Deciphering the Bifunctional Role of Disulfide Oxidoreductase DsbA2 in
Legionella pneumophila

by

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Dedication

This dissertation is dedicated in loving memory to my beloved brother, Massaquoi M. Kamara Jr.; who taught me that a fulfilled life is a life of service. Thanks for showing me what happiness, loyalty, and love look like. May you rest in blessed peace!

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My dearest mother, Argbah K. Momolu; your life has provided me with a wonderful, day-by-day, lifetime demonstration of a godly mother who understands the true value of family, hard work, dedication, humility, giving and higher education.

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My amazing father, Selma D. Kpadeh; thanks for your encouragement and belief in the importance of me being a well-rounded lady in life.

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List of Abbreviations and Symbols

%	percent
μ	micro (10^{-6})
α	alpha
º	degree
~	approximately
2D	2-Dimension
ACES	2-(2-amino-2-oxoethyl)-amino] ethanesulfonic acid
Amp^R	ampicillin resistant
AMS	4-acetamido-4'- maleimidylstilbene-2,2'-disulfonic acid
BCYE	buffered charcoal-yeast extract
bp	base pair
BSA	bovine serum albumin
BYE	buffered yeast extract
C	Celsius
Cm^R	chloramphenicol resistant
CO_2	carbon dioxide
CSGID	Center for Structural Genomics of Infectious Diseases
Cys	cysteine
Da	Dalton
ddH$_2$O	double-distilled water
DMEM	Dulbecco's minimal essential medium
DFA	direct fluorescent antibody

DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
Dot	defect in organelle trafficking
Dsb	disulfide bonds
DsbA1P150T	*cis*-proline at position 150 was changed to threonine
DsbA2P198T	*cis*-proline at position 198 was changed to threonine
DsbA2ΔN	DsbA2 minus the dimerization domain sequences
EDTA	ethylenediaminetetraacetic acid
ELISAs	enzyme-linked immunosorbant assays
ER	endoplasmic reticulum
g	gram
g	centrifugal force
Gent^R	gentamicin resistant
h	hour
His$_6$	hexameric histidine tag
Icm	intracellular multiplication
IPTG	isopropyl-β-D-galactoside
Km^R	kanamycin resistant
l	liter
LB	Luria-Bertani
LCV	*Legionella* containing vacuole
LD	Legionnaires' disease
Lpn	*Legionella pneumophila*

LPS   lipopolysaccharide

m   meter or milli (10^3)

M   molar

MalPEG   *N*-ethylmaleimide covalently bound to polyethylene glycol

MH   Mueller-Hinton

MIF   mature intracellular form

Min   minute

MOMP   major outer membrane protein

MW   molecular weight

N^{++} nickel

OD   optical density

Omps   outer membrane proteins

PAGE   polyacrylamide gel electrophoresis

PBS   phosphate-buffered saline

PDI   protein disulfide isomerase

PCR   polymerase chain reaction

PHBA   poly β-hydroxybutyric acid

RF   replicative form

rpm   revolutions per minute

ROIs   reactive oxygen intermediates

RT   reverse transcriptase

S   seconds

S-S   disulfide

SD   standard deviation

SDS   sodium dodecyl sulfate

SH   thiol

Strep^R streptomycin resistant

T3SS   type III secretion system

T4SS   type IV secretion system

TAE   tris acetate EDTA

TCA   trichloroacetic acid

TEMED   N,N,N',N'-tetramethylethylenediamine

V   volt

VBNC   viable but non-culturable

X-gal   5-bromo-4-chloro-3-indolyl-β-D-galactopyronidase

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First, and foremost, God is the creator of all things and when it comes to expressing the gratitude in my heart, for me that is where it begins. He gives me strength, hope, joy, peace… - Phil 4:13-19, and He is unfolding the plans for my life - Jer 29:11.

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Abstract

Extracytoplasmic formation of disulfide bonds (DSB) is often required for proper folding of proteins, many of which are important for the assembly of virulence factors such as the Dot/Icm type IVb secretion system (T4SS) of *Legionella pneumophila* (Lpn), the major virulence system that delivers multiple effector proteins into the cytoplasm of host cells. In *Escherichia coli*, DsbA catalyzes the formation of disulfide bonds, while DsbC repairs inappropriate disulfides through protein disulfide isomerase (PDI) activity. DsbA is a monomer that is maintained in a fully oxidized state by the cytoplasmic membrane protein DsbB, whereas DsbC is a homodimer and kept reduced by a second cytoplasmic membrane protein, DsbD.

In contrast, Lpn expresses two DsbA-like proteins (DsbA1 and DsbA2), but no orthologue of DsbC. Both DsbA proteins are capable of catalyzing DSB formation; however, *dsbA1* is dispensable for viability and virulence, while *dsbA2* is essential and required for disulfide bond formation in core proteins of the Dot/Icm type IVb secretion system. DsbA2 is a homodimer that is phylogenetically distinct from DsbA and DsbC lineages. We hypothesized that in a *dsbA1* mutant of Lpn, DsbA2 must be responsible for both oxidase and PDI activities; i.e., a single player system as compared to the two player DsbA/DsbC system of *E. coli*.

In this study we confirmed that DsbA2 possesses PDI activity using a PDI detector assay in which DsbA2 replaces DsbC in *E. coli*. Consistent with a single player bifunctional system, we showed that DsbA2 exists in the periplasm of Lpn as a mixture of disulfides (S-S) and free thiols (SH). This equilibrium can only occur if the two DsbB oxidases (DsbB1 and DsbB2) cooperate with the two DsbD reductases (DsbD1 and DsbD2). To test this hypothesis, we reconstituted the Lpn Dsb system in various *dsb* mutants of *E. coli*, including a *dsb* null mutant strain. Our studies showed that DsbA2 did not restore motility to a *dsbA* mutant of *E. coli*, whereas a monomeric dimerization domain mutant (DsbA2ΔN) restored motility. We showed that both DsbBs of Lpn were able to oxidize DsbA2ΔN, but not DsbA2 and that the *E. coli* DsbD reduced DsbA2 to the thiol. Motility was restored to a *dsb* null mutant of *E. coli* expressing Lpn *dsbA2, dsbB1* or *dsbB2* and *dsbD1* or *dsbD2* and this was possible because the reconstituted system maintained DsbA2 as a mixture of S-S/SH forms. This specificity of Lpn DsbB and DsbD proteins for DsbA2 bifunctional activity suggests a distinct evolutionary tract for the DsbA2 system.

In addition, we identified biological roles for nonessential DsbA1 in Lpn, by showing that a *dsbA1* mutant had lost the ability to produce melanin-like pigment and was more infectious of HeLa cells. This mutant was also more virulent by the contact hemolysis assay, and all of these phenotypes were restored to WT through complementation. Finally, overexpression of DsbA1P150T mutant protein in Lpn did not result in a dominant negative effect on DsbA2 function as was seen with either the DsbA2P198T or DsbA2ΔNP198T mutant proteins. Taken together, these studies suggest that DsbA1 might compete for substrate proteins with DsbA2 and perhaps modulate virulence.

These findings indicate that DsbA2 is a bifunctional oxidoreductase in Lpn, which is maintained by the DsbB and DsbD proteins that cooperate in maintaining this single player system. In addition, this protein, which is found in many other pathogens expressing type IV secretion systems, likely provides a selective advantage by economizing disulfide bond management in these slow growing bacteria.

Chapter 1. Introduction

1.1 *Legionella pneumophila* Review

An outbreak of severe respiratory illness, now known as Legionnaires' disease, occurred in the summer of 1976 in Philadelphia, Pennsylvania. On July 30, 1976, Dr. Joseph Campbell realized that he was treating several patients for an atypical pneumonia, chiefly among veterans who attended the American Legion convention (Fraser *et al*., 1977). All of the infected individuals had either stayed as guests at the Bellevue-Stratford hotel, had spent time in the lobby or on the sidewalk immediately outside the building. After returning to their respective hometowns from the convention, over 182 people came down with this unknown illness, 29 of which died. Of the 29 who succumbed to Legionnaires' disease most were males over 55 years of age, who were immunocompromised, or with respiratory problems (smokers), and or other underlying comorbidities. Interestingly, the pneumonic illness was not spread to immediate family members or to healthcare workers in close contact with the infected victims. An extensive laboratory investigation was undertaken by the Pennsylvania State Health Department and the National Centers for Disease Control, where techniques for detecting a wide variety of toxins and for identifying infections caused by chlamydia, fungi, mycoplasmas, parasites, rickettsia and viruses were employed. Despite these efforts, the etiologic agent of the disease was not identified.

