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A Role for the Dsb System in Type III Secretion and Virulence in Salmonella

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A Role for the Dsb System in Type III Secretion and Virulence in *Salmonella*

By

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Doctor of Philosophy

in

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of the

University of California, Berkeley

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Abstract

A Role for the Dsb System in Type III Secretion and Virulence in Salmonella

By

Alexander Chun-Hao Chang

Doctor of Philosophy in Infectious Diseases and Immunity

University of California, Berkeley

Professor Sangwei Lu, Chair

The Disulfide Bond Formation (Dsb) System is important for maintaining the structure and function of periplasmic proteins. Although the biochemistry of various Dsb proteins has been characterized in E. coli, the potential link between this system and virulence mechanisms of pathogenic Gram-negative bacteria has not been elucidated. Deletion mutations generated in dsb genes demonstrated multiple virulence defects in Salmonella enterica serovar Enteritidis, which were in turn rescued by complementation. Analyses revealed that two members of the Dsb system, DsbA and DsbC, were required for invasion of epithelial cells, a process mediated by the Type III Secretion System (T3SS) encoded by Salmonella Pathogenicity Island 1 (SPI-1). The Dsb system was also required for colonization and virulence in vivo. Strains expressing T3SS effectors tagged with a FLAG epitope were transduced with the dsb mutations, and analyses of the supernatant fractions indicated that the Dsb system was involved in the secretion of SipA and SipC, which are critical for ruffling of the host cell membrane and subsequent internalization of the bacteria. A bacterial two-hybrid assay was performed to identify Dsb substrates among the T3SS needle complex proteins, and preliminary results indicated direct binding of the Dsb system with SpaO, a T3SS sorting platform protein. These results suggested that the formation and isomerization of disulfide bonds are critical steps in the function of the T3SS which contributes to the pathogenesis of Salmonella.
Dedicated to my parents
Gus and Amy Chang
for always loving me and supporting me
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Chapter 1
The Disulfide Bond Formation System in *Salmonella enterica*

*Salmonella enterica*
*Salmonella* is a Gram-negative, facultative intracellular bacterial pathogen. It is a major cause of food-borne disease in the world (Wattiau et al., 2011). Outbreaks of nontyphoidal salmonellosis have been associated with contaminated raw eggs, poultry products, and dairy products (Voetsch et al., 2004). The clinical manifestations of nontyphoidal *Salmonella* strains typically cause a self-limiting gastroenteritis resulting in nausea, vomiting, abdominal cramps, and diarrhea. *Salmonella enterica* serovar Typhi and related serotypes can cause enteric fever. In severe cases such as with immunocompromised individuals, any *Salmonella* serotype may result in bacteremia and cause septic shock and death (Ryan and Ray, 2004).

*Salmonella enterica* is responsible for an estimated 1.4 million illnesses, 15,000 hospitalizations, and more than 400 deaths in the United States each year (Jones et al., 2008). The fiscal impact of salmonellosis, including costs of medical care, lost productivity, and contaminated food recalls, is at least $14.6 billion annually (Voetsch et al., 2004; Reinberg, 2010). *Salmonella enterica* is further divided into over 2,500 serovars based on its lipopolysaccharide O, capsular K, and flagellar H antigens (Jacobsen et al., 2011). The average genome conservation among these serovars is between 96 to 99%, and most genes present in both *Salmonella enterica* and *Escherichia coli* share between 80% to 85% identity (Edwards et al., 2002).

Pathogenesis and Type III Secretion
Upon ingestion of contaminated food products, *Salmonella enterica* passes through the stomach acid and the intestinal mucosa before adhering to the enterocytes and M cells of the small intestines (Ryan and Ray, 2004). However, in order to multiply and persist in animal hosts, it must traverse the epithelial layer and then survive in macrophages. To that end, it encodes two Type III Secretion Systems (T3SS) on *Salmonella* Pathogenicity Islands (SPI) 1 and 2 to invade non-phagocytic epithelial cells and persist in professional phagocytes, respectively (Miki et al., 2004; Steele-Mortimer, 2008).

The T3SS is a multi-molecular needle-like structure that spans the bacterial cell wall to deliver effector proteins into host cells (Figure 1.1). The T3SS itself consists of several components: the basal body composed of oligomeric inner membrane rings, the export apparatus imbedded in the inner membrane, the regulatory and chaperone proteins that assist in assembly, the outer membrane rings, the central cylinder that forms the inner rod spanning the periplasm and extending outward toward the host cell, and the translocon that inserts to form a pore in the host cell membrane (Kimbrough and Miller, 2002; Gerlach and Hensel, 2007; Steele-Mortimer, 2008). Through the needle complex, T3SS effector proteins are secreted or translocated from the bacterial cytosol directly into the host cell cytoplasm. While the structural proteins are well-conserved amongst all T3SS, the effector proteins are specific to each bacterial species, and in the case of *Salmonella enterica*, are specific to each SPI (Galan and Wolf-Watz, 2006).
Once in the host cell cytosol, the SPI-1 T3SS effector proteins induce ruffling of the host cell membrane, which facilitates the uptake of the bacteria (Zhou and Galan, 2001; Marlovits et al., 2006; Gerlach et al., 2007; Steele-Mortimer, 2008). Likewise, the SPI-2 effectors modulate the *Salmonella* containing vacuole (SCV) for survival within phagocytes (Waterman and Holden, 2003; Bueno et al., 2005).
Figure 1.1. The Type III Secretion System (T3SS) of Gram-negative bacteria.
A simplified diagram of the SPI-1 T3SS, a multi-molecular needle-like structure that spans the bacterial cell wall to deliver effector proteins into host cells. Adapted from various sources (Kimbrough and Miller, 2002; Galan and Wolf-Watz, 2006; Gerlach and Hensel, 2007; Lara-Tejero et al., 2011).
**Disulfide Bond Formation**

*Salmonella enterica* utilizes a family of periplasmic proteins termed the Disulfide Bond Formation (Dsb) System to maintain the structure and function of periplasmic proteins with multiple cysteine residues. Given the oxidizing environment of the periplasm, and the critical functionality of virulence factors such as the T3SS in regards to ability of *Salmonella* to invade and persist, it can be hypothesized that Dsb may be required for assembly of the T3SS needle complex. Although the various Dsb proteins have been characterized biochemically in *E. coli*, the potential link between this system and the virulence mechanisms of *Salmonella enterica* has not been well studied (Collet and Bardwell, 2002; Lasica and Jagusztyn-Krynicka, 2007).

*Salmonella enterica* has at least six Dsb proteins, labeled DsbA, DsbB, DsbC, DsbD, DsbE, and DsbG (Figure 1.2). DsbA oxidizes an unfolded protein with at least two cysteine residues, forming a disulfide bond. However, once DsbA has been reduced, it must be re-oxidized before it can oxidize another protein. DsbB is a transmembrane inner membrane protein that oxidizes DsbA, returning it to its active state. Together, DsbA and DsbB form the oxidative pathway of the Dsb system (Collet and Bardwell, 2002; Lasica and Jagusztyn-Krynicka, 2007).

However, DsbA may misfold a protein with more than two cysteine residues by forming disulfide bonds between incorrect cysteines. In this case, there are two dimeric proteins, DsbC and DsbG, that may isomerize or shuffle the disulfide bond to its correct conformation. Alternatively, DsbC and DsbG may also reduce the incorrect disulfide bond and return the protein to native, unfolded state, allowing DsbA to have another chance to correctly folding the protein (Rietsch et al., 1996; Nakamoto and Bardwell, 2004). It should also be noted that DsbC and DsbG, while performing similar roles, have different substrate specificities (Collet and Bardwell, 2002; Hiniker and Bardwell, 2004). After reducing the incorrect disulfide bond, DsbC and DsbG must be re-reduced. DsbD is a transmembrane inner membrane protein that reduces DsbC and DsbG, returning them to their active state. Together, DsbC, DsbD, and DsbG form the isomerization pathway of the Dsb system (Bessette et al., 1999; Collet and Bardwell, 2002; Lasica and Jagusztyn-Krynicka, 2007).

In addition to the genes encoding for DsbA, DsbB, DsbC, DsbD, and DsbG, *Salmonella enterica* also has two copies of the ccmG (dsbE) gene, which encodes for the DsbE protein. This protein is involved in cytochrome c biogenesis and transfers electrons from DsbD to CcmH, which in turn reduces the heme binding site of apocytochrome c and prepares it for heme binding (Feissner et al., 2006; Christensen et al., 2007; Richard-Fogal et al., 2007).

It should be noted that although the Dsb proteins are within the same periplasmic compartment, the oxidative and isomerization pathways are distinct, and DsbB does not oxidize DsbC or DsbG. This is due to the dimeric natures of DsbC and DsbG, which protects their active site cysteines from DsbB (Bader et al., 2001; Collet and Bardwell, 2002). As a result, cross-talk is limited and the Dsb system is able to function despite the opposing mechanisms of action of the oxidative and isomerization pathways.
All of the members of the Dsb system contain at least two cysteine residues arranged in a CXXC motif, which allows for electron transfer in the form of an intramolecular disulfide bond (Collet and Bardwell, 2002; Kadokura et al., 2003; Nakamoto and Bardwell, 2004; Messens and Collet, 2006; Lasica and Jaguszyn-Krynicka, 2007). DsbA, DsbC, and DsbG also possess a chaperone activity independent of the CXXC motif (Zheng et al., 1997; Chen et al., 1999; Kadokura et al., 2003; Messens and Collet, 2006).
Figure 1.2. The Disulfide Bond Formation (Dsb) System of Gram-negative bacteria.
The Dsb system is a family of at least six periplasmic proteins. The oxidative pathway consists of DsbA and DsbB, and forms disulfide bonds in proteins with multiple cysteine residues. The isomerization pathway consists of DsbC, DsbG, and DsbD, and may shuffle incorrectly formed disulfide bonds or reduce the disulfide bond entirely. DsbE is involved in cytochrome c (cyt c) biogenesis. Dark bold arrows indicate electron transfer. Dotted light lines indicate protein folding, from an unfolded protein (purple) to either a misfolded (yellow) or a correctly folded (green) state (Collet and Bardwell, 2002; Nakamoto and Bardwell, 2004; Stirnimann et al., 2006; Lasica and Jagusztyn-Krynicka, 2007).
Dsb Substrates
To further the notion that the Dsb system may be responsible for maintaining the structure and function of the protein systems important for the pathogenesis of Salmonella enterica, such as the T3SS, it is reported that impairment of the Dsb system leads to reduced virulence of other pathogenic Gram-negative bacterial species (Lasica and Jagusztyn-Krynicka, 2007). For example, the dsbA mutant of Pseudomonas aeruginosa has reduced intracellular survivability in infection of HeLa cells (Ha et al., 2003). Also, the dsbA mutant of Yersinia pestis secretes reduced amounts of Yops, virulence proteins secreted by a T3SS (Jackson and Plano, 1999). Furthermore, the dsbA mutant of Shigella flexneri is defective in cell-to-cell spread (Yu et al., 2000). In all of these cases, the reduced virulence is due to impairment of the extracytoplasmic protein transport machinery (Lasica and Jagusztyn-Krynicka, 2007).

Moreover, there are studies that have explored the direct interactions between Dsb and periplasmic virulence factors. In Salmonella enterica serovar Typhimurium, DsbA has been shown to directly bind to SpaA, a SPI-2 T3SS outer membrane protein, and to Flgl, a flagellar P ring component (Miki et al., 2004; Lin et al., 2008). In Pseudomonas aeruginosa, DsbA has been shown to directly bind to ExsA, a T3SS transcriptional regulator (Ha et al., 2003). In Yersinia pestis, DsbA has been shown to directly bind to YscC, which is an outer membrane secretin and a homolog of the SpaA from the SPI-2 T3SS (Koster et al., 1997; Jackson and Plano, 1999). And in Shigella flexneri, DsbA has been shown to directly bind to Spa32, a T3SS protein that regulates the length of the extracellular needle (Yu et al., 2000; Parsot, 2005).

Hypothesis
Despite the previous studies regarding the Dsb system, direct interactions between the Dsb system and the SPI-1 T3SS structural proteins of Salmonella remain unexplored. In fact, while the known non-SPI-1 substrates listed above have homologs among the SPI-1 T3SS proteins, none of them have cysteine residues and are not viable direct binding partners of the Dsb system (Lasica and Jagusztyn-Krynicka, 2007). Due to the critical function of the SPI-1 T3SS to facilitate invasion of the intestinal epithelium and in the pathogenesis of Salmonella enterica, and because a number of SPI-1 T3SS structural proteins have cysteine residues and may be substrates for the Dsb system, it is of interest to determine how the Dsb system interacts with the SPI-1 T3SS structural proteins and which SPI-1 T3SS proteins are direct binding partners of the Dsb system.


Sequence analysis of Salmonella enterica serovar Enteritidis strain P125109 on GenBank of the structural proteins InvH, InvA, SpaS, SpaR, SpaQ, SpaO, OrgA, and OrgB indicate the presence of two or more cysteine residues after cleavage of the signal sequences (Thomson et al., 2008). Also, PrgH and PrgK each have one cysteine
residue, and allow for the possibility of intermolecular disulfide bonds among the oligomeric inner membrane rings of the basal body (Table 1.1).

Given the numerous reports in the literature examining the various substrates of DsbA, it is interesting to note that there are no direct interactions between the Dsb system and the SPI-1 T3SS structural proteins that have been reported. Our preliminary results have indicated that mutations in the Dsb system result in reduced virulence in vitro and in vivo, and that the phenotypes observed are related to the function of the SPI-1 T3SS. Therefore, the hypothesis that the Dsb system plays a role in the secretion of virulence effectors by binding the T3SS structural proteins was further analyzed.
Table 1.1. Potential SPI-1 T3SS substrates of the Dsb system.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Location</th>
<th>Number of Cysteines</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>OrgB</td>
<td>Inner membrane</td>
<td>2</td>
<td>(Kimbrough and Miller, 2002; Galan and Wolf-Watz, 2006; Gerlach and Hensel, 2007; Thomson et al., 2008; Lara-Tejero et al., 2011)</td>
</tr>
<tr>
<td>OrgA</td>
<td>Inner membrane</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>PrgK</td>
<td>Inner membrane</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>PrgH</td>
<td>Inner membrane</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>SpaS</td>
<td>Inner membrane</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>SpaR</td>
<td>Inner membrane</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>SpaQ</td>
<td>Inner membrane</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>SpaO</td>
<td>Inner membrane</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>InvA</td>
<td>Inner membrane</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>InvH</td>
<td>Periplasm</td>
<td>4</td>
<td></td>
</tr>
</tbody>
</table>
**Scope**
Elucidation of the Dsb system and its various roles in pathogenesis will provide insight into how *Salmonella enterica* and other Gram-negative human pathogens facilitate invasion and colonization of hosts in order to establish a replicative niche, and may allow for the construction of attenuated strains of pathogenic bacteria for use as vaccine candidates. Furthermore, analysis of the binding capacity of the Dsb proteins may identify critical motifs and substrates as potential targets for antibacterial drugs.

