA interactions at multiple loci. Proc. Natl. Acad. Sci. U. S. A. *95*, 12456–12461.

Lennon, C.W., Ross, W., Martin-Tumasz, S., Toulokhonov, I., Vrentas, C.E., Rutherford, S.T., Lee, J.-H., Butcher, S.E., and Gourse, R.L. (2012). Direct interactions between the coiled-coil tip of DksA and the trigger loop of RNA polymerase mediate transcriptional regulation. Genes Dev. *26*, 2634.

Livak, K.J., and Schmittgen, T.D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods *25*, 402–408.

Majdalani, N., Cunning, C., Sledjeski, D., Elliott, T., and Gottesman, S. (1998). DsrA RNA regulates translation of RpoS message by an anti-antisense mechanism, independent of its action as an antisilencer of transcription. Proc. Natl. Acad. Sci. U. S. A. *95*, 12462–12467.

Majdalani, N., Chen, S., Murrow, J., St. John, K., and Gottesman, S. (2001). Regulation of RpoS by a novel small RNA: The characterization of RprA. Mol. Microbiol. *39*, 1382–1394.

Mastroeni, P., and Grant, A.J. (2011). Spread of Salmonella enterica in the body during systemic infection: unravelling host and pathogen determinants. Expert Rev. Mol. Med. *13*, e12.

Mills, D.M., Bajaj, V., and Lee, C.A. (2006). A 40 kb chromosomal fragment encoding Salmonella typhimurium invasion genes is absent from the corresponding region of the Escherichia coli K-12 chromosome. Mol. Microbiol. *15*, 749–759.

Mogull, S.A., Runyen-Janecky, L.J., Hong, M., and Payne, S.M. (2001). dksA is required for intercellular spread of Shigella flexneri via an RpoS-independent

mechanism. Infect. Immun. *69*, 5742–5751.

Monteiro, C., Papenfort, K., Hentrich, K., Ahmad, I., Le Guyon, S., Reimann, R., Grantcharova, N., and Römling, U. (2012). Hfq and Hfq-dependent small RNAs are major contributors to multicellular development in Salmonella enterica serovar Typhimurium. RNA Biol. *9*, 489–502.

Nakanishi, N., Abe, H., Ogura, Y., Hayashi, T., Tashiro, K., Kuhara, S., Sugimoto, N., and Tobe, T. (2006). ppGpp with DksA controls gene expression in the locus of enterocyte effacement (LEE) pathogenicity island of enterohaemorrhagic Escherichia coli through activation of two virulence regulatory genes. Mol. Microbiol. *61*, 194–205.

Paul, B.J., Barker, M.M., Ross, W., Schneider, D.A., Webb, C., Foster, J.W., and Gourse, R.L. (2004). DksA: A critical component of the transcription initiation machinery that potentiates the regulation of rRNA promoters by ppGpp and the initiating NTP. Cell *118*, 311–322.

Rabsch, W., Andrews, H.L., Kingsley, R.A., Prager, R., Tschäpe, H., Adams, L.G., and Bäumler, A.J. (2002). Salmonella enterica serotype Typhimurium and its host-adapted variants. Infect. Immun. *70*, 2249–2255.

Rathman, M., Sjaastad, M.D., and Falkow, S. (1996). Acidification of phagosomes containing Salmonella typhimurium in murine macrophages. Infect. Immun. *64*, 2765–2773.

Riesenberg-Wilmes, M.R., Bearson, B., Foster, J.W., Curtis, R., and 3rd (1996). Role of the acid tolerance response in virulence of Salmonella typhimurium. Infect. Immun. *64*, 1085–1092.

Ryan, D., Ojha, U.K., Jaiswal, S., and Padhi, C. (2016). The Small RNA DsrA Influences the Acid Tolerance Response and Virulence of Salmonella enterica Serovar Typhimurium. *7*, 1–8.

Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989). Molecular Cloning: A Laboratory Manual. Cold Spring Harbor laboratory press.