Six months after the initial report of the epidemic, Dr. Joseph McDade from the Centers for Disease Control, first noted that histological slides of human lung biopsy material and spleen tissue from experimentally infected guinea pigs contained a common bacterial "contaminant" (McDade *et al*., 1977). Convalescent serum confirmed the Gram-negative bacterium as the etiological agent of the disease, and was subsequently named *Legionella pneumophila* honoring

the victims who brought national attention to the disease. Since the bacterium had not been isolated on routine bacteriological media, special medium was developed for its isolation and cultivation (Feeley *et al*., 1979). These developments allowed epidemiologists to eventually identify the air-conditioning system of the Bellevue-Stratford hotel as responsible for the dissemination of the organisms to the veterans (Blackman *et al*., 1978). Interestingly, the sera of the hotel employees, who had experienced none of the symptoms of the disease, tested positive for previous exposure to the organism. This indicated that the bacterium was an opportunistic pathogen, causing illness primarily in physically debilitated or immunocompromised individuals. Retrospective studies revealed that other epidemics of Legionnaires' Disease (LD) had occurred prior to 1976, and that the disease Pontiac Fever was caused by *Legionella pneumophila* (Kaufmann *et al*., 1981). Scientific research over the last 40 years has yielded vast amount knowledge into the etiology and pathogenesis of this disease; yet, detailed information about the role of disulfide bonds proteins in the periplasm for the folding of virulence factors for the pathogenesis of *Legionella* still remains a mystery.

1.2 Clinical relevance

Legionella pneumophila is also responsible for causing a less severe disease – a mild flu-like illness called Pontiac Fever (Glick *et al*., 1978), and Legionnaires' disease (LD), a more severe multi-system infection that causes severe pneumonia (Fraser *et al*., 1977), and despite antibiotic intervention it is often fatal. *L. pneumophila* is the predominant cause of legionellosis in humans, accounting for ~80-90% of all reported cases in the United States with a predominance for serogroup 1 (Fields *et al*., 2002).

Pontiac fever was first documented from a flu-like outbreak that struck workers in 1968 in Pontiac, Michigan, but *L. pneumophila* was only identified as the etiological agent ten years after the incident (Glick *et al.*, 1978). Infected individuals displayed mild symptoms such as malaise, myalgia, and headaches, and usually recovered without hospitalization within five days (Glick *et al.*, 1978). In contrast, LD presents as an atypical pneumonia with diffuse radiographic infiltrates within 2 to 10 days after initial exposure to *Legionella*, and with more severe symptoms that may include fever, muscle aches, chest pain, dry cough, abdominal pain, diarrhea, neurological problems and urinary retention (McDade *et al.*, 1977; Fraser *et al.*, 1977). The annual incidence of LD in the United States is estimated at >100,000 cases (Fields, 1996; Bitar *et al.*, 2004). This number may underestimate the true number of cases, as up to 50% of pneumonia is of unknown etiology. Outbreaks of disease can be traced to cooling towers, hot water systems, potable water, humidifiers, hot tubs, and many more other sources (Wadowsky *et al.*, 1982). LD is always transmitted from the environment to people via *L. pneumophila*-laden aerosols and there are no reported cases of human-human transmission. *L. pneumophila* remains a significant threat to the public because it continues to cause random epidemics that often involve hundreds of people over distances up to 14 kilometers from the point source (Ngyen *et al.*, 2006). The mortality rate can exceed 25% in debilitated individuals (cancer patients, elderly, immunocompromised persons and solid organ transplant patients) (Wadowsky *et al.*, 1982; Winn, 1988; Marrie, 1993; Fields *et al.*, 2002). In addition to the morbidity and mortality it causes, *Legionella* is studied because its intracellular life cycle is similar to other existing and emerging human pathogens, and for its weapon potential in studying transmission by aerosol.

1.3 *Legionella* Family and Taxonomy

To date, more than 50 species of *Legionella* have been identified, and at least 24 of these have been associated with human disease (Newton *et al*., 2010). Lpn is the predominant cause of legionellosis in humans, accounting for ~90% of cases, with serogroup 1 accounting for 84% of *L. pneumophila* pneumonia (Yu *et al*., 2002). Some species of *Legionella* can be further differentiated into serotypes, of which there are at least 15 for *L. pneumophila* but so far no more than two for any other species. Within the type species Lpn, 15 serogroups have been identified (Benson & Fields, 1998). The variability between the serogroups has largerly been attributed to the diversity in antigens (lipopolysaccharide, LPS) expressed on the surface of the bacterium. Since Lpn accounts for 90% of human cases of disease (Marston *et al*., 1994), this *Legionella* species has received the most attention. While *Legionella* serogroup 1 is found in the environment, it is not the dominant species worldwide. In France, studies have found that serogroup 1 comprises 28% of environmental isolates but accounts for 94% of clinical isolates (Doleans *et al*., 2004). Of the remaining cases, the majority are caused predominantly by *L. longbeachae* (4%) and *L. bozemanii* (2.4) (Muder and Yu, 2002). Based on a single reported human infection, some of these species are classified as pathogenic (Newton *et al*., 2010).

Lpn, a Gram-negative coccobacillus belongs to the gamma-subgroup of proteobacteria. Comparison of the DNA relatedness of the Legionnaires' disease bacterium, Lpn to known bacterial isolates led researchers to propose that *Legionella* be placed in a new family, the *Legionellaceae* (Brenner *et al*., 1979). Later 16S rRNA analysis demonstrated that *Rickettsia* and *Coxiella* species show distinct evolutionary relatedness with *Legionella*. In 2004, the completed *Legionella* genome sequence revealed that Lpn shares distinct evolutionary relatedness by sharing ~42% of its genome (3.4Mbp) with the *Coxiella burnetii* genome (1.9Mbp), an intracellular

pathogen causing Q fever (Chien *et al*, 2004). Several known and putative virulence factors are among the shared genes (Seshadri *et al*., 2003; Seshadri and Samuel, 2005). The novel DsbA2 protein of Lpn, which is futher characterized in this study was originally annotated as Com1, an orthologue of the Com1 protein of *Coxiella* (Jameson-Lee *et al*., 2011). *Coxiella* is genetically related to Lpn, including genes encoding the highly conserved Dot/Icm T4SS, and orthologues of *dsbB*, *dsbD*, and DsbA2. Since *Coxiella* does not encode DsbA1, by default, DsbA2 must be bifunctional in this bacterium.

1.3.1. Microbiology of *Legionella pneumophila*

Legionella pneumophila is an aerobic, non-sporulating Gram negative intracellular pathogen that is 0.3 to 0.9µm in width and 2 to more than 20µm in length (Brenner *et al*., 1979; Brenner, 1986); it is responsible for most cases of Legionnaires' disease worldwide (Fraser *et al*, 1977; Horwitz, 1983 a and b). The bacteria possess flagella and are thus considered motile. Except for *L. oakridgenesis*, *L. brunesis*, *L. cincinnatiensis*, and *L. longbeachae* serogroup 1, the other named species are motile by one, or two polar or lateral flagella (Chandler *et al*., 1980; Rodgers *et al*., 1980; Bornstein *et al*., 1991).

In natural environments, *L. pneumophila* multiplies in protozoa and acquires traits that increase its virulence (Cirillo *et al*., 1994; Fields *et al*., 2002; Greub and Raoult, 2004). In fact, *L pneumophila* displays a developmental cycle in protozoa and some mammalian cell lines, where the organisms differentiate into metabolically dormant cyst-like forms that enable the bacteria to survive for extended periods in a highly infectious state (Garduno *et al*., 2002; Greub and Raoult, 2004). Transmission to susceptible humans occurs via inhalation of *Legionella*-contaminated aerosols where *L. pneumophila* infects alveolar macrophages (Fields *et al*., 2002). By virtue of

virulence factors such as the Dot/Icm type IVB secretion system, *L. pneumophila* avoids lysosomal degradation by generation of a replication-permissive endosome that fails to mature via the endocytic pathway (Berger and Isberg, 1993; Horwitz, 1983 a and b; Marra *et al*., 1992). Evasion of phagolysosomal fusion is a particularly apt strategy to avoid being consumed by protozoa or killed by human alveolar macrophages and as such will be discussed in a future section (1.3.3).

In terms of their laboratory growth requirements, the organisms are fastidious. They do not grow on blood agar or other standard laboratory media. Their source of carbon and energy is derived from amino acids (George *et al*., 1980). The bacteria rely on cysteine (cys) and iron, and an optimal pH of between 6.0 and 6.9 for growth (Feeley *et al*., 1979; Edelstein, 1981) The growth medium that is currently used is buffered charcoal yeast extract (BCYE) agar supplemented with cys and α-ketoglutarate, and has a pH of 6.9 (Feeley *et al*., 1979, Edelstein, 1981). The charcoal in the media tends to neutralize toxic compounds such as hydrogen peroxide and superoxide radicals that are found upon exposure of the media to fluorescent light, and whose levels are accelerated upon autoclaving (Hoffman *et al*., 1983). The organisms are generally grown at 35 to 37ºC in a CO_2 incubator. *Legionella* colonies, which are bluish-grey in color, are usually visible on BCYE plate after three to four days after inoculation.