This study examines the role of the Dsb system in effector protein secretion and pathogenesis of *Salmonella enterica*. Chapter 2 analyzes the effects of mutations in the Dsb system on fitness of the bacteria. Chapter 3 determines the invasiveness of the *dsb* mutants of *Salmonella in vitro* and their virulence in a mouse model of infection. Chapter 4 characterizes the impact of the *dsb* mutant strains on SPI-1 T3SS effector secretion and seeks to identify which structural proteins are direct substrates of the Dsb system. The final chapter provides a discussion concerning the current study and what it adds to the field of pathogenesis of enteric bacteria and protein-protein interactions.
Chapter 2
Characterizing the Impact of Mutations in the Dsb System on Bacterial Fitness

Abstract
*Salmonella enterica* is a food-borne pathogen that has a significant public health impact. Elucidation of the virulence mechanisms of *Salmonella enterica* and other Gram-negative human pathogens is an area of intense study in the field. Prior to this study, a transposon mutant library of *Salmonella* was constructed and screened for sensitivity to reactive oxygen intermediates to identify potential genes that were important for virulence. One transposon mutant of interest had an insertion in the *dsbD* gene, which encoded a member of the Disulfide Bond Formation (Dsb) System, which maintains the structure of periplasmic proteins. In order to study the potential role of the Dsb system in virulence of *Salmonella*, mutant strains were constructed with deletions in the *dsb* genes. The *dsb* mutant strains were found to have increased amount of intracellular proteins present in the culture media. Likewise, the *dsb* mutant strains were less motile than the wild-type strain. Complementation of the *dsb* mutations restored or partially restored the wild-type phenotype. In addition, the *dsb* mutant strains were found to have varying susceptibility to hydrogen peroxide stress. These results suggested that the Dsb system has an important role in bacterial fitness.
Introduction

*Salmonella enterica* is a major food-borne bacterial pathogen. The ramifications of salmonellosis in infected populations and subsequent recalls of contaminated food products are extremely detrimental to public health. Of the over 2,500 serovars of *Salmonella enterica*, the serovar Enteritidis has in recent years become a leading serotype reported in the United States (Lu *et al.*, 1999; Jones *et al.*, 2008).

To further examine the ability of *Salmonella enterica* serovar Enteritidis to cause disease, a set of clinical isolates was obtained from the California Department of Health Services and were analyzed for bacterial virulence phenotypes *in vitro* and *in vivo*. The lethal dose, 50% (LD$_{50}$) of the clinical isolates was calculated in a mouse infection to determine their pathogenicity *in vivo*, and then the results were correlated with the resistance of the clinical isolates to killing by reactive oxygen intermediates (ROI) and reactive nitrogen intermediates (RNI). One clinical isolate, SE2472, was found to be extremely virulent *in vivo*, and highly resistant to killing by both ROI and RNI (Lu *et al.*, 1999).

For the purposes of this study, the clinical isolate SE2472 was used as the wild-type strain. To identify genes that would be important for virulence of *Salmonella enterica* serovar Enteritidis, a transposon mutant library was constructed as described earlier (Clavijo *et al.*, 2006). The library consists of approximately 7,000 independent mutants, each with a single, random transposon insertion in the genome.

The transposon mutant strains were then grown in the presence of 3 mM H$_2$O$_2$, which may directly cause damage to the bacterial membrane (Janssen *et al.*, 2003). However, H$_2$O$_2$ may also readily diffuse across the bacterial membrane, and once inside the bacterial cell, it may react with iron or copper ions to form highly cytotoxic hydroxyl radicals (OH•) via the Fenton reaction (Das and Essman, 1990; Buchmeier *et al.*, 1995). It is these hydroxyl radicals that cause DNA damage and are a major cause of cell death (Imlay and Linn, 1988; Janssen *et al.*, 2003). Therefore, it is of particular interest to identify those transposon mutant strains that are more susceptible to H$_2$O$_2$ exposure than the wild-type strain, an indication that the transposon inserted into a gene of importance to ROI resistance.

The transposon mutant strains that did not grow in the presence of 3 mM H$_2$O$_2$ were then selected and sequenced to determine where the transposon inserted and which genes were disrupted. One mutant, HS32, was found to have a transposon insertion in the 3' end of the *dsbD* gene (Clavijo and Lu, unpublished data). A deletion mutant of the *dsbD* gene was made using the Red recombinase system (Datsenko and Wanner, 2000). The Δ*dsbD* mutant was also found to be more susceptible to H$_2$O$_2$ than the wild-type strain and was less virulent than the wild-type strain in a competitive infection of BALB/c mice (Clavijo and Lu, unpublished data).

The DsbD protein is part of the Disulfide Bond Formation (Dsb) System, a family of proteins that maintains the structure and function of periplasmic proteins with multiple cysteine residues. In the oxidizing environment of the periplasm, maintaining the correct
structures of virulence factors, including those of the T3SS, is critical for the bacteria to survive and persist inside the host. There are reports that putative disulfide bond containing proteins in *Pseudomonas aeruginosa* are key responders to oxidative stress induced by exposure to H$_2$O$_2$ (An *et al.*, 2010). However, such oxidative stress responses in *Salmonella enterica* and the role that the Dsb system plays in the response are not well characterized.

In order to further examine the role of the Dsb system in bacterial fitness, a series of deletion mutants were made in the other genes of the Dsb system besides *dsbD* (Table 2.1). The panel of mutant strains was then tested for membrane integrity by determining levels of an exclusively intracellular protein in the culture supernatant, and for motility by measuring the colony growth on semi-solid medium, and for resistance to ROI by exposure to H$_2$O$_2$. Taken together, these assays will characterize the function of the Dsb system in the fitness of *Salmonella enterica*. 
Materials and Methods

Reagents
Growth media for all bacteria strains were purchased from BD Diagnostics (Sparks, MD). Chemicals and antibiotics were obtained from Sigma-Aldrich (Saint Louis, MO) unless otherwise indicated. Restriction and modifying enzymes for manipulation of DNA were obtained from New England BioLabs (Ipswich, MA). Custom oligonucleotides were purchased from Sigma-Aldrich (St. Louis, MO).

Bacterial strains
Salmonella enterica serovar Enteritidis clinical isolate SE2472 was used as the parental strain for all experiments (Lu et al., 1999). Escherichia coli DH5α (Invitrogen, Carlsbad, CA) was used as the host for all recombinant DNA manipulations. A panel of knockout deletion mutants of Salmonella was constructed by replacing the dsb genes with an antibiotic resistance cassette (Table 2.1) (Datsenko and Wanner, 2000). In brief, knockout primers amplified an antibiotic resistance cassette conferring resistance to either kanamycin or chloramphenicol with homology regions flanking the PCR amplicon (Table 2.2). The amplicons were transformed into a strain expressing a plasmid encoding the Red recombinase system, allowing for replacement of the dsb gene with the antibiotic resistance cassette. Antibiotic-resistant transformants were selected, and screened, and then transduced using bacteriophage P22 into a fresh wild-type background. Phage-free transductants were screened by selecting light-colored colonies on Evans blue uranine plates, selected by growth on LB agar plates supplemented with either 75 μg/ml kanamycin or 34 μg/ml chloramphenicol, screened by PCR using verification primers, and then sequence-verified. For the ΔdsbA/C double mutant, the ΔdsbA mutation was transduced using bacteriophage P22 into the ΔdsbC background, and phage-free transductants were selected, screened, and sequence-verified. The dsb mutants were complemented with plasmids expressing the dsb genes on a pRB3-273C vector (Table 2.1). In brief, verification primers amplified the dsb genes from SE2472 (Table 2.2), and the PCR amplicons were digested with HindIII, and cloned into the pRB3-273C vector at the HindIII site. The resulting plasmids were sequence-verified and the transformed into their respective dsb mutant strains (Lu et al., 2002).

Growth curve
Strains were inoculated into 3 ml of Difco Luria-Bertani (LB) medium supplemented with antibiotics and incubated at 37°C with shaking at 225 rpm for 16 hours. A 1:1,000 dilution was inoculated into 15 ml of fresh LB medium and incubated at 37°C with shaking for 24 hours. Aliquots were taken at 0, 1, 2, 4, 6, 8, and 24 hours, and dilutions were plated onto LB agar plates. The plates were then incubated at 37°C for 16 hours and bacterial CFU determined. Strains were also inoculated into 15 ml of M9 minimal medium, prepared with 1X Difco M9 Minimal Salts supplemented with 0.4% glucose, 2 μM MgSO₄, and 0.1 mM CaCl₂, and incubated at 37°C with shaking for 24 hours. Aliquots were taken at 0, 1, 2, 4, 6, 8, and 24 hours, and dilutions were plated onto LB agar plates. The plates were then incubated at 37°C for 16 hours and bacterial CFU determined.
Total extracellular protein gel
Strains were inoculated into 3 ml of LB medium supplemented with antibiotics and incubated at 37°C with shaking at 225 rpm for 16 hours. Culture supernatant proteins were isolated by precipitation in 6.25% trichloroacetic acid (TCA) for 30 minutes on ice. The samples were then washed three times with cold acetone to remove TCA, and the resulting pellet resuspended in urea sample buffer containing 8 M urea and 10 mM Tris pH 7.5. Culture supernatant proteins were quantified by DC Protein Assay (Bio-Rad, Hercules, CA), and 75 μg of each protein sample were separated by SDS-PAGE. The gel was fixed in 10% glacial acetic acid and 40% methanol for 30 minutes, and then rinsed in water for 5 minutes. The gel was stained with a Coomassie Blue G solution containing 10% phosphoric acid, 10% ammonium sulfate, 0.12% Brilliant Blue G, and 20% methanol at room temperature with shaking for 16 hours. The gel was destained in water with shaking until the desired contrast was achieved.

β-galactosidase release assay
Strains were transformed with plasmids expressing the lacZ gene on a pRB3-273C vector (Table 2.1). Strains were inoculated into 5 ml of LB medium supplemented with antibiotics and incubated at 37°C with shaking at 225 rpm for 16 hours. Overnight cultures were centrifuged and culture supernatant was filtered through 0.2 μm filter to remove any carryover bacteria. The filtered culture supernatant was then concentrated in a 10,000 MWCO filter (EMD Millipore, Billerica, MA) by centrifuging at 7,360 x g for 10 minutes in a SL-50T rotor in a Sorvall Super T21 centrifuge (Thermo Fisher Scientific, Waltham, MA). The culture pellets were resuspended in 200 μl PBS, chilled on ice for 10 minutes, then sonicated three times at 10% power for 20 seconds each using a Branson S-450D sonicator (Branson Ultrasonics, Danbury, CT). The sonicated sample was then chilled on ice for 30 minutes, and centrifuged at 16,000 x g for 15 minutes in an Eppendorf 5417R centrifuge (Eppendorf, Hauppauge, NY), and the resulting supernatant containing whole cell lysate was isolated. Then the concentrated supernatant protein and the sonicated whole cell lysate were quantified by DC Protein Assay (Bio-Rad, Hercules, CA). The presence of β-galactosidase in the culture supernatant and culture pellet fraction was then tested for using the substrate 4-methyl-umbelliferyl β-D-galactopyranoside (MUG). The associated buffers are described in a recent study on Streptococcus mutans (Honeyman et al., 2002). In brief, MUG Assay Buffer was composed of 60 mM K2HPO4, 40 mM KH2PO4, and 100 mM NaCl, and the MUG Solution was composed of 0.8 mg MUG diluted in 2 ml DMSO. Then, 700 μg of the concentrated supernatant protein or the sonicated whole cell lysate was combined with 40 μl MUG Assay Buffer and 10 μl MUG Solution, and incubated at room temperature for 30 minutes. Then 200 μl MUG Assay Buffer was added to slow, but not stop, the reaction for reading. Then 200 μl of the sample was added to an opaque 96-well plate and fluorescence was read at 460 nm (355 nm excitation) in a SpectraMax M2 plate reader (Molecular Devices, Sunnyvale, CA).

Motility assay
Strains were inoculated into 3 ml of LB medium supplemented with antibiotics and incubated at 37°C with shaking at 225 rpm for 16 hours. A sterile inoculation needle was
dipped into liquid culture and then stabbed onto BBL Motility Test Medium agar plates. The plates were then incubated at 37°C until colonies had grown to a sufficient size and diameters of the bacterial zones of growth were measured.

**Over-expression assay**
The Δ*dsbA* mutant strain was transformed with pRB3-*dsbC*, and the Δ*dsbC* mutant strain was transformed with pRB3-*dsbA*. The Δ*dsbA/C* double mutant strain was transformed with either pRB3-*dsbA* or pRB3-*dsbC* (Table 2.1). These over-expression strains were then tested for motility as described above.

**H₂O₂ resistance assay**
Strains were inoculated into 3 ml of LB medium supplemented with antibiotics and incubated at 37°C with shaking at 225 rpm for 16 hours. A 1:1,000 dilution was inoculated into 2 ml of fresh LB medium and hydrogen peroxide was added to a final concentration of 2 mM. Cultures were incubated at 37°C with shaking for 6 hours. Aliquots were taken at 0, 1, 2, 4, and 6 hours, and dilutions were plated onto LB agar plates. The plates were then incubated at 37°C for 16 hours and bacterial CFU determined.
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Results

The Dsb system was not essential for growth in vitro.