Sittka, A., Pfeiffer, V., Tedin, K., and Vogel, J. (2007). The RNA chaperone Hfq is essential for the virulence of Salmonella typhimurium. Mol. Microbiol. *63*, 193–217.

Steenackers, H., Hermans, K., Vanderleyden, J., and De Keersmaecker, S.C.J. (2012). Salmonella biofilms: An overview on occurrence, structure, regulation and eradication. Food Res. Int. *45*, 502–531.

Tomljenovic-Berube, A.M., Mulder, D.T., Whiteside, M.D., Brinkman, F.S.L., and Coombes, B.K. (2010). Identification of the regulatory logic controlling Salmonella pathoadaptation by the SsrA-SsrB two-component system. PLoS Genet. *6*.

Vogel, H.J., and Bonner, D.M. (1956). Acetylornithinase of Escherichia coli: partial purification and some properties. J. Biol. Chem. *218*, 97–106.

Watson, B.W., Meldrum, S.J., Riddle, H.C., Brown, R.L., and Sladen, G.E. (1972). pH profile of gut as measured by radiotelemetry capsule. Br. Med. J. *2*, 104–106.

Webb, C., Moreno, M., Wilmes-Riesenberg, M., Curtiss Iii, R., and Foster, J.W. (1999). Effects of DksA and ClpP protease on sigma S production and virulence in Salmonella typhimurium. Mol. Microbiol. *34*, 112–123.

Yun, J., Jeon, B., Barton, Y.W., Plummer, P., Zhang, Q., and Ryu, S. (2008). Role of the DksA-like protein in the pathogenesis and diverse metabolic activity of Campylobacter jejuni. J. Bacteriol. *190*, 4512–4520.

Supplementary Information

ATR assay: 14028s and ssrB::Km^R

N =1	WT		*ssrB*::Km^R	
T = 0				
	10^5	10^4	10^5	10^4
Duplicate 1	15	89	32	clustered
Duplicate 2	20	143	21	191
T = 1				
	10^5	10^4	10^5	10^3
Duplicate 1	11	59	22	187
Duplicate 2	4	74	37	clustered
T = 2				
	10^5	10^4	10^5	10^3
Duplicate 1	no colonies	31	11	160
Duplicate 2	7	53	16	113

N =2	WT		*ssrB*::Km^R	
T = 0				
	10^5	10^4	10^5	10^4
Duplicate 1	28	clustered	17	190
Duplicate 2	26	302	14	195
T = 1				
	10^5	10^4	10^5	10^3
Duplicate 1	no colonies	81	31	clustered
Duplicate 2	13	74	41	clustered
T = 2				
	10^5	10^4	10^5	10^3
Duplicate 1	no colonies	62	2	141
Duplicate 2	2	44	no colonies	97

N =3	WT		*ssrB*::Km^R	
T = 0				
	10^5	10^4	10^5	10^4
Duplicate 1	6	52	19	207

Duplicate 2	61	clustered	25	187
T = 1				
	10^5	10^4	10^5	10^3
Duplicate 1	3	32	53	clustered
Duplicate 2	21	clustered	47	clustered
T = 2				
	10^5	10^4	10^5	10^3
Duplicate 1	3	27	17	clustered
Duplicate 2	7	90	26	clustered

N =4	WT		*ssrB*::Km^R	
T = 0				
	10^5	10^4	10^5	10^4
Duplicate 1	76	193	11	151
Duplicate 2	3	129	8	154
T = 1				
	10^5	10^4	10^5	10^3
Duplicate 1	10	83	51	clustered
Duplicate 2	7	49	18	157
T = 2				
	10^5	10^4	10^5	10^3
Duplicate 1	3	26	26	clustered
Duplicate 2	9	54	12	clustered

N =5	WT		*ssrB*::Km^R	
T = 0				
	10^5	10^4	10^5	10^4
Duplicate 1	3	225	21	209
Duplicate 2	7	220	7	341
T = 1				
	10^5	10^4	10^5	10^3
Duplicate 1	19	81	35	389
Duplicate 2	22	84	89	clustered
T = 2				