1.3.2. Diagnosis

Post-mortem human lung tissue was used to infect guinea pigs and spleen homogenates were inoculated into embryonated yolk sacs, resulting in the original discovery of the etiological agent of Legionnaires' disease (McDade *et al*., 1977; Brenner *et al*., 1979). The resulting bacteria were visualized with Gimenez stain, which uses carbol fuchsin to intensely stain Gram-negative

bacteria and *Rickettsia*. The use of the Gimenez stain was critical, as traditional staining methods failed to detect any bacteria despite the tremendous numbers of bacteria present in the lung in fatal cases (Greer *et al*., 1980). In addition, early attempts to culture *Legionella* on common laboratory media such as Mueller-Hinton (MH) agar were unsuccessful (Feeley *et al*., 1979). A better diagnostic technique was established when a growth medium supporting Lpn growth was discovered. Addition of hemoglobin and IsoVitaleX permitted growth, resulting in the identification of soluble iron and L-cysteine as essential components of the Feeley-Gorman agar (Feeley *et al*., 1979).

Since then, routine laboratory culture of virulent Lpn was made possible using charcoal yeast extract agar in which starch was replaced with charcoal and essential amino acids were provided in yeast extract (Feeley *et al*., 1979). Iron salts like ferric pyrophosphate establish equilibrium between cysteine and the oxide dipeptide cystine, maintaining a steady-state level of cysteine that promotes growth (Ewann and Hoffman, 2006). The α-ketoglutaric acid also serves as a primary carbon source for *Legionella* (Tesh and Miller, 1981). Further refinements like addition of ACES buffer and charcoal led to the currently used buffered charcoal-yeast extract (BCYE) medium (Pascule *et al*., 1980). Charcoal, α-ketoglutaric acid, and cysteine were shown to scavenge reactive oxygen intermediates (ROIs) generated in the medium during aerobic growth (Hoffman *et al*., 1983; Pine *et al*., 1986).

Legionella can be defined in culture by its requirement for L-cysteine, serologically by direct fluorescent antibody (DFA), enzyme-linked immunosorbant assays (ELISAs), polymerase chain reaction (PCR), real-time PCR, and by DNA sequencing (Fields *et al*., 2002).

1.3.3. Microbial Ecology

Paradoxically, the natural habitat of *Legionella* is aquatic, where the nutrient levels and the general growth conditions are expected to be poor. They have been isolated from both natural aquatic environments, i.e. rivers, lakes, streams, and thermally polluted waters (Nguyen *et al.*, 1991) as well as manmade aquatic environments, i.e. cooling towers, hot-water systems, whirlpool spa baths, clinical humidifiers in respiratory equipment, and a supermarket vegetable sprayer (Lee and West, 1991). Also, in an outbreak of Legionnaires' disease in Australia where *L. longbeachae* was identified to be the causative agent, this organism was isolated from potting soil compost used by the patients, who were all avid gardeners (Steel *et al.*, 1990). In the aquatic environment, *Legionella* have been isolated from water temperatures ranging from 5.7°C to 63°C (Fliermans *et al.*, 1981).

Legionella are able to survive for extended periods of time in aquatic biofilms (Rogers *et al.*, 1994) where they fall prey to grazing amoeba (Greub and Raoult, 2004). Bacterial-protozoa interactions not only represent a shelter against stress, but also serve as a niche for replication and selection of virulence traits that prime the pathogen for human infection (Greub and Raoult, 2004). Excluding environmental protozoa, biofilms can provide nutrients and shelter from environmental stresses and thus could provide a suitable environment for persistence (Fields *et al.*, 2002). Lpn can survive for extended periods of time as a viable but non-culturable (VBNC) form; however, it is less clear whether the bacterial is able to replicate in biofilms without protozoa (Steinert *et al.*, 1997).

1.3.4. The Developmental cycle of *Legionella*

As a means of ensuring survival, many intracellular organisms have adopted a variety of mechanisms to allow persistence within their adapted niches (Finlay & Falkow 1997). Intracellular parasitism is not unique to *Legionella*. Despite what is most likely to be an inhospitable environment, several bacteria including *Salmonella, Shigella, Yersinia, Mycobacteria, Chlamydia, Coxiella* and *Listeria* all prefer, or seem to have adapted to intracellular lifestyles (Moulder *et al*., 1984; Finlay & Falkow, 1997). Selective pressures distinct to each niche favor certain traits that maximize survival of the organism in its habitat. One mechanism to allow survival in aquatic environments is transition to a cyst-like form when environmental conditions are poor; when the proper nutrients become available, the organism then differentiates into a metabolically active vegetative form (Sadoff, 1976). A number of Gram-negative intracellular pathogens have evolved a similar differentiation mechanism to allow their persistence, though the mechanism is modified slightly for each of their individual intracellular lifestyles. Like *Legionella* and *Chlamydia*, *C. burnetii* possesses a developmental cycle that was recognized as early as the 1950's (Heinzen, 1997) when density gradient centrifugation yielded a variety of bacterial forms with different buoyant densities. *Coxiella* alternates between a replicative large-cell variant and the environmentally resistant small-cell variant (Samuel et al., 2003). Similarly, the differentiation of Lpn in protozoa and mammalian cells has been well documented (Figure 1). Byrne and Swanson (1998) provided a detailed investigation of the changes associated with Lpn growth *in vitro*. During exponential phase, Lpn
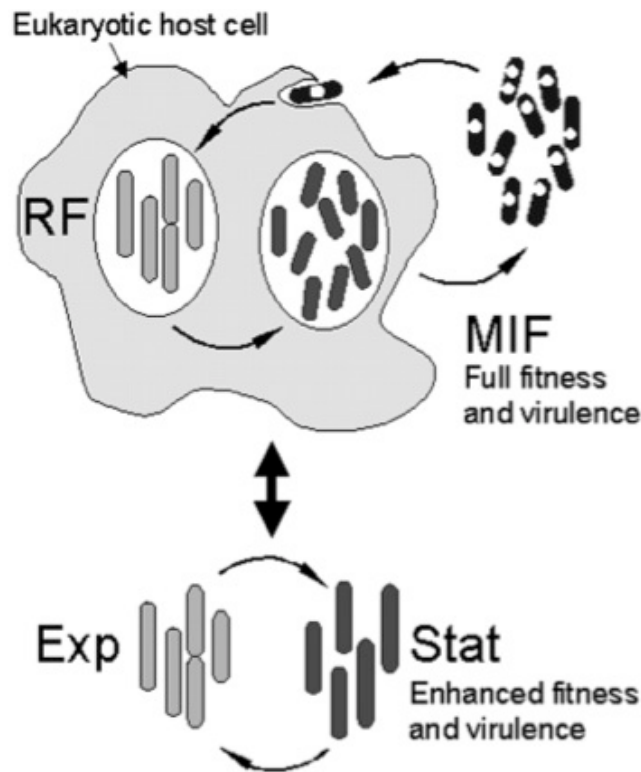
Figure 1. Simplified model illustrating the intracellular and extracellular growth cycles of L. pneumophila. Only the intracellular growth cycle produces the fully differentiated mature intracellular form (MIF), which is more infectious and environmentally fit than the *in vitro*-grown SP bacteria (Stat). Presumably, host-derived signals (inducers) are required for post-stationary-phase differentiation of MIFs. Intracellular replicative forms (RFs) would be equivalent to exponentially growing bacteria (Exp) *in vitro*, as they display similar morphological traits, but it remains to be determined whether or not they are physiologically different. The central double arrow indicates that Lpn can move from one cycle to the other when the proper conditions are met. Notice that, to initiate a new intracellular cycle, Lpn has to exit into the aquatic environment, where it is possible for MIFs to continue their maturation in response to extracellular signals (inducers). Adapted from Garduño *et al*., 2002.

was most resistant to salt (a phenotype often associated with avirulent Lpn strains) (Catrenich & Johnson, 1989), less cytotoxic, less osmotically tolerant, less motile, and less infectious. Based on *in vitro* growth studies, virulence was shown to increase post-exponentially. Similar changes were observed when Lpn were intracellular; intracellular forms were salt-tolerant and non-motile, but upon host cell lysis, the bacteria were observed to be flagellated and sodium sensitive.