To determine the role of the Dsb system in the pathogenesis of *Salmonella enterica*, a panel of deletion mutant strains was constructed with mutations in the *dsb* genes (Table 2.1). Each *dsb* open reading frame was replaced by an antibiotic resistance cassette, either Kan’ or Cm’ (Datsenko and Wanner, 2000). In addition, a double mutant strain was constructed by transducing the Δ*dsbA* mutation using bacteriophage P22 into the Δ*dsbC* background. The single mutant strains were also complemented by introducing the respective *dsb* gene on a pRB3-273C expression vector. Also, empty vector was transformed into the single mutant strains as a control.

In order to ascertain the effects of the *dsb* mutations on bacterial fitness and pathogenesis, it is first necessary to establish a background phenotype to which any virulence defects can be compared. This background was established by measuring the growth of the *dsb* mutant strains in complete (LB) and minimal (M9) media over the course of logarithmic phase (Su *et al.*, 2009). Growth in minimal medium was tested due to previous reports of the *dsb* mutant strains of *Escherichia coli* growing poorly in minimal medium lacking cysteine (Vertommen *et al.*, 2008).

The Δ*dsbA*, Δ*dsbC*, and Δ*dsbA/C* mutant strains were grown in LB medium at 37°C with shaking for 24 hours. Aliquots were taken at 0, 1, 2, 4, 6, 8, and 24 hours and bacterial CFU was recovered. The Δ*dsbA*, Δ*dsbC*, and Δ*dsbA/C* mutant strains had similar growth kinetics as the wild-type strain throughout log phase and until at least 24 hours (Figure 2.1 A). The single mutants’ respective complemented strains also had similar growth kinetics as the wild-type strain. Likewise, when grown in M9 medium, the Δ*dsbA*, Δ*dsbC*, and Δ*dsbA/C* mutant strains had similar growth kinetics as the wild-type strain throughout log phase and until at least 24 hours (Figure 2.1 B). The single mutants’ respective complemented strains also had similar growth kinetics as the wild-type strain. It should be noted that in minimal medium, the onset of logarithmic growth was delayed by 2 hours when compared to rich medium; however, the curves are consistent with that of the wild-type strain in minimal medium.

The Δ*dsbB*, Δ*dsbD*, Δ*dsbE1/2*, and Δ*dsbG* mutant strains, as well as the complemented strains for Δ*dsbB* and Δ*dsbD*, as well as the empty vector controls, were also tested for growth defects in LB and M9 media, but there were no observable differences in growth kinetics (data not shown). It should be noted that while the Δ*dsbE1/2* and Δ*dsbG* mutant strains were constructed, the corresponding complemented strains were not made due to a variety of factors. The complex nature of the Dsb system necessitated narrowing the focus of this study. Since the role of DsbE in cytochrome c biogenesis is well characterized and DsbG has similar function to DsbC, albeit with different substrate specificity, it was decided to focus on DsbA-D, with a greater emphasis on DsbA and DsbC as the major oxidoreductases of the Dsb system (Hiniker and Bardwell, 2004; Feissner *et al.*, 2006).
Figure 2.1. The *dsb* mutants of *Salmonella* had similar growth kinetics as the wild-type strain.

The wild-type strain, the *dsb* mutant strains, and their respective complemented strains were grown at 37°C with shaking for 24 hours in LB (A) and M9 (B) media. Bacterial concentrations were determined by plating. At least three experiments were performed, and the results of a representative experiment performed in triplicate were shown. The error bars indicate standard deviations.
The Dsb system had a role in outer membrane integrity. The ability of the Salmonella bacterium to survive in hostile environments can be attributed to the numerous transmembrane virulence factors, such as flagella, secretion systems, and adherence factors (Lasica and Jagusztyn-Krynicka, 2007). It was observed that the dsb mutant strains had drastically different secretion profiles when their culture supernatant were run on SDS-PAGE and stained with Coomassie Blue G. More specifically, it was observed that the ΔdsbA, ΔdsbC, and ΔdsbA/C mutant strains had a greater overall protein content present in the culture supernatant than that of the wild-type strain (Figure 2.2). On the other hand, strains such as ΔdsbD had very similar banding patterns to that of the wild-type strain. Upon consulting the literature, it appears there are multiple reports regarding the Dsb system and a possible role in folding outer membrane proteins that are critical for outer membrane integrity and protein secretion (Ruiz et al., 2010; Wager et al., 2010). However, there were no concrete explanations as to why only some dsb mutant strains, and not others, had greater overall protein content in the culture supernatant.

In order to assess the outer membrane integrity of the dsb mutant strains, a pRB3-273C expression vector expressing the lacZ gene was transformed into the wild-type, ΔdsbA, ΔdsbC, and ΔdsbA/C strains. The lacZ gene encodes β-galactosidase, a protein that is exclusively intracellular and is not trafficked outside the bacterial cell. Moreover, β-galactosidase can be readily detected by the addition of an enzymatic substrate, such as 5-bromo-4-chloro-indoly-β-D-galactopyranoside (X-gal), chlorophenol red-β-D-galactopyranoside (CPRG), or 4-methyl-umbelliferyl β-D-galactopyranoside (MUG). As a result, measurable levels of β-galactosidase in the culture supernatant were used as readouts for outer membrane integrity.

The lacZ-expressing transformed strains were then grown in liquid culture, and their supernatant and whole cell lysate were separated. Initial tests using CPRG as the substrate and measuring for colorimetric changes in absorbance at 574 nm were unsuccessful due to a lack of signal sensitivity (data not shown). Next, MUG was used as the enzymatic substrate due to the increased discriminatory power of the fluorescent signal (Honeyman et al., 2002).

The fluorescent readings from the wild-type strain (without lacZ) were subtracted from those readings obtained from the lacZ-expressing transformed strains. The subtracted fluorescent readings in the whole cell lysate fractions of the dsb mutant strains were comparable to the readings from the SE2472 [lacZ] strain (data not shown). These readings indicated that the amount of β-galactosidase in the cytosol of the lacZ-expressing transformed strains was consistent.

The subtracted fluorescent readings in the supernatant fractions of the ΔdsbA [lacZ] and ΔdsbA/C [lacZ] strains were significantly higher than that of the readings from the SE2472 [lacZ] strain, by approximately 18-fold and 27-fold, respectively (p < 0.05). Likewise, the readings for ΔdsbC [lacZ] were nearly the same as that of the SE2472 [lacZ] (Figure 2.3). However, these readings were not as high as those recorded in the whole cell lysate fractions (data not shown). This suggests that there was a substantial
amount of β-galactosidase in the culture supernatant, which is interesting as β-
galactosidase is an exclusively intracellular protein and there are no known secretion
mechanisms.
Figure 2.2. The ΔdsbA, ΔdsbC, and ΔdsbA/C mutants of *Salmonella* had greater overall extracellular protein content.

Total protein from the culture supernatant of the wild-type strain and the *dsb* mutant strains were isolated by TCA precipitation, quantified, and 75 μg was separated by SDS-PAGE. BenchMark Protein Ladder (Invitrogen, Carlsbad, CA) was used as ladder. The gel was stained with a Coomassie Blue G solution and destained to achieve optimal contrast. At least three experiments were performed, and the results of a representative experiment were shown.
Figure 2.3. The ΔdsbA and ΔdsbA/C mutations resulted in increased β-galactosidase levels in the culture supernatant.
Culture supernatant and whole cell lysate from the lacZ-expressing transformed SE2472 strain and the dsb mutant strains were added to MUG substrate and fluorescence readings were obtained. At least three experiments were performed, and the results of a representative experiment performed in triplicate were shown. The error bars indicate standard deviations. Asterisks denote a p-value < 0.05 calculated by Student's t-test.
The Dsb system was required for motility.

The ability of the bacteria to swim through the extracellular milieu is critical to finding a niche in which to replicate and avoid the host's immune system. There have been several reports in *Escherichia coli* of the requirement for DsbA to fold the FlgI protein, which is a component of the P ring of the flagellar periplasmic structure (Hiniker and Bardwell, 2004; Macnab, 2004). Electron microscopy images of an *E. coli* strain with a mutated *dsbB* gene show basal bodies lacking L and P rings as well as the hook and filament proteins (Dailey and Berg, 1993). Furthermore, it was empirically observed that the *dsb* mutant strains tended to settle in their culture tubes after several hours without shaking; this phenomenon was not observed in the wild-type strain (data not shown). These observations led to the notion that the Dsb system be required for the ability of the bacteria to remain motile in liquid culture.

In order to assess the motility of the *dsb* mutant strains, inoculation needles were immersed in liquid cultures and stabbed into semi-solid motility test agar plates, and growth and motility of the bacteria was monitored. The *dsb* mutant colony sizes were measured and compared to that of the wild-type strain, and the ratios were plotted.

For the Δ*dsbA*, Δ*dsbC*, and Δ*dsbA/C* strains, their ratios compared to wild-type were significantly less than 1.0 (*p* < 0.05). The greatest differences were observed with the Δ*dsbA* and Δ*dsbA/C* mutants with ratios of 0.22 and 0.15, respectively, and even the Δ*dsbC* mutant had a measurably reduced ratio of 0.61. The complemented strains returned the colony sizes similar to that of the wild-type strain (Figure 2.4).

These results support the observation of the *dsb* mutants of *Salmonella* settling in their culture tubes on the bench top. In fact, the mutant strain lacking both the *dsbA* and *dsbC* genes yielded the smallest colony size. The combination of qualitative and quantitative data suggests that the Dsb system was necessary for the motility of the bacteria in a liquid environment.
Figure 2.4. The dsb mutations resulted in decreased colony sizes. The wild-type strain, the dsb mutant strains, and their respective complemented strains were stabbed into semi-solid motility test media and growth was monitored. At least three experiments were performed, and the results of a representative experiment performed in triplicate were shown. The error bars indicate standard deviations. Asterisks denote a $p$-value < 0.05 calculated by Student’s $t$-test.
Complementation of the \( dsb \) mutations.

It is noteworthy that in the previous assays, the most severe phenotypes were observed in the \( \Delta dsbA \) and \( \Delta dsbA/C \) mutant strains, while the \( \Delta dsbC \) mutant strain had a more intermediate phenotype compared to the wild-type strain (Figures 2.3 and 2.4). These results seemed to imply that the DsbA protein still produced by the \( \Delta dsbC \) mutant was able to partially compensate for the loss of DsbC. This might be possible, despite DsbA being an oxidase and DsbC being a reductase, as the two proteins are part of the same overall system with the same goal of folding cysteine-containing proteins.

To investigate any potential cross-complementation in the \( dsb \) mutant strains, the pRB3-273C plasmids expressing \( dsbA \) and \( dsbC \) were transformed into the opposing mutant strain; that is, pRB3-\( dsbA \) was transformed into the \( \Delta dsbC \) mutant strain, and vice versa. These newly transformed strains were effectively over-expressing the other Dsb protein. Furthermore, each plasmid was transformed separately into the \( \Delta dsbA/C \) mutant strain, to partially complement the double mutation. These partial complements also effectively over-expressed a Dsb protein when compared to the untransformed \( \Delta dsbA/C \) mutant strain.

In order to determine any effect on the bacterial fitness of these over-expression strains, a motility assay was performed. Inoculation needles were immersed in liquid cultures and stabbed into semi-solid motility test agar plates, and growth of the bacteria was monitored. The over-expression strains’ zone of growth were measured and compared to that of the wild-type strain, and the ratios were plotted.

For the \( \Delta dsbA [dsbC] \) strain, the ratio rose from 0.22 in the \( \Delta dsbA \) mutant strain to 0.66, which was similar to the \( \Delta dsbA [dsbA] \) (ratio of 0.64) complemented strain. Likewise, for the \( \Delta dsbC [dsbA] \) strain (ratio of 1.04), the ratio was approximately the same as the wild-type strain, which was similar to the \( \Delta dsbC [dsbC] \) complemented strain (ratio of 1.12) (Figures 2.4 and 2.5). For the over-expression strains to have a similar phenotype as the complemented strains, it can be suggested that over-expression of Dsb proteins can have a comparable effect on bacterial motility as complementing the mutation.

Moreover, both the \( \Delta dsbA/C [dsbA] \) (ratio of 0.78) and \( \Delta dsbA/C [dsbC] \) (ratio of 0.65) strains had substantially higher ratios than the \( \Delta dsbA/C \) mutant (ratio of 0.15) (Figures 2.4 and 2.5). These results suggested that increased amount of a given Dsb protein can partially rescue the defect of a mutation in another \( dsb \) gene.
Figure 2.5. Over-expression of the *dsb* genes partially rescued the defect in motility.

The wild-type strain and the over-expression *dsb* mutant strains were stabbed into semi-solid motility test media and growth was monitored. At least three experiments were performed, and the results of a representative experiment performed in triplicate were shown. The error bars indicate standard deviations. Asterisks denote a *p*-value < 0.05 calculated by Student's *t*-test.
The Dsb system had a role in resistance to reactive oxygen intermediates.

Once *Salmonella* has traversed the host's epithelial layer, it encounters professional phagocytes such as macrophages, and is subjected to, among other defense mechanisms, oxidative burst which may damage or destroy the bacteria. Therefore, the bacteria must be able to resist the action of reactive oxygen intermediates to survive and persist. It was found that a transposon insertion in the 3' end of the *dsbD* gene resulted in lack of growth of the mutant strain in the presence of 3 mM H$_2$O$_2$. Furthermore, a clean deletion mutant that replaced the *dsbD* gene with a kanamycin resistance cassette was unable to be recovered on solid medium after growth at 37°C with shaking for 4 hours in liquid LB medium supplemented with 3 mM H$_2$O$_2$ (Clavijo and Lu, unpublished data). It was the original intent of the transposon mutant screen to identify novel genes which would be associated with the wild-type strain's ability to resist ROI and RNI stress.

The *dsb* mutant strains of *Salmonella* were tested for resistance to H$_2$O$_2$. The *dsb* mutant strains were grown at 37°C with shaking for 6 hours in the presence of 2 mM H$_2$O$_2$. Aliquots were taken at 0, 1, 2, 4, and 6 hours and bacterial CFU was recovered. The Δ*dsbD* mutant was more susceptible to 2 mM H$_2$O$_2$ than the wild-type strain from 4 to 6 hours post-exposure (p < 0.05), a similar trend to previously unpublished data (Figure 2.6 A).