	10^5	10^4	10^5	10^3
Duplicate 1	13	74	3	55
Duplicate 2	12	61	3	204

ATR assay: 14028s, *dsrA*::Cm^R, *dsrA*::Cm^R pET-*dsrA*

N = 1	WT		*dsrA*::Cm^R		*dsrA*::Cm^R pET-*dsrA*	
T = 0						
	10^5	10^6	10^5	10^6	10^5	10^6
Duplicate 1	219	24	149	15	231	28
Duplicate 2	264	20	161	19	225	24
T = 1						
	10^5	10^4	10^5	10^4	10^5	10^4
Duplicate 1	127	clustered	94	clustered	189	clustered
Duplicate 2	131	clustered	98	clustered	187	clustered
T = 2						
	10^5	10^6	10^5	10^6	10^5	10^6
Duplicate 1	101	No colonies	87	No colonies	125	No colonies
Duplicate 2	99	No colonies	81	No colonies	103	No colonies

N = 2	WT		*dsrA*::Cm^R		*dsrA*::Cm^R pET-*dsrA*	
T = 0						
	10^5	10^6	10^5	10^6	10^5	10^6
Duplicate 1	241	27	210	25	199	28
Duplicate 2	251	24	199	21	221	22
T = 1						
	10^5	10^6	10^5	10^6	10^5	10^6
Duplicate 1	228	31	151	27	209	34
Duplicate 2	246	26	181	18	189	24
T = 2						
	10^5	10^4	10^5	10^4	10^5	10^4
Duplicate 1	149	clustered	101	clustered	127	clustered
Duplicate 2	143	clustered	94	clustered	148	clustered

N = 3	WT		*dsrA*::Cm^R		*dsrA*::Cm^R pET-*dsrA*	
T = 0						
	10^5	10^6	10^5	10^6	10^5	10^6
Duplicate 1	247	20	253	21	181	25
Duplicate 2	248	28	198	17	235	28
T = 1						
	10^5	10^6	10^5	10^6	10^5	10^6
Duplicate 1	111	19	135	24	158	16
Duplicate 2	187	31	131	15	171	14
T = 2						
	10^5	10^6	10^5	10^6	10^5	10^6
Duplicate 1	127	18	117	16	141	16
Duplicate 2	142	21	119	12	161	12

N = 4	WT		*dsrA*::Cm^R		*dsrA*::Cm^R pET-*dsrA*	
T = 0						
	10^5	10^6	10^5	10^6	10^5	10^6
Duplicate 1	121	13	129	21	115	13
Duplicate 2	103	14	162	25	159	15
T = 1						
	10^5	10^6	10^5	10^6	10^5	10^6
Duplicate 1	110	12	144	11	111	15
Duplicate 2	107	19	156	12	141	18
T = 2						
	10^5	10^6	10^5	10^6	10^5	10^6
Duplicate 1	139	No colonies	127	No colonies	101	No colonies
Duplicate 2	111	No colonies	139	No colonies	121	No colonies

Set that showed a *dsrA*-dependent ATR phenomenon:

N = 5	WT		*dsrA*::Cm^R		*dsrA*::Cm^R pET-*dsrA*	
T = 0						
	10^5	10^4	10^5	10^4	10^5	10^4

Duplicate 1	259	clustered	261	clustered	295	clustered
Duplicate 2	282	clustered	276	clustered	288	clustered
T = 1						
	10^5	10^4	10^3	10^4	10^5	10^4
Duplicate 1	21	254	17	4	81	clustered
Duplicate 2	27	255	22	3	59	clustered
T = 2						
	10^5	10^4	10^3	10^4	10^5	10^4
Duplicate 1	No colonies	4	No colonies	No colonies	1	56
Duplicate 2	No colonies	5	No colonies	No colonies	9	55