As depicted in Fig. 1; throughout the infection of host cells, Lpn is shown to alternate between a replicative form (RF) and a metabolically dormant, highly infectious cyst-like form termed the mature intracellular form (MIF) (Garduño et al., 2002). The stage-specific transition is emphasized by the various intermediate (RF-to-MIF or MIF-to-RF) morphological forms (Faulkner and Garduño, 1998). RFs and MIFs could be distinguished by Giménez staining (Fig. 2), where MIFs retained the carbol fuschin red color (Giménez-positive) whereas RFs, being unable to retain the stain, stained green by the malachite green (Giménez-negative) counterstain (Garduño *et al*., 2002). Compared to RFs, MIFs were metabolically dormant and displayed numerous ultrastructural differences, including poly β-hydroxybutyric acid (PHBA) inclusions, a thickened cell-wall architecture, and multi-laminated intra-cytoplasmic membranes, which were all reflected by increased resistance to various stress (Garduño *et al*., 2002). It should also be noted that due to early demise of the host, Lpn does not differentiate into MIF-like forms very efficiently in infected macrophages, which may partly explain why LD is not a communicable disease (Garduño *et al*., 2002). Nonetheless, Lpn progeny arising from intracellular growth in macrophages express numerous transmission traits that could facilitate their uptake by other phagocytes (Molofsky and Swanson, 2004). It is also important to note that *in vitro*, MIFs do not form because development is arrested in stationary phase. For intracellular pathogens that have a
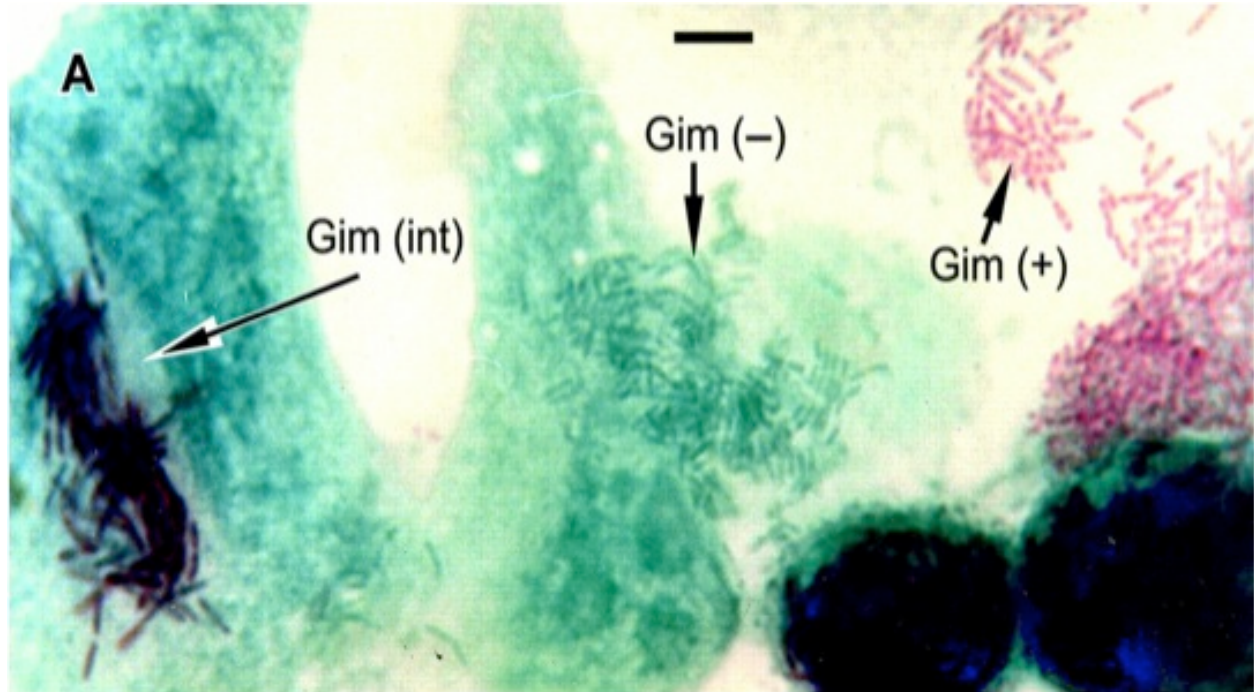
Figure 2. Morphological differences between the two principal forms of *L. pneumophila in vivo.* Giménez phenotypes observed in HeLa cell monolayers infected with purified MIFs. Light micrograph showing three different Giménez phenotypes (+, −, and int) in a single microscopical field from a specimen fixed and stained 24 h after infection. The Giménez negative (green, -) replicative forms are believed to be the form of Lpn that replicates within the host cell. The intermediate (int) forms are believed to represent a transition phase between the replicative forms and the mature intracellular forms (MIFs), which stain red with the Giménez stain, but lack the discontinuous staining pattern of the MIFs (+) formed during later stages of infection. Scale bar = 10 µm. Adapted from Garduño *et al*., 2002.

developmental cycle, there is probably no well-defined stage for stationary phase – they might just go right to differentiation.

1.4 Virulence of *Legionella* in protozoa and Human hosts

The resistance of *Legionella* to these harsh environmental conditions may be explained by the observation that these bacteria can maintain a commensal association with other aquatic microorganisms. The first evidence for symbiosis between *Legionella* species and other organisms was presented by (Tison *et al*., 1980), where Lpn was isolated from an algal mat in thermally polluted water. In the same year, Rowbotham (1980) reported that *Legionella* can infect amoebal trophozoites and then become incorporated in their cysts. To date, Lpn has been found to replicate in numerous amoeba, ciliates, and slime mold (Abu Kwaik *et al*., 1998; Fields *et al*., 2002; Steinert *et al*., 2002). *Acanthameoba castellanii* has been shown to resuscitate Lpn from its environmental VBNC state, revealing important ecological significance (Steinert *et al*., 1997). Growth with protozoa protects Lpn from chlorination, which may explain why elimination of *Legionella* from water systems is so difficult. Indeed, continuous treatment with chlorine dioxide was identified as the most efficient method for controlling Lpn in water systems; however, presence of protozoa resulted in quick re-colonization by Lpn (Thomas *et al*., 2004). In 1986, Rowbotham suggested that *Legionella*-infected amoeba might represent an important vehicle for human transmission. This hypothesis was further confirmed in a murine model where co-infection of Lpn and protozoa resulted in a more severe pneumonia than with either organism alone (Brieland *et al*., 1996). Rowbotham (1986) also suggested that *Legionella*-filled vacuoles could represent a transmissible form. As further demonstrated in *A. castellanii* or *polyphaga*, Lpn was expelled in vesicles that could easily be inhaled ("respirable"), and deliver a substantial dose of

organisms (Berk *et al*., 1998). Latter studies showed that the release of vesicles from protozoa was attributed to two proteins, LepA and LepB (Chen *et al*., 2004) that were translocated into the host cells by the Dot/Icm system (to be described in detail in section 1.5) (Fig. 3). It is important to note that intracellular growth in environmental protozoa is known to select for traits promoting environmental survival (Greub & Raoult, 2004; Rowbotham, 1986) such as resistance to biocides, cold, antibiotics, or traits required for the intracellular lifestyle within human macrophages (Brüggemann *et al*., 2006; Greub & Raoult, 2004)