The Δ*dsbA*, Δ*dsbA/C*, and Δ*dsbE1/2* mutant strains were more resistant to 2 mM H$_2$O$_2$ than the wild-type strain; Δ*dsbA* was more resistant from 2 to 4 hours post-exposure, Δ*dsbA/C* was more resistant from 1 to 4 hours post-exposure, and Δ*dsbE1/2* was more resistant from 4 to 6 hours post-exposure (p < 0.05) (Figure 2.6 B). In contrast, the Δ*dsbB*, Δ*dsbC*, and Δ*dsbG* mutant strains were more susceptible to 2 mM H$_2$O$_2$ than the wild-type strain from 4 to 6 hours post-exposure (p < 0.05). Of particular interest are the Δ*dsbC* and Δ*dsbG* mutant strains, which did not have any measurable bacteria recovered at 6 hours post-exposure to H$_2$O$_2$ (Figure 2.6 A).

It should be noted that the concentration of H$_2$O$_2$ used to inflict stress upon the bacterial strains is rather subjective. For example, one study of *E. coli* determined the optimal concentration for a bactericidal effect to be 1 mM (Imlay and Linn, 1988). However, another study in *Salmonella enterica* serovar Typhimurium used a higher concentration of 4 mM to obtain a measurable phenotype (Buchmeier et al., 1995). It is also interesting to note that the physiological concentration of H$_2$O$_2$ released by the innate immune cells during oxidative burst is not known due to the rapid breakdown of H$_2$O$_2$ in vivo (Das and Essman, 1990).

However, due to the disparate phenotypes among the various *dsb* mutant strains, the complemented strains were not tested for their resistance to ROI stress. As it is, the data obtained from the *dsb* mutant strains exposed to 2 mM H$_2$O$_2$ indicate somewhat contradictory results, which suggest a much more complicated stress response mechanism that transcends the role of a single Dsb protein. The roles of the Dsb system and each of the Dsb proteins in the resistance of *Salmonella* to reactive oxygen intermediates needs to be further characterized.

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Figure 2.6. The *dsb* mutants of *Salmonella* had varying of resistance to H$_2$O$_2$.

The wild-type strain and the *dsb* mutant strains were grown at 37°C with shaking for 6 hours in LB medium supplemented with 2 mM H$_2$O$_2$. Bacterial concentrations were determined by plating, indicating those strains that were more susceptible to H$_2$O$_2$ exposure (A) and those that were more resistant to H$_2$O$_2$ exposure (B). At least three experiments were performed, and the results of a representative experiment performed in triplicate were shown. The error bars indicate standard deviations. Asterisks denote a *p*-value < 0.05 calculated by Student's *t*-test.
Discussion
The Dsb system of Gram-negative bacteria is important for maintaining the structure and function of periplasmic proteins. Due to the importance of the periplasm and outer membrane in regards to the multitude of virulence factors of Salmonella, such as flagella, secretion systems, and adherence factors, it can be theorized that the Dsb system in Salmonella is involved in multiple virulence mechanisms. The assays presented in this chapter sought to establish the impact of the Dsb system on bacterial fitness.

Each dsb gene of Salmonella was deleted individually and the resulting mutant strains' ability to grow in vitro was not affected (Figure 2.1 A and B). This provided a suitable baseline to which future assays can be compared, since any virulence phenotypes observed would likely be specific rather than a gross inability of the strain to grow. Moreover, since the introduction of complementing plasmids also did not affect the growth curves of the strains, it can be concluded that the pRB3-273C expression vector did not adversely interfere with the bacteria's fitness.

The focus on the Dsb system was initially due to the discovery of a transposon mutant with a disruption of the dsbD gene that was more susceptible to H$_2$O$_2$ than the wild-type strain. It was thought that characterizing the mechanism by which the pathogenic clinical isolate might resist oxidative stress would provide insight into the ability of the bacteria to survive and replicate. However, when the panel of dsb mutants was exposed to H$_2$O$_2$ in a similar assay, the results were ambiguous. Some strains, such as ΔdsbB, ΔdsbC, ΔdsbD, and ΔdsbG were more susceptible to H$_2$O$_2$ than the wild-type strain (Figure 2.6 A). However, other strains such as ΔdsbA, ΔdsbA/C, and ΔdsbE1/2 were more resistant to the same concentration of H$_2$O$_2$ than the wild-type strain (Figure 2.6 B). These seemingly contradictory results would seem to suggest that some Dsb proteins were more important than others regarding the ability of the bacteria to resist oxidative stress. It is possible that this mechanism of resistance occurs externally to the Dsb system, such as with the electron transport chain in the inner membrane and cytosol, and only peripherally involves the various Dsb proteins.

Nevertheless, the H$_2$O$_2$ resistance assay provided several critical lessons. First, there are many virulence mechanisms that are potentially impacted by the dsb mutations, a fact that is clear since the ΔdsbD mutant was also less able to colonize spleen and liver than the wild-type strain in a competitive infection of BALB/c mice (Clavijo and Lu, unpublished data). Second, the large number of dsb genes necessitated a narrowing of the focus. The function of DsbE was already well characterized in the biogenesis of cytochrome c (Feissner et al., 2006). Furthermore, DsbG had a similar function, as a reductase, as an isomerase, and as a chaperone, to DsbC, albeit with different substrate specificity (Collet and Bardwell, 2002; Hiniker and Bardwell, 2004). To enhance the quality of the study of the Dsb system, the focus was placed on DsbA, DsbB, DsbC, and DsbD, as the four major members of the Dsb system. Of particular note were the two major enzymes, DsbA and DsbC, which had the greatest potential to affect virulence as they would likely bind substrates associated with virulence.
To that end, those respective mutant strains, as well as the exceedingly interesting \( \Delta dsbA/C \) double mutant were tested for outer membrane integrity by determining the extracellular levels of an exclusively intracellular protein. The \( \Delta dsbA \) and \( \Delta dsbA/C \) mutant strains tested had levels of \( \beta \)-galactosidase in the culture supernatant significantly higher than that of the wild-type strain (Figure 2.3). Since there is no active mechanism by which \( \beta \)-galactosidase is secreted by the bacteria, it can be suggested that the protein leaked out of the cell due to compromised inner and outer membranes. These results are not unexpected given some reports regarding the folding of outer membrane proteins by DsbA in \( E. coli \) (Ruiz et al., 2010). However, while outer membrane integrity is nevertheless an important component for bacterial fitness, it is not an active virulence mechanism that can explain the preliminary evidence of reduced virulence \textit{in vivo}. Therefore, the Dsb system must be performing another function that is more relevant to the overall ability of the bacteria to cause disease.

Again, attention was turned to the plethora of periplasmic virulence factors. One such prominent feature is the flagellar motor complex that spans the periplasm (Macnab, 2004). Evolutionarily similar to the T3SS, the flagella has important roles in allowing the bacteria to find a replicative niche in which to propagate. In order to determine a role for the Dsb system in flagellar function, the \( dsb \) strains were grown on semi-solid motility test medium and their growth was monitored. The \( \Delta dsbA \), \( \Delta dsbC \), and \( \Delta dsbA/C \) mutant strains were significantly less motile than the wild-type strain. The respective complemented strains partially or completely restored the wild-type phenotype (Figure 2.4). These results suggested that the Dsb proteins were indeed required for proper function of the flagellar motor complex. This is unsurprising given previous reports that indicate the importance of DsbA in binding FlgI (Hiniker and Bardwell, 2004). In addition, it was found that the DsbC protein was also involved in the function of the flagella, an observation that has not been reported previously. This suggested that while DsbA is indeed important, the overall status of the Dsb system is crucial for the proper function of the virulence mechanisms.

Further evidence to the interplay among the various Dsb proteins could be seen when the \( dsb \) mutant strains were transformed with the plasmid expressing another Dsb protein, i.e. \( \Delta dsbA \) [\( dsbC \)] and \( \Delta dsbC \) [\( dsbA \)]. These cross-complementing strains had the added benefit of effectively over-expressing a Dsb protein. To ascertain their impact on bacterial fitness, those strains were tested for growth on semi-solid motility test medium and the results compared to the previous motility assay. In all cases, the over-expression strains had partially or completely restored the phenotypes to wild-type levels (Figures 2.4 and 2.5). These results confirmed the notion that there is a certain redundancy in the Dsb system, where a defect in one member can be at least be partially compensated by the presence of another member. This may explain why the \( \Delta dsbA/C \) double mutant had a more severe phenotype in Figures 2.3 and 2.4, since both the oxidative and isomerization pathways of the Dsb system were effectively rendered nonfunctional.

Taken together, the results presented in this chapter have established the importance of the Dsb system in bacterial fitness. The \( dsb \) mutant strains will be examined in future
chapters with more specialized virulence assays. Upon analyzing those results, a more discrete mechanism for the Dsb system can be elucidated in regards to the pathogenesis of *Salmonella enterica*. 
Chapter 3
Determining the Role of the Dsb System in the Virulence of Salmonella

Abstract
Salmonella enterica is a successful human pathogen primarily because it is able to colonize and persist within a multitude of animal hosts, especially those which are integral to the human food supply. In order to study how the Dsb system contributes to this aspect of the Salmonella infectious cycle, the \textit{dsb} mutant strains were assayed for their invasiveness in HeLa cells, and were found to be less invasive than the wild-type strain. Furthermore, the \textit{dsb} mutant strains were used in a mouse model of infection, and were found to be less competitive than the wild-type strain in colonization of spleen and liver. The \textit{dsb} mutant strains also had significantly higher LD$_{50}$s than the wild-type strain. Complementation of the \textit{dsb} mutations restored or partially restored the virulence that of wild-type level. These results suggested that the bacterium relies on the Dsb system for its full virulence and ability to replicate in animal hosts.
Introduction

*Salmonella enterica* is a major cause of food-borne disease in the world, and outbreaks of nontyphoidal salmonellosis are typically associated with food products from animal sources or contaminated with animal fecal matter (Voetsch *et al*., 2004). Due to the modern advent of distribution of pre-packaged food products, the prospect of a wide-reaching outbreak stemming from a single contamination incident is of great public health concern. In industrialized nations, food products are susceptible to improper handling all the way from the "farm to table," and each step represents a potential avenue for pathogens and contaminants to enter the human food supply (Centers for Disease Control and Prevention, 2011).

In a normal route of infection, the contaminated food is ingested by the host and *Salmonella enterica* serovar Enteritidis passes through the stomach and into the intestinal tract. The bacteria are then able to swim through the intestinal mucosa and adhere to enterocytes and M cells of the intestinal epithelium. Upon contacting the apical surface of the epithelial cells, the bacteria then inject effector molecules via the T3SS encoded by SPI-1, which hijack the host cell actin cytoskeletal machinery to induce ruffling of the epithelial membrane. The bacteria then transcytose through the cell to the basolateral membrane, and they enter the lamina propria. In this thin layer of connective tissue beneath the epithelium, the bacteria are taken up by macrophages into a modified phagosome termed the *Salmonella* containing vacuole (SCV). Survival inside the macrophages is mediated by a separate T3SS encoded by SPI-2, and allows the bacteria to traffic through the bloodstream to the spleen and liver, where they replicate and persist (Vazquez-Torres *et al*., 1999; van der Velden *et al*., 2000; Ryan and Ray, 2004).

The initial steps of this infectious route are shared among many other Gram-negative pathogens that must actively invade the host in order to replicate. For instance, *Shigella spp.* and enteroinvasive *E. coli* also invade the human intestinal M cells and multiply in adjacent enterocytes (Parrot, 2005). Likewise, *Yersinia spp.* invade the M cells by injecting *Yersinia* outer membrane proteins (Yops) in an analogous manner to the *Salmonella* T3SS (Cornelis, 2002). Furthermore, *Campylobacter jejuni* adheres to endothelial cells, and invades and replicates once inside; however despite being the cause of many bacterial food-borne infections in the United States, the virulence mechanisms of *Campylobacter* are currently not well known (Madigan and Martinko, 2006; Lasica *et al*., 2010). Therefore, a critical first step for *Salmonella enterica* and other pathogenic bacteria to survive within the host is to cross the epithelial barrier. Otherwise, the bacteria will not be able to establish a replicative niche and will be passed out of the host.

In order to further examine how the Dsb system is involved in the ability of *Salmonella enterica* to find a suitable niche in which to multiply, the *dsb* mutant strains were subjected to a series of virulence assays. The panel of mutants was tested for their invasiveness in a gentamicin protection assay in HeLa cells. Next, competitive infections of mice were used to determine the ability of the *dsb* mutant strains to colonize the spleen and liver as compared to that of the wild-type strain. Additional
infections of mice were used to obtain a quantitative measure of the virulence of the \textit{dsb} mutant strains by calculating their lethal dose, 50\% (LD\textsubscript{50}). Taken together, these analyses will indicate the importance of the Dsb system for the full virulence of \textit{Salmonella enterica}.
Materials and Methods

Reagents
Growth media for all bacteria strains and HeLa cells were purchased from BD Diagnostics (Sparks, MD) and Invitrogen (Carlsbad, CA), respectively. Chemicals and antibiotics were obtained from Sigma-Aldrich (Saint Louis, MO) unless otherwise indicated. BALB/c mice were obtained from Charles River (Wilmington, MA).

Invasion assay
HeLa cells were plated in 24-well plates at 1.2 x 10⁵ cells/well and incubated at 37°C in 5% CO₂ for 16 hours. Strains were inoculated into 3 ml of LB medium supplemented with antibiotics and incubated at 37°C without shaking for 16 hours. A 1:1,000 dilution was inoculated into 5 ml of Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum (HyClone, Logan, UT) and 1X L-glutamine (Gibco, Grand Island, NY). Aliquots were taken from the diluted bacteria and plated onto LB agar plates to determine the inoculum. The diluted bacteria were added to each well at a multiplicity of infection of approximately 5. The plates were then centrifuged at 1,000 rpm in an Eppendorf 5810 R centrifuge (Eppendorf, Hauppauge, NY) for 5 minutes and incubated at 37°C in 5% CO₂ for 1 or 2 hours. The cells were then washed three times with phosphate-buffered saline (PBS), and 1 ml of fresh medium containing 50 μg/ml of gentamicin was added. The plates were then incubated at 37°C in 5% CO₂ for an additional hour, and the cells were washed three times with PBS. Then 0.5 ml 0.1% Triton X-100 was added and the monolayer was lysed with vigorous pipetting. Aliquots were diluted, plated onto LB agar plates, and incubated at 37°C for 16 hours. Intracellular bacterial CFU was quantified and compared to the inoculum.

Competitive infection
Strains were inoculated into 2 ml of LB medium supplemented with antibiotics and incubated at 37°C with shaking at 225 rpm for 16 hours. A 1:2,000 dilution of wild-type and an equal dilution of dsb mutant bacteria were mixed in PBS and groups of ten 7-week-old female BALB/c mice were infected with 0.25 ml of diluted bacteria mixture orally via a feeding needle (Lu et al., 1999). The infected mice were monitored daily and their morbidity was observed over two weeks. Spleen and liver were harvested and homogenized in 5 ml PBS. Homogenates were then diluted and plated onto LB agar plates with and without antibiotics. The plates were then incubated at 37°C for 16 hours and bacterial CFU determined. Mutant bacteria were quantified by CFU grown on LB plates supplemented with antibiotics, and wild-type bacteria were quantified by the total bacteria grown on LB plates minus the number of mutant bacteria. Ratios of mutant to wild-type bacteria were calculated.

Mouse infection
Strains were inoculated into 2 ml of LB medium supplemented with antibiotics and incubated at 37°C with shaking at 225 rpm for 16 hours. Ten-fold dilutions were prepared in PBS and groups of five 7-week-old female BALB/c mice were infected with 0.25 ml of diluted bacteria orally via a feeding needle (Lu et al., 1999). The infected mice were monitored daily and their morbidity was observed over two weeks. Lethal
dose, 50% (LD₅₀) was calculated by the method of Reed and Muench (Reed and Muench, 1938).
Results

The Dsb system was required for invasion in vitro.
To examine the ability of Salmonella enterica to cause disease in humans, the dsb mutants of Salmonella were analyzed for their ability to traverse the epithelial layer in vitro. A gentamicin protection assay was performed in which the dsb mutant strains were added to confluent HeLa cell monolayers. The bacteria were allowed to invade before the monolayers were treated with gentamicin, a bactericidal protein synthesis inhibitor that cannot penetrate eukaryotic cell membranes, thereby killing only those bacteria that were unable to invade, and allowing those that had already invaded to survive. The HeLa cells were then washed to remove all extracellular debris, before being lysed to release intracellular bacteria. The intracellular bacteria were then recovered, quantified, and invasiveness of each bacterial strain was calculated as (intracellular bacteria / input bacteria) x 100%. The invasiveness of each dsb mutant strain was compared to that of the wild-type strain (Su et al., 2009).

The bacteria were allowed to invade the HeLa monolayers for 1 to 2 hours at 37°C in 5% CO₂. At each timepoint, infected cells were washed three times with PBS and fresh media containing gentamicin was added and the dsb mutant intracellular bacteria were recovered and compared to that of the wild-type strain. The HeLa cells infected with the ΔdsbA, ΔdsbC, and ΔdsbA/C mutant strains all contained significantly less intracellular bacteria compared to the wild-type strain, at both 1 and 2 hours of invasion (p < 0.05). The ΔdsbA and ΔdsbA/C mutant strains had the most severe phenotype, as the percentage of relative invasion at 1 hour was 8.07% and 1.92%, respectively, with the wild-type strain’s invasiveness set to 100%. At 2 hours, the wild-type strain’s invasiveness jumped to 201.44%, while ΔdsbA and ΔdsbA/C only increased to 15.46% and 4.21%, respectively. The ΔdsbC mutant strain also had diminished invasiveness, albeit not to the severe extent as the other mutant strains, with 46% invasion at 1 hour and 102.22% at 2 hours. Nevertheless, the ΔdsbC mutant strain was at best only half as invasive as the wild-type strain (Figure 3.1).

Complementation of the dsb mutant strains was moderately effective at rescuing the invasion defects. While the ΔdsbA [dsbA] complemented strain was not able to perfectly match wild-type levels after 1 hour (63.9% vs. 100%), the amount of intracellular bacteria was the same in both strains after 2 hours (191.16% vs. 201.44%). In contrast, the ΔdsbC [dsbC] complemented strain had slightly more intracellular bacteria than the wild-type after 1 hour (158.38% vs. 100%), but comparable levels were seen in both strains after 2 hours (213.32% vs. 201.44%). Thus, complementation was able to rescue the reduced invasiveness phenotype seen in the dsb mutant strains (Figure 3.1).
Figure 3.1. The dsb mutations resulted in decreased invasiveness in vitro.
The wild-type strain, the dsb mutant strains, and their respective complemented strains of Salmonella were added to confluent HeLa monolayers for 1 to 2 hours, and intracellular bacteria was recovered. The invasiveness of each bacterial strain was calculated as (intracellular bacteria / input bacteria) x 100%. The invasiveness of the wild-type strain at 1 hour was set as 100% and the invasiveness of each dsb mutant strain was measured relative to that of the wild-type strain at 1 hour. At least three experiments were performed, and the results of a representative experiment performed in triplicate were shown. The error bars indicate standard deviations. Asterisks denote a p-value < 0.05 calculated by Student's t-test.
The Dsb system was required for effective colonization of mouse organs. This study used a mouse bacteremia model to determine how effectively *Salmonella enterica* colonizes mice and to characterize the molecular determinants for its virulence. The ability of the *dsb* mutants of *Salmonella* to successfully colonize mouse organs was compared to the wild-type strain to determine if the *dsb* genes were necessary for the replication and persistence of *Salmonella enterica* in an animal infection. Conversely, if the *dsb* mutant does not affect virulence in an animal model, then the corresponding Dsb protein would not be a virulence determinant.

In order to directly compare the *dsb* mutant strains with the wild-type strain, each *dsb* mutant was co-infected with an equivalent amount of wild-type bacteria in a competitive infection assay. With equal amounts of each strain, the bacteria are in direct competition for nutrients and other resources to evade the host immune system. As the bacteria spread systemically throughout the mouse, they will drain into the spleen and liver (van der Velden et al., 2000; Holden, 2002). These are the two largest organs and are normally sterile sites in uninfected BALB/c mice (Janeway et al., 2005). Infected organs were then harvested and bacteria were recovered. The *dsb* mutant strains and the wild-type strain were then differentiated by antibiotic selection and a competitive index was calculated. If the ratio of *dsb* mutant to wild-type was 1, then the *dsb* mutant strain was equally adept at colonizing organs as the wild-type strain. However, if the ratio of *dsb* mutant to wild-type was less than 1, then there was less *dsb* mutant recovered from the organ and the *dsb* mutant strain was not able to compete with the wild-type strain *in vivo* (Auerbuch et al., 2001).

The Δ*dsbA*, Δ*dsbC*, and Δ*dsbA/C* mutant strains were each paired with the wild-type strain, and equivalent amounts of each strain (approximately 6.5 x 10⁵ CFU/ml) were mixed in PBS and aliquots were fed orally to BALB/c mice. The mice were monitored for onset of symptoms, which included lethargy, ruffled fur, hunched posture, and weight loss of more than 15% over 24 hours. The onset of symptoms has been observed in previous experiments to accurately predict death; likewise, mice that never develop symptoms will not die from infection (Lu et al., 1999). Therefore, it is not necessary to wait until a sick mouse succumbs on its own, avoiding unnecessary suffering on the part of the animal. Rather, upon observation of symptoms, sick mice were sacrificed and their spleen and liver were removed and homogenized. The homogenates were plated on LB plates with and without antibiotics to recover *dsb* mutant bacteria and total bacteria.

The Δ*dsbA*, Δ*dsbC*, and Δ*dsbA/C* mutant strains were all significantly less competitive than the wild-type strain in colonizing organs (*p* < 0.05). The Δ*dsbA/C* mutant strain had the most severe phenotype, as the ratio of mutant to wild-type bacteria was 1.19 x 10⁻⁴ and 8.2 x 10⁻⁶ in the spleen and liver, respectively. The Δ*dsbC* mutant strain had a more moderate phenotype, as the ratio of mutant to wild-type bacteria was 7.44 x 10⁻³ and 6.2 x 10⁻³ in the spleen and liver, respectively. Surprisingly, the Δ*dsbA* mutant strain had a mild phenotype, a ratio of mutant to wild-type bacteria of 2.15 x 10⁻¹ and 3.25 x 10⁻¹ in the spleen and liver, respectively (Figure 3.2).
However, it should be noted that the absolute amount of $dsb$ mutant recovered from all organs was spread over the entire spectrum. Indeed, at least one organ in each group was colonized entirely by the wild-type strain and had no detectable amount of the $dsb$ mutant bacteria at all. This spread is especially apparent in the $\Delta dsbA$ and wild-type co-infection. Nevertheless, all of the average ratios were significantly less than 1 ($p < 0.05$), indicating a substantial defect in the ability of the $dsb$ mutant strains to colonize the organs of mice when in competition with the virulent wild-type strain (Figure 3.2).
Figure 3.2. The dsb mutations resulted in deficiency in competitive infection of mice.
The wild-type strain and the dsb mutant strains were orally co-infected in equivalent amounts to BALB/c mice and their morbidity was monitored. Sick mice were sacrificed and bacteria were recovered from their spleen and liver. At least two experiments were performed, and the results of a representative experiment performed with replicates were shown. The dark bars indicate average ratios. All data points are significantly less than 1, $p$-value < 0.05 calculated by Wilcoxon Rank Sum Test.
The Dsb system was required for full virulence in vivo.
In addition to calculating the bacterial concentration recovered from colonized spleen and liver, the lethal dose, 50% (LD$_{50}$) was calculated for each mutant strain to obtain a definitive, quantitative measure of virulence. The LD$_{50}$ assessment is complementary to the competitive infection, as it allows for cross-comparison of multiple experiments without having to conduct a new competitive infection each time new comparisons are warranted. More importantly, this quantitative data allows for comparisons with other studies of Gram-negative pathogens, or even other dsb mutant strains in different backgrounds. The implications for study of the Dsb system are readily evident.

The wild-type strain, the $\Delta$dsbA, $\Delta$dsbC, and $\Delta$dsbA/C mutant strains, as well as their respective complemented strains were each diluted ten-fold in PBS and aliquots were fed orally to BALB/c mice. The inocula were adjusted for each strain, depending on the expected virulence of the various strains. For example, the wild-type strain is a known virulent clinical isolate, and as such, higher dilutions were administered to obtain a LD$_{50}$. However, the $\Delta$dsbA/C mutant strain was observed to have severe defective phenotypes, and lower dilutions were administered accordingly. The mice were monitored for onset of symptoms, which included lethargy, ruffled fur, hunched posture, and weight loss of more than 15% over 24 hours. The onset of symptoms has been observed in previous experiments to accurately predict death; likewise, mice that never develop symptoms were not likely to die from infection (Lu et al., 1999). Following the guidelines of the institutional Animal Care and Use Committee, death was not used as an endpoint, and therefore all mice were sacrificed before they were moribund. Mice that had not developed symptoms by 2 weeks had cleared the infection and would not succumb (Lu et al., 1999).

In order to obtain a discrete LD$_{50}$, the method of Reed and Muench was applied. Originally developed to obtain the optimal dilution of a hypothetical protective serum, the algorithm was modified to calculate the corresponding inoculum at which 50% of a given population was killed. To calculate the LD$_{50}$ for an infectious agent, the summation of the deaths and survival columns were merely reversed in direction; otherwise, the method remained the same (Reed and Muench, 1938).

The $\Delta$dsbA, $\Delta$dsbC, and $\Delta$dsbA/C mutant strains were all significantly less virulent than the wild-type strain. The dsb mutant strains had LD$_{50}$s of at least 2 to 3 logs higher than that of the wild-type strain. Indeed, the LD$_{50}$s of the $\Delta$dsbA and $\Delta$dsbA/C mutant strains were beyond the upper limit of detection (greater than $10^8$ CFU); even when the mice were given undiluted overnight culture ($\sim 10^8$ CFU/ml), they did not develop symptoms. The $\Delta$dsbC mutant strain had an LD$_{50}$ of 9.74 x $10^7$ CFU, which was still 2 logs higher than that of the wild-type at 7.7 x $10^5$ CFU (Figure 3.3).

Complementation with plasmid DNA was partially effective at rescuing the virulence defect. Both the $\Delta$dsbA [dsbA] (2.38 x $10^6$ CFU) and $\Delta$dsbC [dsbC] (4.09 x $10^6$ CFU) complemented strains had LD$_{50}$s of only 1 log higher than that of the wild-type strain (7.7 x $10^5$ CFU). Nevertheless, this indicates that the complemented strains were able to cause symptoms in 50% of the mice tested with less bacterial organisms than any of
the dsb mutant strains. Thus, the complemented strains were able to partially rescue the reduced virulence phenotype seen in the dsb mutant strains (Figure 3.3).
The *dsb* mutations resulted in reduced virulence *in vivo*. The wild-type strain, the *dsb* mutant strains, and their respective complemented strains were orally infected in ten-fold dilutions in BALB/c mice and their morbidity was monitored. Sick mice were sacrificed and the number of mice alive and dead were tabulated, and used to calculate the LD$_{50}$ using the method of Reed and Muench (Reed and Muench, 1938). At least three experiments were performed, and the results were averaged among all experiments.