Legionella's ability to replicate within many different protozoa has equipped the bacteria with the capacity to replicate in human alveolar macrophages (Abu Kwaik, 1996). *Legionella* growth in environmental hosts has been studied with *Acanthamoeba castellani*, *Hartmanella vermiformis*, *Naegleria* spp., and *Dictostelium discoideum* (Valster, *et al.*, 2010). After replicating in these hosts, disseminated Lpn invades and replicates within human alveolar macrophages, monocytes, and alveolar epithelial cells (Gao *et al.*, 1998). After phagocytosis, the facultative intracellular pathogen Lpn is found within a membrane-bound compartment, termed the *Legionella*-containing vacuole (LCV), which diverges from the endosomal-lysosomal pathway within five minutes (Horwitz, 1983b; Roy *et al.*, 1998). The LCV fuses transiently with mitochondria, subsequently becoming surrounded by ribosomes and does not fuse with primary or secondary lysosomes (Horwitz, 1983b). Formation of the LCV involves recruiting secretory vesicles from the endoplasmic reticulum (ER) (Kagan and Roy, 2002), which then proceed to remodel the LCV to resemble an ER-like vacuole (Horwitz, 1983; Swanson and Isberg, 1995; Tilney *et al.*, 2001). Egress from spent hosts is thought to occur via cell death (Gao and Abu Kwaik, 1999) caused by pore formation in the host plasma membrane (Alli *et al.*, 2000; Kirby *et al.*, 1998). In addition to possessing traditional bacterial virulence determinants such as
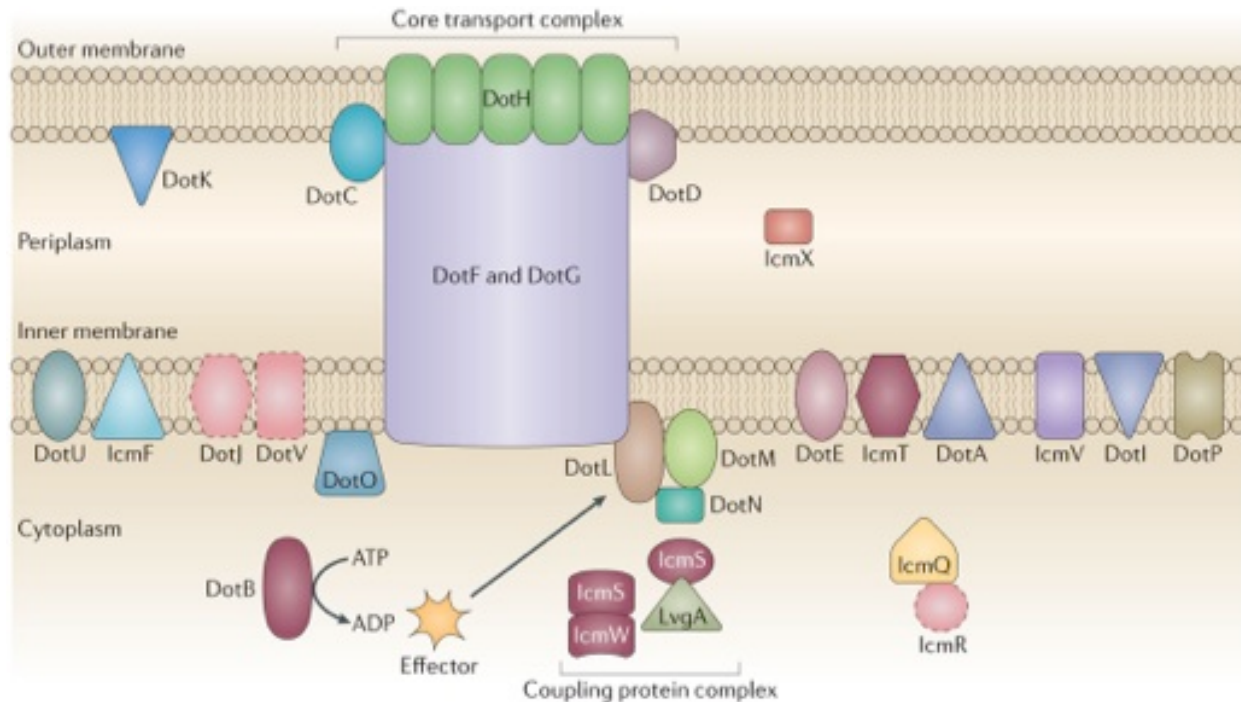
Figure 3. The type IVB secretion systems of *Legionella pneumophila.* The proposed structure of Lpn T4bSS based on mutational analysis encoding *dot* (defect in organelle trafficking) and *icm* (intracellular multiplication) proteins. Studies using Lpn identified two subcomplexes: first termed the core transport complex, which links the inner and outer membranes and is composed of DotC, DotD, DotF, DotG and DotH. A second subcomplex consists of the coupling protein DotL (providing a link between the substrates and the transport complex), DotM, DotN, IcmS and IcmW. Adapted from van Schaik *et al*., 2013.

lipopolysaccharide (LPS) and flagella, *Legionella* has a unique Dot/Icm type IV secretion system (T4SS) (Fig. 3), which serves as a conduit that injects over 300 effector proteins into the host to manipulate host cell processes.

1.5 Dot/Icm Type IV Secretion System (T4SS)

Genetic analysis has revealed Lpn contains two T4SSs: T4aSS, which is more similar to the *Agrobacterium tumafaciens* Vir system, and the T4bSS, first studied in *Legionella* that is more similar to Incl plasmids (Newton *et al*., 2010). Of these two systems, only Dot/Icm T4bSS is known to play a crucial role in virulence (Marra *et al.*, 1992; Berger & Isberg, 1993; Segal *et al*., 1999). Despite their diversity, all T4SS are thought to be evolutionarily related and probably functionally similar (Lessi & Lanka, 1994). For the interest of this report, the Dot/Icm T4bSS is discussed below.

The Dot/Icm T4SS (Fig. 3) of Lpn was discovered simultaneously by two independent groups (Marra *et al*., 1992; Berger & Isberg, 1993), and is now known to be encoded by 26 genes of various nomenclatures found on two distinct regions of the *Legionella* chromosome designated *dot* for "defect in organelle trafficking" and *icm* for "intracellular multiplication" (Vogel *et al*., 1998, Segal *et al*., 1998, Sexton & Vogel, 2002). Phagosomes containing *Legionella dot/icm* mutants fail to evade the endocytic pathway and quickly acquire endosome markers producing an avirulent phenotype. Though the global architecture of the Dot/Icm system is yet to be elucidated, the macromolecular complex is thought to act as a molecular syringe to inject effector proteins that modulate host cell functions into the host cell upon contact activation within minutes of bacterial internalization (Roy *et al*., 1998; Wiater *et al*., 1998; Coers *et al*., 1999). The T4SSs are important for the virulence of several pathogens, including *Agrobacterium tumafaciens*,

Helicobacter pylori, Bordetella pertussis, Bartonella sp., Brucella sp., Ricketssia sp., Coxiella burnetii, and *Wolbachia sp*. (Backert & Meyer, 2006). Lpn utilizes the Dot/Icm T4SS to inject effector proteins into host cells, most of which have unknown effects or may be reductant, rendering them unsuitable to mutational analysis (Hubber & Roy, 2010). Functions for some Dot/Icm effector proteins have been reported; *sdeA* regulates uptake of Lpn into host cells (Bardill *et al*., 2005), AnkX allows Lpn to evade the host endocytic maturation pathway (Pan *et al*., 2008), LegC3 (de Felipe *et al*., 2008), and Dot F (Paumet *et al*., 2009), recruit ER-derived vesicles to the LCV (DrrA (Machner & Isberg, 2006), LepB (Ingmundson *et al*., 2007), SidD (Tan & Luo, 2011)), and inhibit host cell apoptosis (SdhA and SidF (Banga *et al*., 2007; Laguna *et al*., 2006)). The formation of correct DSB in the proteins required for the assembly of the Dot/Icm T4SS apparatus is essential for proper folding and function (to be discussed in detail in section 1.6). While the core proteins require for the assembly of the Dot/Icm T4SS apparatus have been described (Vincent *et al*., 2006), the roles of the disulfide bonding proteins involved in the proper folding of these core proteins in *Legionella* have not been elucidated.

1.6 Protein Folding and Disulfide Bond Formation

In Gram-negative bacteria, membrane or secreted proteins are first expoerted to the periplasm. They are initially synthesized in the cytoplasm, and then translocated across the inner membrane by different export mechanisms (Wandersman, 1992). As newly synthesized polypeptides emerge into the periplasmic space, they must fold into their three dimensional configuration in order to achieve their native structure and be functional later in their final location (Missiakas & Raina, 1997a and b). For proteins that contain intramolecular disulfide bonds, such as the core proteins (DotC (2 cys), DotG (15 cys), DotK (3 cys) and IcmX (3 cys)) of the Dot/Icm T4SS apparatus,

proper folding is dependent on correct formation of new bonds or isomerization of existing disulfide bonds. These bonds are often essential for the stability of the tertiary and quaternary protein structures and their function (Bardwell & Beckwith, 1993; Luckey *et al*., 1991; Gething & Sambrook, 1992). Reduction or breakage of disulfide bridges leads to unstable or unfolded proteins and inactive catalytic enzymes (Bardwell *et al*., 1991; Bardwell *et al*., 1993; Bardwell & Beckwith, 1993; Dailey & Berg, 1993; Missiakas *et al.,* 1993b). These bonds are formed by the linkage of pairs of cysteines in unfolded polypeptides and involve the transfer of a disulfide bond from an external donor that then becomes reduced. The disulfide bond donor in Gram-negative bacteria is an oxidase, which is located in the bacterial periplasm. A disturbing factor in the kinetics of renaturation of reduced proteins was that it took them hours to fold *in vitro*, but only minutes in a cell (Anfinsen *et al*., 1961; Canfield & Anfinsen *et al*., 1963). This discrepancy led to the search for and discovery of an enzyme eventually identified as protein disulfide isomerase (PDI), which catalyzes the proper formation of DSBs (Venetianer & Straub, 1963; Canfield & Anfinsen *et al*., 1963) in proteins. It is exciting to note that Bruno Straub later became the President of Hungary, and Christian Anfinsen won a Nobel Prize for his work on protein folding.

As previously stated above, protein folding has been studied for many years, and has been shown to occur spontaneously and slowly *in vitro* (Anfinsen, 1973). Specifically, *in vivo*, protein folding occurs so rapidly that it must be assisted by accessory proteins, especially in response to environmental conditions such as changes in temperature, pH or osmolarity (Gross, 1996). The role of these accessory proteins is not only to assist folding into native structure, but also to prevent degradation of unfolded proteins. Although PDI had been discovered many years previously, no one had looked for an enzyme similar to PDI in bacteria; it was assumed that the oxidizing environment of the periplasm was enough to cause DSB to form spontaneously. By the

1990's, two labs discovered *dsb* A (DsbA) in *E. coli* and *Vibrio cholerae* (Bardwell *et al*., 1991; Peek & Taylor, 1992), leading to the characterization of these processes in bacteria.