**Figure 3.3. The *dsb* mutations resulted in reduced virulence *in vivo*.**

<table>
<thead>
<tr>
<th>Strains</th>
<th>Lethal Dose 50% Oral Infection (CFU)</th>
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<tr>
<td>SE2472</td>
<td>7.7 x 10$^5$</td>
</tr>
<tr>
<td>Δ<em>dsbA</em></td>
<td>&gt;10$^8$</td>
</tr>
<tr>
<td>Δ<em>dsbA</em> [<em>dsbA</em>]</td>
<td>2.38 x 10$^6$</td>
</tr>
<tr>
<td>Δ<em>dsbC</em></td>
<td>9.74 x 10$^7$</td>
</tr>
<tr>
<td>Δ<em>dsbC</em> [<em>dsbC</em>]</td>
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<tr>
<td>Δ<em>dsbA/C</em></td>
<td>&gt;10$^8$</td>
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Discussion

We have shown here that the Dsb system of *Salmonella enterica* is critical for the full virulence in the infection of an animal host. The *dsb* mutant strains were first tested for their ability to invade HeLa monolayers. It was determined that mutations in the Dsb system resulted in a significant loss of the bacteria's ability to invade epithelial cells. This phenotype was especially pronounced in the Δ*dsbA/C* mutant strain, which had almost no invasive ability, even after 2 hours after addition of the bacteria. While the Δ*dsbC* mutant strain had a slightly less dramatic defect, it was still significantly less invasive than the wild-type strain. This indicated the possibility that DsbA was playing a greater role than DsbC in the ability of the bacteria to invade, and that removal of both of these main enzymes was remarkably detrimental to the bacteria.

Although cervical epithelial cells are not normally encountered by the bacteria in the digestive tract, HeLa cell are routinely used to determine the invasiveness of *Salmonella*. There are more relevant *in vitro* models used in the field, such as transepithelial migration assay using confluent monolayers of polarized T84 intestinal epithelial cells. Such an assay would have the distinct advantage of being part of the natural infectious cycle of *Salmonella enterica*, and for having an apical surface through which *Salmonella* could adhere to and induce its internalization (McCormick et al., 1993). Nevertheless, HeLa cell invasions assays are useful in the field for their consistent susceptibility to invasion by pathogenic organisms, such as the clinical isolate used as the wild-type strain.

In order to establish a direct comparison with the wild-type strain, each *dsb* mutant strain was orally co-infected with an equivalent amount of the wild-type bacteria in a competitive infection assay. It goes without saying that an oral route of infection was necessary to establish a path by which the bacteria would traverse the intestinal epithelia. As the bacteria progressed through the lympatics to reach the spleen and liver, the *dsb* mutants and the wild-type would be in direct competition for nutrients and other resources. The bacteria recovered from the spleen and liver were then selectively differentiated and a ratio of *dsb* mutant to wild-type bacteria was calculated. In all recovered organs, the ratio of *dsb* mutant bacteria to wild-type was significantly less than 1, and in several instances across all *dsb* mutant strains, there was no detectable *dsb* mutant bacteria. These results suggest that the *dsb* mutant bacteria were not as able as the wild-type strain to establish a replicative niche in a mouse model of infection. This is substantial because it indicates a marked decrease in fitness of the *dsb* mutants compared directly to the wild-type strain. A logical conclusion is that the absence of a completely functional Dsb system prevents the mutant strain from effectively colonizing mouse organs. Moreover, in a normal infection, *Salmonella* is not the only organism present in the intestinal tract; there are millions of other organisms, commensal and otherwise, that represent direct competitors for the same limited nutrients and resources required for growth. Indeed, since the *dsb* mutants were unable to effectively colonize organs in naive mouse, it is doubtful that the same mutants would be able to colonize organs in the wild.
The clear establishment of bacterial fitness defect \textit{in vivo} necessitates a metric for comparing the virulence characteristics of the \textit{dsb} mutant strains to the wild-type strain and other pathogenic organisms. To this end, the \textit{dsb} mutant strains were assessed for their ability to kill 50\% of a target population. Serial dilutions of the \textit{dsb} mutant strains were orally infected into mice and their morbidity was monitored. By tabulating the number of mice in each group that survived or succumbed to infection, a LD\textsubscript{50} was calculated for each strain. For the \( \Delta\text{dsbA} \), \( \Delta\text{dsbC} \), and \( \Delta\text{dsbA/C} \) mutant strains, their LD\textsubscript{50}s were 2 to 3 logs higher than that of the wild-type, indicating that more mutant organisms were required to cause the same amount of mortality in a given population than the wild-type strain. Of particular note are the \( \Delta\text{dsbA} \) and \( \Delta\text{dsbA/C} \) mutant strains, which did not kill 50\% of the mice despite the extremely high inoculum used (~10\textsuperscript{9} CFU/ml). Therefore, these \textit{dsb} mutant bacteria were less virulent than the wild-type strain. Complementing the mutations partially restored the wild-type phenotype.

Interestingly, while it appeared that the \( \Delta\text{dsbA} \) mutant had a more severe phenotype than the \( \Delta\text{dsbC} \) mutant in the invasion assay (Figure 3.1) and virulence assay (Figure 3.3), it is the \( \Delta\text{dsbC} \) mutant that had a lower ratio of mutant to wild-type bacteria in the spleen and liver than the corresponding ratio of \( \Delta\text{dsbA} \) mutant to wild-type bacteria. Perhaps this indicates a greater role for DsbA in invasion of epithelial cells and a similarly greater role for DsbC in survival in macrophages while colonizing distal organs, with both having the effect of increasing morbidity as compared to when those proteins are absent. This supposition is not improbable, as it is known that various Dsb proteins differ in their substrate specificities (Katzen \textit{et al.}, 2002; Hiniker and Bardwell, 2004; Lasica and Jagusztyn-Krynicka, 2007).
Chapter 4
Identifying the SPI-1 T3SS Substrates of the Dsb System

Abstract
Salmonella enterica utilizes the Type III Secretion System (T3SS) encoded by the Salmonella Pathogenicity Island 1 (SPI-1) to translocate effector proteins into host cells and facilitate invasion. The periplasmic components of the T3SS must be correctly folded to allow for proper assembly and function of the needle complex. The hypothesis is that the Dsb system helps fold the SPI-1 T3SS structural proteins in the periplasm. Previous experiments indicated that the dsb mutant strains were less virulent than the wild-type strain in vivo, and that this defect is potentially related to a reduced ability to invade epithelial cells. In order to characterize the interactions between the Dsb system and SPI-1 T3SS, the dsb mutations were transduced into strains expressing SipA and SipC tagged with a FLAG epitope. The dsb mutations were found to secrete less SipA and SipC compared to the wild-type strain. Next, the dsb and SPI-1 T3SS genes were cloned into a bacterial two-hybrid system, and interaction assays were performed to identify the potential SPI-1 T3SS substrates of the Dsb system. Preliminary results indicated direct binding of the Dsb system with SpaO, a sorting platform protein. These results suggested that the Dsb system has a critical role in the assembly and function of the SPI-1 T3SS which contributes to the pathogenesis of Salmonella.
Introduction

*Salmonella enterica* is a successful pathogen because of its ability to survive and persist in a wide range of hosts. Among the distinguishing characteristics that set it apart from other non-pathogenic Gram-negative organisms are virulence factors encoded by large regions of DNA termed *Salmonella* pathogenicity islands (SPI). These clusters of chromosomal virulence genes comprise large regions of 10-200 kb in size, and are present in pathogenic strains but absent from related non-pathogenic strains. The G+C content of these pathogenicity islands (between 37 and 47%) also differs from that of the rest of the genome (approximately 52%), and are associated with tRNA genes, suggesting that these SPIs were acquired via horizontal transfer (Hacker and Kaper, 2000; Marcus *et al*., 2000; Amavisit *et al*., 2003).

There are at least seventeen different SPIs that have been described in the literature, although not all SPIs are necessarily present in any one serovar of *Salmonella enterica*. In fact, SPI-15, SPI-16, and SPI-17 are only predicted by a novel computational method to be present in *Salmonella enterica* serovar Typhi (Vernikos and Parkhill, 2006). Of the remaining SPIs, perhaps the two most well characterized are SPI-1 and SPI-2, which are both present within the genome of *Salmonella enterica* serovar Enteritidis. Both of these SPIs encode for distinct Type III Secretion Systems (T3SS) that play different roles in the infectious cycle of *Salmonella enterica* (Hacker and Kaper, 2000).

In brief, upon ingestion by the host, the bacteria make their way into the intestinal tract where it adheres to enterocytes and M cells of the intestinal epithelium. Upon making contact with the epithelial cells, the bacteria express the T3SS encoded by SPI-1. The SPI-1 T3SS spans the periplasm and forms a straw-like needle through which effector proteins are transported directly into the cytoplasm of the host cell, and once there, these effectors facilitate the uptake of the bacteria (Kimbrough and Miller, 2002).

The bacteria then enter the lamina propria where they are taken up by macrophages. The bacteria are able to persist in a modified phagosome termed the *Salmonella* containing vacuole (SCV), and survival in the SCV is mediated by the T3SS encoded by SPI-2. From within the SCV, the bacteria form the SPI-2 T3SS and inject effector proteins into the cytoplasm of the macrophage. These effector molecules, which are distinct from those transported by the SPI-1 T3SS, continue to modify the SCV and help the bacteria survive the macrophages’ antimicrobial mechanisms, and ultimately help form a suitable replicative niche in which to propagate (Steele-Mortimer, 2008).

Together, these two T3SS are critical for the successful invasion and eventual colonization of the host by *Salmonella enterica*. The SPI-1 T3SS is induced in the extracellular environment of the gut and the SPI-2 T3SS is induced once inside a SCV. Although there are some reports that the SPI-1 T3SS may have some additional overlapping functions in the biogenesis of the SCV, these two systems have evolved to play largely distinct roles in the pathogenesis of *Salmonella enterica* (Steele-Mortimer, 2008). Therefore, it is of vital importance to first establish the impact of the Dsb system on the invasive qualities of the bacteria through study of the interactions with the SPI-1 T3SS. Such an analysis would provide a possible mechanism by which mutations in the
Dsb system would adversely affect invasion \textit{in vitro} and the first few hours of the mouse infection in as seen in Chapter 3.

In order to assess the impact of the Dsb system on SPI-1 T3SS function, it is necessary to analyze the outcome of the effector protein secretion. Invasion of host cells by \textit{Salmonella enterica} is strictly dependent on the function of the host actin cytoskeleton, and the bacteria induce actin cytoskeletal rearrangements at the site of contact with the host cell. Upon translocation of the SPI-1 T3SS effector proteins into the host cell cytoplasm, they stimulate the host signal transduction pathways in several ways (Ly and Casanova, 2007; Srikanth \textit{et al.}, 2011). Effectors SopE and SopE2 are guanine nucleotide exchange factors that activate Rho family GTPases Cdc42 and Rac1 that promote actin polymerization (Hardt \textit{et al.}, 1998). Effectors SipA and SipC are actin-binding proteins; SipA decreases the critical concentration required for actin polymerization and inhibits depolymerization of actin filaments, and SipC nucleates and bundles actin, as well as serving as a component of the translocon (Giacomodonato \textit{et al.}, 2007). Once the bacteria has been internalized, effector SptP, a GTPase activating protein, is secreted to deactivate Cdc42 and Rac1, shutting down membrane ruffling and allowing the host cell to rebuild its actin cytoskeleton (Stebbins and Galan, 2000).

While there are other effector molecules, with both known and unknown functions, effectors SipA and SipC are well-characterized in regards to invasion of \textit{Salmonella enterica}. Furthermore, they are among the most abundant of the effector proteins. As such, they are useful tools in assessing the function of the SPI-1 T3SS. In brief, recombinant \textit{Salmonella} strains were constructed with a FLAG epitope at the C-terminal end of SipA or SipC (Gong \textit{et al.}, 2009; Su \textit{et al.}, 2009). The function of the effector proteins were intact, and allowed for the tracking of the proteins by the FLAG tag. The \textit{dsb} mutations were transduced into the tagged strains, and secretion of SipA and SipC were measured as an assessment of SPI-1 T3SS secretion. Decreased secretion of SipA and SipC in the \textit{dsb} mutant strains would indicate a requirement for Dsb in the secretion by SPI-1 T3SS.

Even if the secretion of SipA or SipC is affected in the \textit{dsb} mutants of \textit{Salmonella}, they are unlikely to be the substrates of the Dsb system, since SipA and SipC are translocated from the bacterial cytosol directly to the host cell cytoplasm. Rather, the potential substrates of the Dsb system are likely among the SPI-1 T3SS needle complex proteins. Specifically, the SPI-1 T3SS in \textit{Salmonella enterica} serovar Enteritidis is comprised of at least 35 proteins, roughly grouped into the basal body and the export apparatus in the inner membrane, the regulatory and chaperone proteins that assist in assembly, the outer membrane rings, the central inner rod spanning the periplasm and extending outward toward the host cell, the translocon at the end of the needle, and the secreted effector proteins themselves (Kimbrough and Miller, 2002; Gerlach and Hensel, 2007).

While some of these proteins have been previously studied, other components of the SPI-1 T3SS are still being elucidated. The various models for assembly of the SPI-1 T3SS are summarized as follows. Initially, SpaO, OrgA, and OrgB form a sorting
platform in the inner membrane and interact with proteins PrgK and PrgH forming two oligomeric rings and inner rod protein PrgJ. Next, additional proteins are recruited to the growing substructure, including the ATPase InvC, and export apparatus proteins InvA, SpaQ, SpaP, SpaR, and SpaS. Meanwhile, the outer membrane rings are comprised of hundreds of InvG secretins to form another oligomeric pore substructure with the assistance of chaperone InvH. The two substructures are joined with the help of IagB, a muramidase that helps penetrate the peptidoglycan layer. This newly formed base structure allows the SpaO-OrgA-OrgB sorting platform to begin secreting PrgJ to extend the inner rod to the outer membrane, then PrgI to extend the outer rod outward from the bacterial cell, and regulator InvJ to modulate the overall length of the needle itself. Once the needle is fully assembled, the sorting platform switches substrate specificity and begins to secrete protein translocases SipB, SipC, and SipD to form the translocon at the needle tip, which then forms a pore in the target eukaryotic cell membrane (Kimbrough and Miller, 2002; Galan and Wolf-Watz, 2006; Gerlach and Hensel, 2007; Lara-Tejero et al., 2011) (Figure 4.1). The process is regulated by HilD, HilC, and HilA in response to environmental signals, presumably in the vicinity of intestinal epithelial cells (Ellermeier et al., 2005).
Figure 4.1. Putative model for assembly of the SPI-1 T3SS.
A simplified model for the assembly of the SPI-1 T3SS needle. The SpaO-OrgA-OrgB sorting complex associates with the inner membrane ring proteins PrgK and PrgH (A). While the sorting complex assembles the export apparatus comprised of InvC, InvA, SpaQ, SpaP, SpaR, and SpaS, the outer membrane ring protein InvG assembles with the help of chaperone InvH (B). The two structures join through the peptidoglycan layer with the assistance of muramidase IagB, and secretes needle proteins PrgJ and PrgI (C). Upon completion of the needle, the sorting platform switches substrate specificity and begins to secrete the translocon proteins SipB, SipC, and SipD, and then ultimately protein effectors directly into the host cell (D). Adapted from various sources (Kimbrough and Miller, 2002; Galan and Wolf-Watz, 2006; Gerlach and Hensel, 2007; Lara-Tejero et al., 2011).
Although much work has been done to characterize the SPI-1 T3SS, there are still questions concerning the role of other proteins, such as the Dsb system, due to the significant amount of transiently associated proteins with the needle complex. The multitude of cysteine-containing proteins in the SPI-1 T3SS needle complex provides a plethora of potential Dsb substrates to study. Some proteins have multiple cysteine residues, such as SpaS (6 cysteines), SpaR (4), SpaQ (2), InvA (2), InvH (2), SpaO (4), OrgA (4), and OrgB (2). While PrgK and PrgH have only a single cysteine residue each, they form oligomeric rings and have the potential to form intermolecular disulfide bonds. In order to study these potential interactions with Dsb, the corresponding genes were cloned into the BacterioMatch II Two-Hybrid System Vector Kit (Agilent Technologies, Santa Clara, CA).