DSB is the covalent link formed between the thiol groups of two cysteine residues when they are in an oxidizing environment, and when the redox potential of the cysteine residues is below that of the biological system it is in. The redox potential of any species is a measure of its affinity for electrons compared against a standard hydrogen electrode with a half-cell potential of 0.0 mV. As electrons are gained by thiol residues, they are simultaneously protonated, leading to the tradition of labeling redox active thiol groups as "SH" when reduced or "S-S" when oxidized. DSBs are known to contribute to the stability, activity, and folding of many proteins, including bacterial proteins that are destined to reside outside the cytosol (Kadokura & Beckwith, 2010). In prokaryotes, this includes outer membrane proteins, flagella, and secreted proteins. This complex but essential process of protein folding and DSB formation is not unique to prokaryotes. Cysteine oxidation also occurs in eukaryotes as proteins are being translocated from the cytosol (reducing compartment) toward the extracellular environment. DSB can also be formed in proteins in the oxidizing environment of the endoplasmic reticulum (Hwang *et al*., 1992) by PDI, which can oxidize, reduce, and isomerize DSB proteins (Freedman *et al*., 1994; Luz & Lennarz, 1996). Oxidases/isomerases accelerate the rate-limiting step of DSB formation by directly catalyzing them or rearranging them. Members of these groups have been well studied in *E. coli* (as described below). In *L. pneumophila,* proteins similar to these DSB proteins in *E. coli* are the subjects of the study presented here.

1.7 The DSB System in *Escherichia coli*

Almost all organisms, from bacteria to humans, are equipped with enzymes involved in disulfide bond generation and isomerization (Inaba, 2010). These processes have been studied in *E. coli* (Ito & Inaba, 2008), the endoplasmic reticulum of yeast (Sevier & Kaiser, 2008), and the intermembrane space of mitochondria (Chacinska *et al*., 2009). The discovery of the first bacterial DSB protein (DsbA) happened similarly to any great scientific discovery; discovering something novel while searching for something else entirely. In the case of DsbA discovery, a reporter fusion protein was constructed and subjected to a genetic screen in *E. coli*, looking for mutations in bacterial secretion. Astonishingly, the reporter protein accidently contained DSB, and the mutants obtained did not interfere with translocation from the cytosol to the periplasm, but mapped to some unknown locus. The mutant was frozen in glycerol for four years until a talented postdoctoral fellow took on the project and proved that the disrupted gene (*dsbA*) catalyzed the formation of DSBs in the bacterial periplasm (Beckwith, 2007; Bardwell *et al*., 1991).

Over 250 proteins are expressed by *E. coli* that contain two or more DSBs and are predicted to enter the periplasm (Hiniker & Bardwell, 2005; Dutton *et al*., 2010), and DsbA is thought to interact with many of them (Frech *et al.,* 1996). Since then, a model for DSB formation in the *E. coli* periplasm has been developed from studies conducted in several laboratories. Up until this study the current DSB model, as illustrated in Figure 4, has the periplasmic disulfide oxidoreductase DsbA (Bardwell *et al*., 1991; Kamitani *et al*., 1992) as the main enzyme responsible for disulfide bond formation. It is the functional equivalent of PDI that is found in the endoplasmic reticulum of eukaryotes (Freedman *et al*., 1994). However, DsbA has only one disulfide active site (CXXC) while PDI has two (Luz & Lennarz, 1996). This
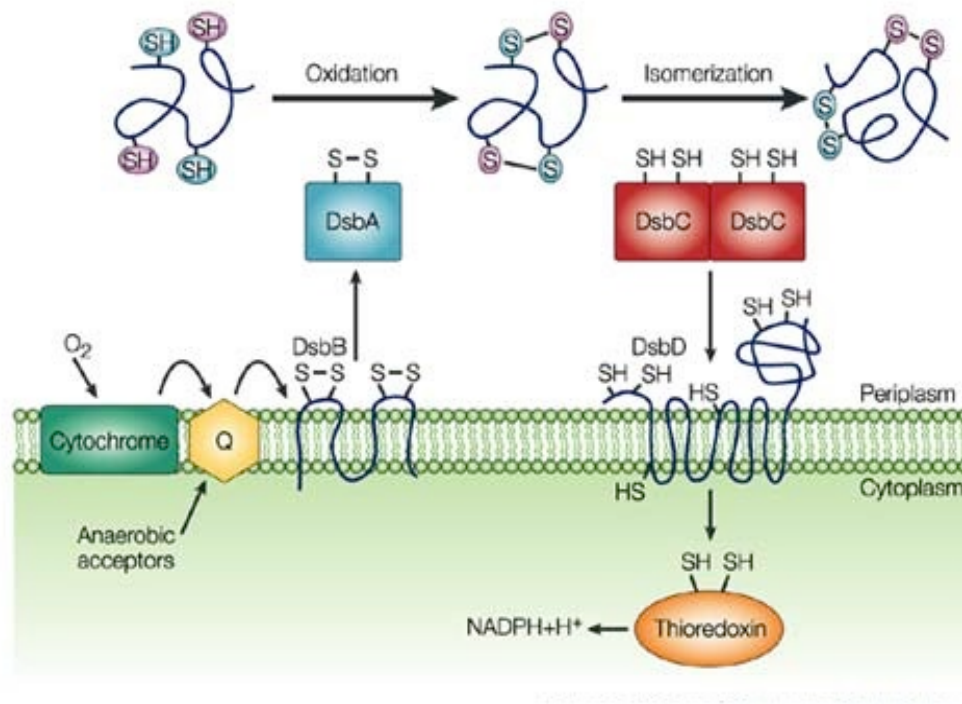
Figure 4. The Disulfide bonds system in *E. coli*. The oxidative pathway. Disulfides are introduced into substrate proteins by disulfide exchange with DsbA, which is in turn reoxidized by the inner membrane protein DsbB. DsbA is a soluble periplasmic protein while DsbB is an inner membrane protein. **The isomerase pathway**. Substrates with more than two cysteines may form incorrect disulfides, causing them to misfold. Non-native disulfides are corrected by DsbC. These two proteins are maintained in their active reduced state by the inner membrane protein DsbD. DsbC is a soluble homodimer in the periplasm and DsbD is a membrane protein. S–S indicates cysteines are in the oxidized disulfide bond form while SH indicates cysteines in the reduced dithiol form. Adapted from Sevier *et al*., 2002.

is probably why PDI, a multifunctional protein, is much more active than DsbA in isomerization reactions and does not need to be re-oxidized by another component (Zapun & Creighton, 1994) while DsbA displays primarily oxidoreductase activity. The redox potential of the active site of DsbA has been measured and is one of the most potent biological oxidants known (Grauschopf *et al*., 1995). DsbA catalyzes disulfide bond formation by oxidizing the sulfhydryl groups on free cysteine residues of reduced, unfolded polypeptides as soon after they are translocated to the periplasm (see Fig. 4) (Frech *et al*., 1996; Kadokura & Beckwith, 2009). Once it has donated its own disulfide bond to its substrate, DsbA itself is reduced. In order to continue its disulfide bond forming function, DsbA need to be re-oxidized. The integral, inner membrane protein DsbB specifically oxidizes DsbA so that it is able to participate in another oxidation reaction (Bardwell *et al*., 1993; Dailey & Berg, 1993; Missiakas *et al*., 1993; Jander *et al*., 1994). Guilhot *et al.* (1995) as well as Kishigami *et al*. (1995) have provided direct evidence of a disulfide-bonded complex between a reduced DsbA and DsbB. In addition, Darby and Creighton (1995) as well as Ruddock *et al.* (1996) have demonstrated the rate-limiting factor in an oxidation reaction between DsbA and short peptides is the reoxidation of DsbA.

   E. coli DsbA is one of the most oxidizing proteins identified in biology (Zapun *et al*., 1993), with a redox potential of -122 mV (Carvalho *et al*., 2006). Its redox potential, coupled with the greater thermodynamic stability in its reduced form, is thought to give rise to its highly oxidizing activity (Philipps & Glockshuber, 2002). The hydrophobic surface that surrounds the active site of *E. coli* DsbA aids in binding unfolded proteins, and when this surface is perturbed through mutation, the substrate repertoire of DsbA narrows (Heras *et al*., 2008; Grimshaw *et al*., 2008; Vivian *et al*., 2009). DsbB is an intergral membrane protein that straddles the cytoplasm and periplasm with a four-helix bundle linked through on cytoplasmic and two periplasmic loops

(Inaba *et al*., 2004). Each of the periplasmic loops contains a pair of cysteines that act in concert to transfer electrons from DsbA through DsbB onto ubiquinones (Kishigami & Ito, 1996), which are then reoxidized by terminal oxidases (Bader *et al*., 2001). The periplasmic loop of DsbB undergoes a conformation change in order to complete the disulfide exchange (Bader *et al*., 2001). *E. coli* DsbB has been demonstrated to have a strict substrate specificity for DsbA, and does not participate in direct disulfide bond reactions with reduced nascent polypeptides emerging into the periplasm (Heras *et al*., 2009).

The DsbA-mediated oxidase system, which is known to introduce disulfide bonds between consecutive cysteine residues in the primary sequence of protein, may not always result in correct DSB formation (Wunderlich & Glockshuber, 1993), especially in proteins with more than two cysteines, or with proteins that contain non-consecutive disulfide bonds. The isomerase activity of DsbC is required for correct protein folding (Shevchik *et al*., 1994; Rietsch *et al*., 1996). DsbC, another periplasmic protein, was first thought to be a disulfide oxidoreductase-like DsbA (Missiakas *et al*., 1994) because defects in disulfide bond formation of several proteins were seen in *dsbC* null mutants. However, work demonstrated by *Zapun et al*. (1995) and supported by Rietsch *et al*. (1996) revealed that DsbC is actually an isomerase. It is only when it is overexpressed that DsbC can substitute for the loss of DsbA function and can reverse most of the *dsbA*⁻ phenotypic defects (Missiakas et al., 1994). DsbC also has a single disulfide bond Cys-Gly-Tyr-Cys (CXXC) in each arm of the homodimer (Zapun *et al*., 1995). In order for DsbC to act as an isomerase, it needs to be in a reduced state. DsbC is kept in a reduced state by its membrane bound redox partner, DsbD (Missiakas *et al*., 1995; Rietsch *et al*., 1996). DsbD is a membrane protein with eight transmembrane helices and two periplasmic domains (Katzen & Beckwith, 2000). DsbC has a C-terminal thioredoxin fold connected to an N-terminal dimerization domain,

forming a V-shaped homodimer (McCarthy *et al.*, 2000). Dimerization serves two purposes: to form a hydrophobic pocket where misfolded proteins can bind, and secondly to prevent interaction with DsbB (Inaba *et al.*, 2006). This supports the current existing Dsb machinery hypothesis that the oxidative and isomerase pathways are separated to prevent futile cycling (Heras *et al.*, 2009).

1.8 The Role of DsbA in virulence

EcDsbA, a 23 kDa periplasmic protein, was first described by Beckwith and Bardwell (Bardwell *et al.*, 1991). Martin et al. (1993) have presented the crystal structure of oxidized DsbA, and demonstrated that structurally it closely resembles the cytoplasmic redox protein thioredoxin despite a low general sequence similarity. The most interesting feature of DsbA is its active site which is composed of two cysteine residues separated by a proline and a histidine, which form a very unstable and reactive disulfide bond (Wunderlich & Glockshuber, 1993; Zapun *et al.*, 1994). It is the instability of this bond that facilitates the very rapid transfer of DSB to newly translocated polypeptide substrates during the oxidation reaction (Darby & Creighton, 1995; Frech *et al.*, 1996). The active site of DsbA is surrounded by a relatively extensive uncharged hydrophobic surface, which is proposed to be a substrate-binding domain (Martin *et al.*, 1993; Guddat *et al.*, 1997), where substrates would bind and be stabilized prior to transfer of disulfide bonds.

DSB proteins, especially DsbA-like proteins, play a role in the biogenesis of virulence factors in many bacteria (Yu & Kroll, 1999; Lasica *et al.*, 2007). Even though DsbA is not essential for *E. coli* survival, mutations in the *dsbA* gene can result to loss of virulence or cause more pleiotropic effects such as loss of motility, reduced alkaline phosphatase activity, reduced OmpA

and β-lactamase proteins, increased β-galactosidase activity, increased sensitivity to DTT and inability to assemble F pili (Bardwell *et al*., 1991; Kamitani *et al*., 1992; Missiakas *et al*., 1993). It has been also suggested that DsbA might have a chaperone-like activity that is independent of its disulfide oxidoreductase activity (Jacob-Dubuisson *et al*., 1994; Zheng *et al*., 1997). In Gram-negative bacteria, fimbriae are classified by structure (type I, type P, and type IV (known as bundle-forming pili)) or how they are assembled (chaperone usher pathway or type IV secretion) (Fronzes *et al*., 2009a). These macromolecular structures require DsbA activity in order to have their individual subunits properly formed and assembled correctly in the periplasm. Okamoto *et al*. (1995) have demonstrated that DsbA is also involved in the DSB formation of two *E. coli* heat-stable enterotoxins, which cause increased intestinal secretion and diarrhea. They demonstrated that in a *dsbA* mutant, these extracellular toxins are degraded before they reach the extracellular medium. However, disulfide bond formation is not completely abolished in a *dsbA⁻* strain. Overexpression of DsbC, an isomerase, was found to functionally substitute for a loss of DsbA function and DsbC-mediated disulfide bond formation could therefore be responsible for the residual activity seen (Missiakas *et al*., 1994). Similar bifunctional activity of a DsbA-like protein (DsbA2) in *L. pneumophila* will be further described in this study. The above combined studies have suggested the importance of DsbA in disulfide bond formation in some virulence factors.

1.9 DsbA homologues in other microorganisms

Since the discovery of DsbA in *E. coli* and the definition of its role in the stability and activity of many extracytoplasmic proteins, DsbA homologues have been described in other pathogens, some of which will be described in this section. First, DsbA was shown to be involved in the

biogenesis of *Vibrio cholerae* enterotoxins (Yu *et al*., 1992; Yu *et al*., 1993). Pectate lyase and the cellulose EGZ, both enzymes catalyzing plant cell wall degradation, were rapidly degraded in the periplasm of a *dsbA* mutant in *Erwinia chrysanthemi* (recently renamed as *Dickeya dadantii*) (Shevchik *et al*., 1995). Watarai *et al*. (1995) also demonstrated that in *Shigella flexneri*, DsbA is required for Spa32 to fold correctly. Spa32 is an outer membrane protein involved in the release of Ipa proteins that are essential for the organism to be able to invade epithelial cells. A DsbA homologue has also been found in the Gram-positive bacterium *Bacillus brevis* (Ishihara *et al*., 1995) that complements an *E. coli dsbA*⁻ strain. It was proposed that this DsbA homologue facilitates disulfide bond formation, probably on the cell surface during or immediately after translocation of the substrate proteins across the cytoplasmic membrane. Also, homologs have also been found in *Pseudomonas aeruginosa* (Leipelt *et al*., 1997), *Azobacter vinelandii* (Ng *et al*., 1997), *Klebsiella oxytoca* (Baek *et al*., 1996), Lpn (Sadosky & Shuman, 1994), and in many other pathogens. The overall protein sequence of many of the homologues is highly similar, and the region around the active site (CXXC) is the most well conserved area among all disulfide oxidoreductase that have been identified to date.

Lpn and many Gram-negative microorganisms other than *E. coli* contain more than one DsbA-like protein, but their functions are yet to be properly characterized. Most importantly, no DsbA homologue has been reported to have both oxidase and isomerase functions for disulfide bond formation in proteins prior to this study in *Legionella pneumophila*.
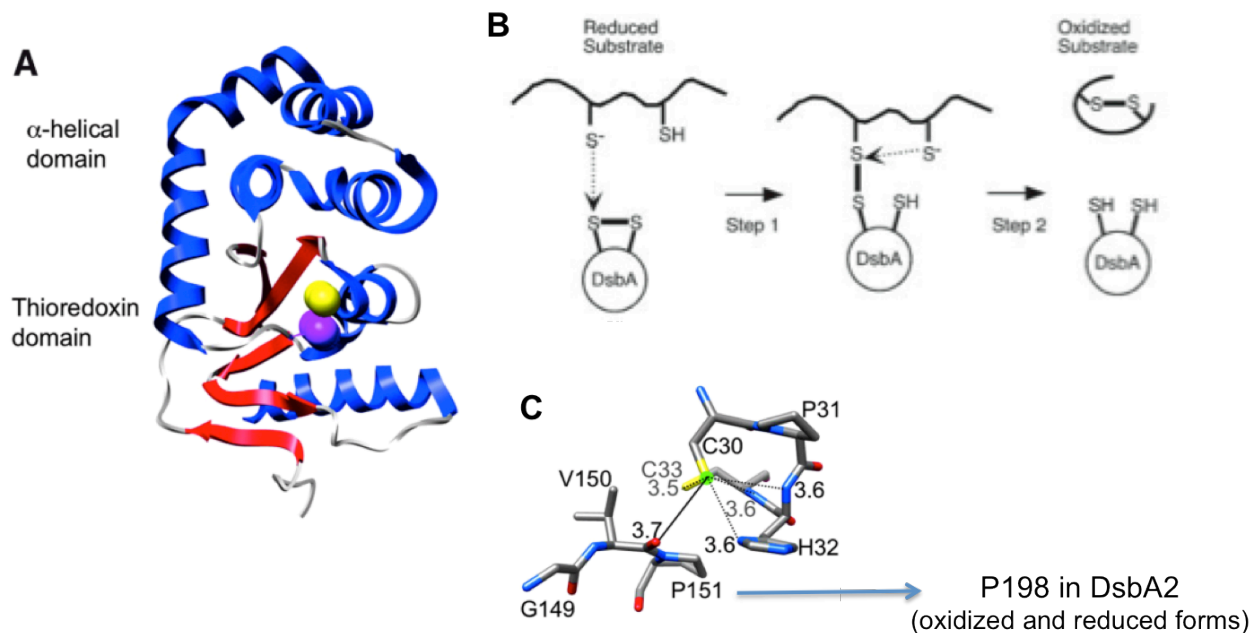
Figure 5. Oxidation of substrate protein by DsbA. A. Ribbon representation of *E. coli* DsbA structure (PDB accession number, 1FVK) generated by PyMol. Yellow spheres represent sulfurs of active site cysteines. Purple spheres also indicate Pro31 and Pro151 residues. **B.** Diagram of DsbA interacting with substrates via S^N2 mechanism adapted from Kadokura et al. (2004). **C.** Active site showing cysteine residue and *cis*-proline that was changed to threonine, which causes a significant defect in resolution of DsbA-substrate complexes. The arrow indicates the conserved proline at position-198 in DsbA2 of Lpn.