Various interaction pairs of bait and target constructs were cotransformed together into the reporter strain. The $\lambda$ cl-bait fusion protein was then tethered to a $\lambda$ operator sequence upstream of a reporter promoter, and given a significant interaction between the bait and target proteins, the RNAP$\alpha$ was brought in proximity to the reporter promoter. This interaction resulted in the recruitment of RNA polymerase at the promoter and the transcription of two reporter genes, $HIS3$ and $aadA$. The $HIS3$ gene product is a component of the histidine biosynthetic pathway that complements a $hisB$ mutation in the reporter strain. The $aadA$ gene product confers streptomycin resistance.

The cotransformation mix was then spread on M9 minimal media without histidine, supplemented with progressively more selective agents. Nonselective Screening Media (NSM) contains only 25 $\mu$g/ml chloramphenicol and 12.5 $\mu$g/ml tetracycline, and only allows growth of reporter cells with both pBT and pTRG constructs. Selective Screening Media (SSM) contains both antibiotics as above, as well as 5 mM 3-amino-1,2,4-triazole (3-AT), which is a competitive inhibitor of the $HIS3$ gene product. In the presence of 5 mM 3-AT, the reporter strain is unable to grow on media lacking histidine. Finally, Dual Selective Screening Media (DSSM) contains all three above compounds, as well as 12.5 $\mu$g/ml streptomycin (Figure 4.2).

Together with the analysis of the SPI-1 T3SS effector secretion, these experiments will demonstrate a critical role for the Dsb system in the assembly and function of the SPI-1 T3SS which contributes to the pathogenesis of Salmonella.
Figure 4.2. **Diagram of the bacterial two-hybrid interaction.**
A simplified diagram of the protein-protein interactions in the reporter strain upon cotransformation of a pair of recombinant bait and target constructs. The λ cl-bait fusion protein binds to a λ operator sequence upstream of a reporter promoter. If there is a significant interaction between the bait and target proteins, the RNAPα is brought in close proximity to the reporter promoter, resulting in the recruitment of RNA polymerase at the promoter. Transcription of the two reporter genes allows for growth of the reporter strain in minimal media lacking histidine and supplemented with streptomycin. Adapted from the manual for the BacterioMatch II Two-Hybrid System Vector Kit (Agilent Technologies, Santa Clara, CA).
Materials and Methods

Reagents
Growth media for all bacteria strains were purchased from BD Diagnostics (Sparks, MD). Chemicals and antibiotics were obtained from Sigma-Aldrich (Saint Louis, MO) unless otherwise indicated. Restriction and modifying enzymes for manipulation of DNA were obtained from New England BioLabs (Ipswich, MA). Custom oligonucleotides were purchased from Sigma-Aldrich (St. Louis, MO).

Dsb secretion profile
Strains expressing SipA and SipC tagged with FLAG were the gift of Dr. Hao Gong at UC Berkeley. In brief, a FLAG epitope and a 6x His epitope were fused in-frame to the C-terminal end of the effector proteins (Gong et al., 2009; Su et al., 2009). The dsb mutations were transduced using bacteriophage P22 into the FLAG tagged strains. Phage-free transductants were screened by selecting light-colored colonies on Evans blue uranine plates, screened by PCR using verification primers (Table 2.2), and then sequence-verified. The dsb mutant SipA and SipC-FLAG-tagged strains were complemented with plasmids expressing the dsb genes on a pRB3-273C vector (Table 2.1) as described previously. Strains were inoculated into 3 ml of LB medium supplemented with antibiotics and incubated at 37°C with shaking at 225 rpm for 16 hours. Bacterial lysate proteins were isolated from culture by resuspending and boiling the pellet in cracking buffer containing 60 μM Tris pH 6.8, 142 μM β-mercaptoethanol (Bio-Rad, Hercules, CA), 1% sodium dodecyl sulfate (SDS), 10% glycerol, and 0.25% bromophenol blue. Culture supernatant proteins were isolated by precipitation in 6.25% trichloroacetic acid (TCA) for 30 minutes on ice. The samples were then washed three times with cold acetone to remove TCA, and the resulting pellet resuspended in urea sample buffer containing 8 M urea and 10 mM Tris pH 7.5. Culture supernatant proteins were quantified by DC Protein Assay (Bio-Rad, Hercules, CA). Previous experiments determined that 25 μg of culture supernatant protein yielded the clearest bands for detection of SipA-FLAG; likewise, 100 μg of culture supernatant protein yielded the best signal for SipC-FLAG. For bacterial lysate protein, 5 μl of protein sample was used for probing with anti-FLAG antibody, and 2 μl of protein sample was used for probing with anti-DnaK antibody. Proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes for Western blotting. The membranes were probed with monoclonal anti-FLAG M2 from mouse (Sigma, Saint Louis, MO) at 1:1,000 dilution and sheep anti-mouse IgG conjugated with HRP (GE Healthcare, Buckinghamshire, UK) at 1:1,000 dilution. The membranes were then visualized by enhanced chemiluminescence. Equivalent amounts of bacterial lysate protein were also probed with monoclonal anti-DnaK from mouse (Enzo Life Sciences, Plymouth Meeting, PA) at 1:10,000 as loading control.

T3SS structural protein mutagenesis
A panel of knockout deletion mutants was constructed by replacing the genes encoding T3SS structural proteins PrgK, PrgH, SpaS, SpaR, SpaQ, InvA, and InvH with an antibiotic resistance cassette (Datsenko and Wanner, 2000). In brief, knockout primers amplified an antibiotic resistance cassette conferring resistance to kanamycin with
homology regions flanking the PCR amplicon (Table 4.3). The amplicons were transformed into a strain expressing a plasmid encoding the Red recombinase system, allowing for replacement of the T3SS structural gene with the antibiotic resistance cassette. Antibiotic-resistant transformants were selected, and screened, and then transduced using bacteriophage P22 into the SipA-FLAG tagged strain as described previously. Phage-free transductants were screened by differential growth on Evans blue uranine plates, screened by PCR using verification primers (Table 4.3), and then sequence-verified. Whole cell and secreted proteins were prepared and Western blots were performed as described previously to demonstrate the necessity of T3SS structural proteins for SipA secretion.

**Bacterial two-hybrid system**

The BacterioMatch II Two-Hybrid System Vector Kit (Agilent Technologies, Santa Clara, CA) was used to generate constructs to test for protein-protein interactions in *E. coli*. In brief, insert primers amplified the *dsb* genes and genes encoding T3SS proteins PrgK, PrgH, SpaS, SpaR, SpaQ, SpaO, InvA, InvH, OrgA, and OrgB with a FLAG epitope fused in-frame to the C-terminal end of the protein, from SE2472 (Table 4.4) for cloning into either pBT (bait) or pTRG (target) expression vectors provided in the kit (Table 4.2). The multiple cloning sites of pBT and pTRG are incompatible with each other, and as a result, distinct primer pairs were used for each construct (Table 4.4). The PCR amplicons were digested with BamHI and EcoRI, and cloned into the pBT and pTRG multiple cloning site regions, and grown in the XL1-Blue MRF' Kan Supercompetent Cells (Agilent Technologies, Santa Clara, CA) as the propagation strain at 30°C. Successful transformants were selected for growth on LB agar plates supplemented with 25 μg/ml chloramphenicol or 12.5 μg/ml tetracycline for recombinant pBT or pTRG constructs, respectively. The resulting plasmids were sequence-verified, and whole cell proteins were prepared and Western blots were performed as described previously to verify expression of chimeric fusion proteins. Various interaction pairs were cotransformed into the BacterioMatch II Validation Reporter Competent Cells (Agilent Technologies, Santa Clara, CA) as the reporter strain at 37°C on Nonselective Screening Media containing 25 μg/ml chloramphenicol and 12.5 μg/ml tetracycline, Selective Screening Media containing those antibiotics with 5 mM 3-amino-1,2,4-triazole (3-AT), and Dual Selective Screening Media containing those antibiotics with 5 mM 3-AT, along with 12.5 μg/ml streptomycin. A significant interaction between bait and target proteins was demonstrated by growth on all screening media; conversely, lack of growth on selective media would suggest a lack of a significant interaction. Interacting protein-pairs were reversed, that is a protein expressed in pBT was subsequently expressed in pTRG, to verify the interaction. The positive control provided in the kit, pBT-LGF2 and pTRG-GAL11P, was utilized to verify the functionality of the system (Table 4.2). A positive control for periplasmic protein interaction was established using a known interacting pair, DsbA and DsbB (Collet and Bardwell, 2002). A negative control utilized empty pBT and pTRG vectors.
Table 4.1. Bacterial strains.

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<th>Characteristics</th>
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</tr>
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<td>SipA(HF) spaQ::kan</td>
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<tr>
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<tr>
<td>Plasmid</td>
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<tr>
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<td>Ap⁺ Cm⁻ oriRγ</td>
<td>(Datsenko and Wanner, 2000)</td>
</tr>
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<td>Ap⁺ Kan⁻ oriRγ</td>
<td>(Datsenko and Wanner, 2000)</td>
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<td>(Datsenko and Wanner, 2000)</td>
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Table 4.3. Oligonucleotide primers for structural protein mutagenesis.

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### Table 4.4. Oligonucleotide primers for protein interaction studies.

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<td><strong>Verification primers</strong></td>
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<tr>
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Results

The Dsb system was important for secretion of SipA and SipC.

Previous experiments have shown the requirement for the Dsb system for virulence in the mouse model of infection, where the *dsb* mutant strains were significantly less able to colonize organs and cause disease symptoms (Figures 3.2 and 3.3). The reduced virulence phenotype was indicated to be related to an invasion defect (Figure 3.1). To further examine the Dsb system's involvement in invasion, the *dsb* mutations were transduced into strains expressing SipA and SipC tagged with a FLAG epitope (Gong et al., 2009; Su et al., 2009). The tagged *dsb* mutant strains were complemented with plasmids expressing the *dsb* genes on a pRB3-273C vector. The logarithmic growth curves of the tagged strains were compared to the parental strains, and no differences were observed, indicating that the tagging did not adversely affect the bacteria (data not shown). Furthermore, the FLAG tag does not affect secretion of the tagged protein (Su et al., 2009). Liquid cultures were separated into supernatant and pellet fractions, and total protein was prepared from both fractions. Equivalent amounts of the protein samples were then separated by SDS-PAGE and transferred to nitrocellulose membranes for probing with an anti-FLAG antibody.

The SipA protein was detected in both the culture supernatant and the bacterial lysate. While there was no discernible difference seen in SipA levels in the culture supernatant in the Δ*dsbA* and Δ*dsbC* mutant strains, there was less SipA in the Δ*dsbA/C* mutant strain compared to the parental strain. The levels of SipA in the bacterial lysate were unchanged in all strains (Figure 4.3 A).

The SipC protein was not detected in the culture supernatants of the Δ*dsbA*, Δ*dsbC*, and Δ*dsbA/C* mutant strains. The complemented strains restored the amount of SipC in the culture supernatant to parental strain's levels. In contrast, the levels of SipC in the bacterial lysate were unchanged in all strains (Figure 4.3 B).
Figure 4.3. The *dsb* mutants of *Salmonella* were defective in secretion of SipA and SipC.

Equivalent amounts of total protein from the culture supernatant or bacterial lysates of the parental FLAG-tagged strains, the *dsb* mutant FLAG-tagged strains, and their respective complemented strains were probed with an anti-FLAG antibody. Protein samples from the culture supernatant were quantified; 25 μg was used for probing for SipA (A), and 100 μg was used for probing for SipC (B). For the bacterial lysate protein, 5 μl was used for probing with anti-FLAG and 2 μl was used for probing with anti-DnaK. At least three experiments were performed, and the results of a representative experiment were shown.
Deletions of T3SS structural genes disrupted secretion of SipA.
A panel of T3SS structural proteins was identified as potential substrates of the Dsb system, due to their localization in the periplasm and the inner membrane, and the presence of cysteine residues. However, prior to studying potential direct binding interactions with the Dsb system, it is imperative to establish the link with these T3SS structural proteins and the function of the T3SS. To that end, knockout deletion mutants were constructed by replacing the genes encoding T3SS structural proteins PrgK, PrgH, SpaS, SpaR, SpaQ, InvA, and InvH as previously described. These mutations were then transduced into the SipA-FLAG tagged strain. Liquid cultures were separated into supernatant and pellet fractions, and total protein was prepared from both fractions. Equivalent amounts of the protein samples were then separated by SDS-PAGE and transferred to nitrocellulose membranes for probing with an anti-FLAG antibody.

The SipA protein was detected in both the culture supernatant and the bacterial lysate of the parental strain. However, the SipA protein was not detected in the culture supernatants of the T3SS mutant strains. In contrast, the levels of SipA in the bacterial lysate were unchanged in all strains (Figure 4.4). These results indicated that these T3SS structural proteins were indispensable for the secretion of the SPI-1 T3SS effector proteins.
Figure 4.4. The SPI-1 T3SS mutant strains were defective in secretion of SipA. Equivalent amounts of total protein from the culture supernatant and bacterial lysates of the parental SipA-FLAG-tagged strain and the SPI-1 T3SS mutant SipA-FLAG-tagged strains were probed with an anti-FLAG antibody. Protein samples from the culture supernatant were quantified; 25 μg was used for probing for SipA. For the bacterial lysate protein, 10 μl was used for probing with anti-FLAG. Two experiments were performed, and the results of a representative experiment were shown.
The Dsb system interacted with SPI-1 T3SS protein, SpaO.

In order to determine the interactions between the Dsb system and the SPI-1 T3SS, the genes for *dsbA*, *dsbB*, *dsbC*, *spaS*, and *spaO* were cloned into the BacterioMatch II Two-Hybrid System Vector Kit (Agilent Technologies, Santa Clara, CA). The genes encoding for these proteins with a FLAG epitope fused in-frame to the C-terminal end of the protein were inserted into either pBT (bait) or pTRG (target) expression vectors immediately downstream of a full-length bacterial phage repressor protein (λ cl) or amino-terminal domain of RNA polymerase α subunit (RNAPα), respectively.

Prior to the interaction assays, individual constructs were transformed separately into the BacterioMatch II Validation Reporter Competent Cells (Agilent Technologies, Santa Clara, CA), and induced to produce the fusion protein. The bacterial lysates from the resulting cultures were then probed with anti-FLAG antibody to verify the expression of the fusion proteins (data not shown).

With a significant interaction between the bait and target proteins (Dsb and T3SS proteins), the reporter strain would grow on both nonselective and selective media. The level of the interaction can be quantified by comparing the number of colonies on the selective media versus the number of colonies on nonselective media. This ratio will then be compared to a similar ratio of a known interaction pair, such as DsbA and DsbB (Collet and Bardwell, 2002). This relative interaction ratio is a useful quantitative indicator of protein-protein binding.

A positive interaction was detected with the pBT-*dsbB* and pTRG-*dsbA* constructs, indicating that the commercial bacterial-two hybrid system was capable of detecting the transient DsbA-DsbB disulfide intermediate. By comparison, a negative control was established by cotransforming empty pBT and pTRG constructs, and was verified to have no colony growth on selective media. The dramatic lack of growth of the negative control on selective media demonstrated that the system was sufficiently discriminating to prevent growth of reporter cells without a true protein-protein interaction *in vivo*. Therefore, the DsbA-DsbB interaction was established as the baseline level of interactions involving the Dsb proteins, and all other interactions were compared to this benchmark (Figure 4.5).

No colonies were detected on selective media that were cotransformed with the pBT-*spaS* and pTRG-*dsbA*, or pBT-*spaS* and pTRG-*dsbC* constructs. However, there were colonies present on the NSM plates. The inability to grow in the presence of 5 mM 3-AT suggested a lack of significant interaction between the Dsb system and SpaS.

On the other hand, colonies were detected on selective media that were cotransformed with pBT-*spaO* and pTRG-*dsbA*, as well as with pBT-*spaO* and pTRG-*dsbC*. Dividing the ratio of colonies on SSM versus NSM for the DsbA-SpaO interaction pair by the baseline ratio for the DsbA-DsbB interaction pair yielded an average relative interaction ratio of 0.4156. Thus, it can be stated that the level of interaction between DsbA and SpaO was 41.56% of the interaction between DsbA and DsbB. For the DsbC-SpaO interaction pair, the average relative interaction ratio was 1.0321, or essentially the
same level of interaction between DsbA and DsbB (Figure 4.5). These results suggested that SpaO is a substrate of the Dsb system.

It should be noted that there were no colonies detected on the DSSM plates, except for a handful of colonies that were cotransformed with the kit's control plasmids, pBT-LGF2 and pTRG-GAL11P (data not shown). Since colonies were seen on SSM and NSM plates with the kit's positive control and the DsbA-DsbB positive control, the cotransformed bacteria were viable and the lack of growth on DSSM plates was not due to a procedural error. Therefore, additional optimization steps of the cotransformation process must be undertaken to obtain colony growth in the presence of streptomycin.
Figure 4.5. DsbA and DsbC interacted with SpaO.
Interacting pairs of bait and target constructs were cotransformed as per the BacterioMatch II Two-Hybrid System Vector Kit (Agilent Technologies, Santa Clara, CA), and cotransformation mixes were spread onto selective screening media (SSM) and nonselective screening media (NSM). The colony counts were obtained on the SSM and NSM plates, and a ratio was calculated. The SSM/NSM ratio was then compared to that of the known interaction pair, DsbA-DsbB, to obtain a relative interaction ratio. Two experiments were performed, and the results were averaged among the two experiments. The error bars indicate standard deviations.
Discussion

*Salmonella enterica* uses the SPI-1 T3SS to secrete effector proteins that facilitate the internalization of the bacteria as a necessary first step to establish a replicate niche. Previous experiments have indicated a requirement for the Dsb system to cause disease in an animal model (Figures 3.2 and 3.3), and mutations in the *dsb* genes resulted in reduced invasion (Figure 3.1). These results suggested that the Dsb system plays a role in the structure and/or function of the SPI-1 T3SS needle complex.

In a *Salmonella* infection, the bacteria induce the assembly of the SPI-1 T3SS needle complex upon contact with the apical surface of host epithelial cells, and secrete extracellular rod proteins and the translocon before a sorting platform in the inner membrane switches specificity and begins to secrete the effectors. However, in liquid bacterial cultures without the presence of host cells, there exists a basal level of expression of the SPI-1 T3SS and effector secretion still occurs. This allows for the tracking of SipA and SipC in strains grown in LB media.

In order to assess the impact of the *dsb* mutations on the SPI-1 T3SS, it is necessary to have a readout for the activity of the secretion system. To that end, the secretion of T3SS effector proteins SipA and SipC was used in this study since SipA and SipC have been shown previously to have important functions in host cell invasion (Su et al., 2009). The levels of SipA and SipC were measured in the culture supernatant and compared to levels in the bacterial lysate, and the presence of SipA or SipC in the lysate but not in the supernatant would indicate a defective secretion system. Here, it is shown that decreased levels of SipA and SipC were seen in the culture supernatants of the *dsb* mutant strains compared to the parental strain, indicating a requirement for the Dsb system in the secretion of the SPI-1 T3SS effector proteins. These decreased levels were especially apparent in the Δ*dsbA/C* mutant strain, with no detectable levels of SipC in the culture supernatant (Figure 4.3).

However, since SipA and SipC are translocated from the bacterial cytosol directly into the host cell cytoplasm, they are not potential binding partners of the Dsb system. Rather, the potential periplasmic substrates with multiple cysteine residues include PrgK, PrgH, SpaS, SpaR, SpaQ, InvA, and InvH. In order to establish the critical importance of these structural proteins in the SPI-1 T3SS needle complex, mutations were made in these genes and the impact on SipA secretion was assessed. SipA was not detected in the culture supernatant in any of the T3SS mutant strains, further supporting the notion that all of these proteins were indispensable for the function of the SPI-1 T3SS (Figure 4.4). After establishing the importance of these structural proteins, three more SPI-1 T3SS proteins were added to the analysis: SpaO, OrgA, and OrgB (Lara-Tejero et al., 2011). For a comprehensive analysis of the potential interactions of the Dsb system with the SPI-1 T3SS, the various genes for the T3SS structural proteins listed above were cloned into the bacterial two-hybrid system for interaction assays (Table 1.1 and 4.2).

The T3SS proteins SpaS and SpaO were tested for interactions with DsbA and DsbC. All interactions were compared to a known interaction pair of DsbA and DsbB (Collet
and Bardwell, 2002). It was shown that while SpaS did not bind to DsbA or DsbC, SpaO was found to interact with both DsbA and DsbC (Figure 4.5). This suggests that the Dsb system is involved in the formation of the SpaO-OrgA-OrgB sorting platform (Lara-Tejero et al., 2011). Given the central function of SpaO-OrgA-OrgB in forming the needle complex and translocon, it is not too clear how possible misfolding of SpaO in the absence of either or both DsbA and DsbC might result in the various reduced virulence phenotypes associated with the SPI-1 T3SS seen in earlier experiments (Figures 3.1, 3.2, 3.3, and 4.1). In addition, the interaction of SpaO with DsbA and DsbC needs to be further confirmed by additional constructs. Furthermore, the bait and target constructs for DsbD, PrgK, PrgH, SpaR, SpaQ, InvA, InvH, OrgA, and OrgB are currently under construction. The interactions involving these proteins will be added to the analysis as soon as the constructs are completed.

In addition to further interaction testing with the other SPI-1 T3SS proteins, the current procedure according to the BacterioMatch II Validation Reporter Competent Cells (Agilent Technologies, Santa Clara, CA) needs to be optimized for interactions involving Dsb proteins, which are more transient than other protein-protein interactions. When completed, the bacterial two-hybrid will demonstrate the interplay between the Dsb system and the T3SS. However, in order to determine if the interaction is mediated by disulfide bridges, it is necessary to introduce point mutations of the various cysteine residues on the Dsb and T3SS proteins. These point mutants would then be cloned into the bacterial two-hybrid system's bait and target constructs. If the interactions are due to disulfide formation as is hypothesized in this study, then the same interactions would not be detected with the point mutant constructs.
Summary

Salmonella enterica is a major cause of food-borne diseases and its impact on patient care and the food industry is of great public health concern. Salmonella enterica is a successful pathogen because it is able to effectively colonize a wide range of animals, including farm animals integral to the human food chain and pest animals that may act as reservoirs. More importantly, it is an effective human pathogen because it is able to colonize the human intestinal tract through the actions of a plethora of virulence factors including the Type III Secretion System (T3SS) encoded by Salmonella Pathogenicity Island 1 (SPI-1).

An examination of a transposon mutant library constructed in a clinical isolate of Salmonella enterica serovar Enteritidis, SE2472, determined that the dsbD gene is a determinant of pathogenicity. A deletion mutant in the dsbD gene was found to be more susceptible to reactive oxygen intermediates in vitro and less virulent than the wild-type strain in a competitive infection of BALB/c mice (Clavijo and Lu, unpublished data). It is significant to note that the Dsb system is a family of periplasmic proteins that have been shown to be involved in the virulence of other Gram-negative pathogens. Pseudomonas aeruginosa utilizes DsbA to survive in host cells (Ha et al., 2003). In Yersinia pestis, DsbA helps fold outer membrane secretins (Jackson and Plano, 1999). And in Shigella flexneri, DsbA is found to bind to a T3SS protein that regulates needle length (Yu et al., 2000). Since the Dsb system has been studied primarily in non-pathogenic Escherichia coli, the role of the Dsb system in the pathogenesis of Salmonella enterica had not been well characterized (Collet and Bardwell, 2002). Moreover, direct interactions between the Dsb system and virulence factors such as those encoded by the SPI-1 T3SS remain unclear. In fact, none of the known substrates of the Dsb system are found in the SPI-1 T3SS (Agudo et al., 2004; Kadokura et al., 2004; Leichert and Jakob, 2004; Lasica and Jagusztyń-Krynicka, 2007; Heras et al., 2009).

The work presented has shown that the Dsb system is involved in the virulence of Salmonella enterica, possibly from when the bacteria swim through the gut and adhere to the host's epithelial cells, to when the bacteria secrete effectors to internalize through the lamina propria, and then to colonization of the liver and spleen. Strains with mutations in the dsb genes were defective in motility, invasion, colonization, and virulence; complementation of those mutations restored the phenotypes of the dsb mutant strains to that of the wild-type strain. However, the mechanisms by which these processes occur are currently a subject of intense study.

A bacterial two-hybrid analysis was used to identify potential substrates of the Dsb system which might explain how disulfide formation affects bacterial virulence. Results showed binding between the Dsb system and SpaO, an inner membrane T3SS protein that forms a sorting complex with OrgA and OrgB (Lara-Tejero et al., 2011). These interactions suggest that the Dsb system helps assemble the T3SS needle complex through the formation of this sorting complex, and then has an impact on bacterial
invasion of host cells when the sorting complex switches specificity to the translocation of effector proteins. This is consistent with the data obtained from the \textit{dsb} mutant strains that showed a reduced invasion of HeLa cells and reduced secretion of effectors SipA and SipC compared to the wild-type strain. Furthermore, the reduced virulence of the \textit{dsb} mutant strains seen in animal infections could be due to a defect in the assembly of the T3SS.

**Future Directions**

The SPI-1 T3SS proteins SpaS and SpaO were tested for their interaction with DsbA and DsbC in the current study, and more SPI-1 T3SS proteins will be tested in the near future. To complete the study, several other constructs are currently undergoing cloning of the candidate genes into the bait and target plasmids. Upon completion of those constructs and screening for interactions of SPI-1 T3SS proteins with DsbA and DsbC using the bacterial two-hybrid system, a better picture of the Dsb-T3SS interactions can be obtained.

Additional parameters must also be considered for future interaction studies of the SPI-1 T3SS proteins with the Dsb system. For example, the interaction pairs must be reversed, where a given protein is expressed from the bait construct in one pairing and from the target construct in another pairing. If the interaction is true, then the reversed interaction pairings will yield consistent results. Also, the current interaction testing procedure needs to be optimized for growth on the dual selective screening media (DSSM), which selects for the transcription of both reporter genes.

Although the bacterial two-hybrid is a first step in identifying the T3SS structural proteins that interact with the Dsb system, much work is still needed to validate the interactions. To that end, it is necessary to introduce point mutations that remove the cysteine residues from the T3SS structural proteins to verify that the protein-protein interactions are mediated by disulfide formation. The assays as performed in this study can be repeated with the T3SS point mutant strains to characterize the importance of the cysteine residues in the virulence of \textit{Salmonella enterica}. This will provide the necessary data to demonstrate the role of disulfide formation in the assembly and function of the T3SS.
References