1.10 Dsb Machinery in *Legionella pneumophila*

Disulfide bonds, both intermolecular and intramolecular, provide structure and rigidity to macromolecular protein complexes such as chaperone/usher pili (Heras *et al*., 2009), flagella motors (Bardwell *et al*., 1991; Zhang & Donnenberg, 1996), dimeric proteins such as alkaline phosphatase (Bardwell *et al*., 1991), type III secretion systems (Heras *et al*., 2009), type IV secretion systems (Jameson-Lee *et al*., 2011), and many toxins including cholera and pertussis toxins (Peek & Taylor, 1992; Zhang & Donnenberg, 1996; Stenson & Weiss, 2002; Kadokura & Beckwith, 2010; Ruiz *et al*., 2010). In *L. pneumophila*, many membrane-spanning and surface-expressed structural proteins such as the OmpS porin, a trimer that is held together by inter-chain DSBs (Hoffman *et al*., 1992), and several Dot/Icm core proteins have disulfide bonds (Conover *et al*., 2003).

Like many pathogens that commonly cause disease in humans, animals, and plants and express a T4SS, *Legionella* contains two DsbA-like proteins, namely DsbA2, which is reported to be essential for virulence and the proper assembly of the Dot/Icm T4SS system (Table 1) (Kpadeh *et al*., 2013), and DsbA1, a second nonessential DSB oxidoreductase.

DsbA proteins with point mutations have shown deficiencies in substrate translocation (Sexton *et al*., 2004). The process of introducing DSBs into newly translocated proteins is thought to occur via formation of mixed disulfide complexes between DsbA-like protein and its substrates. However, these complexes are transient. Kadokura et al (2004) showed that it is possible to detect covalent intermediates *in vivo* by a mutation in DsbA that alters *cis* proline-151 (Fig. 5). Using this mutant they were able to identify substrates of DsbA. Knowing that the *cis*-proline is highly conserved among thioredoxin superfamily members, a *cis*-proline mutant of DsbA2 in Lpn was also constructed as described by (Jameson-Lee *et al*., 2011), and used to

capture substrate proteins and to evaluate bacterial virulence. Substrates captured by DsbA2 P198T mutant revealed that DsbA2 could function as a disulfide bond oxidoreductase for outer membrane proteins and other virulence factors (Jameson-Lee *et al*., 2011).

DsbA2 exists as a mixture of oxidized and reduced forms *in vivo* in *Legionella* (UVA thesis, 2012; Kpadeh *et al*., 2013), and is reported to be similar to *Coxiella burnetii* outer membrane protein 1 (Com1) (Hendrix *et al*., 1993; Jameson-Lee *et al*., 2011). Prior to this study, *Legionella dsbA1* had no discernable phenotype (Jameson-Lee *et al*., 2011). *Legionella* DsbB1 and DsbB2 (oxidant of the oxidase (s)), and DsbD1 and DsbD2 (reductant of the disulfide bond isomerase) have been discovered in screens involving the Dot/Icm T4SS (Conover *et al*., 2003), but not further characterized.

The question of whether or not the two DsbAs (DsbA1 and DsbA2) are required for disulfide bond formation in the periplasm still remained. In this study, we sought to characterize the bifunctional activities of DsbA2, and identify the functional contributions of the DSB proteins in *Legionella pneumophila* for proper assembly of virulence factors, such as the Dot/Icm T4SS.

Table 1. Disulfide bond composition of genera expressing DsbA2

Bacteria	DsbA1	DsbA2	DsbB	DsbD	CcmG	DsbC/G	T4SDS
Legionella	+	+	+	+	+	-	+
Rickettsia	+	+	+	+	+	-	+
Brucella	+	+	+	+	+	-	+
Anaplasma	+	+	+	+	+	-	+
Agrobacterium	+	+	+	+	+	-	+
Coxiella	-	+	+	+	-	-	+
Ehrlichia	+	+	+	+	+	-	+
Afipia	+	+	+	+	+	-	+
Rhodobacter	+	+	+	+	+	-	-
Rhodopseu.	+	+	+	+	+	-	-
Bradyrhizobium	+	+	+	+	+	-	-
Azospirillum	+	+	+	+	+	-	-
Bartonella	+	+	+	+	+	-	+
Caulobacter	+	+	+	+	+	-	-
Rhodospirillum	+	+	+	+	+	-	-
Aeromonas	+	+	+	+	+	-	-
Bordetella	+	-	+	+	+	+	+
Escherichia	+	-	+	+	+	+	-

This is a partial list from more than 60 genomes identified by BLASTP with the 27-amino-acid dimerization domain sequences of DsbA in *Legionella pneumophila*. The *E. coli* protein sequences were used for the searches against each genus of DsbA1, DsbB, DsbD, DsbC, DsbG, and CcmG (a thioredoxin-like periplasmic enzyme associated with cytochrome *c* maturation). The presence or absence of a T4SS was determined from the literature, and the question mark for *Francisella tularensis* indicated that the system might be incomplete (Kpadeh *et al*., 2013).

1.11 Objectives of study

The proper formation of disulfide bonds in bacterial proteins outside of the cytoplasm is aided by specific oxidoreductase, first characterized in *E. coli* as the disulfide bond (DSB) family of proteins. The DSB enzymes are part of the thioredoxin (TRX) super family of proteins, which are defined by the presence of one or more thioredoxin folds and have been described in detail in the above sections. Briefly, these enzymes participate in disulfide bond formation through a conserved Cys-x-x-Cys (CxxC) active site motif, and have evolved to either reduce or oxidize target substrates. Our interest in disulfide bond formation in *L. pneumophila* is based on our discovery that the major outer membrane protein trimer is cross-linked by inter-chain disulfide bonds. In addition, ultrastructural studies of the cyst form indicated substantial remodeling of the cell envelope that included changes to these outer membrane proteins (Anfinsen *et al*., 1961; Anfinsen & Haber, 1961; Bardwell *et al*., 1991). Much of the cell remodeling occurs extracytoplasmically. Therefore, we became interested in investigating DSB proteins that participate in this process.

Over the last decade, a sophisticated system for disulfide bond formation and protein folding in the periplasm of Gram-negative gammaproteobacteria has been described using *E. coli* as a model. However, it is now known that the complement of Dsb proteins in *E. coli* is the "classical" pathway (mostly referred to as the canonical model (Fig. 4)), but not conserved universally (Dutton *et al*., 2008). Many bacterial species have diversified from the canonical model, as bioinformatic and phylogentic analyses indicate that all Gram-negative species expressing T4SS (exception: *Bordetella pertussis*) express DsbA2 and lack orthologues of DsbC and DsbG (see Table 1). DsbA2 is highly conserved in intracellular human, animal and plant pathogens that employ T4SS for virulence and in most aquatic and soil bacteria, many of which

are also dimorphic. Our lab recently identified DSB oxidoreductase DsbA2. In addition to its essential functions, it seemed to be required for proper assembly or function of the T4SS. Also, previous studies showed DsbA2 to exist as a mixture of oxidized and reduced forms in the periplasm of Lpn. A DsbA2P198T mutant protein was used to capture substrates, supporting that DsbA2 was a DsbA oxidase (Jameson-Lee *et al.*, 2011). The existence of a mixture of oxidized and reduced forms suggests that DsbA2 is a naturally occurring bifunctional DSB oxidoreductase, thus suggesting an "alternative" DSB pathway (Fig. 21). In addition, in *E. coli* DsbA is a monomer while DsbC and DsbG are dimers. Because DsbG does not complement isomerase activity in an *E. coli dsbC* mutant (Ren & Bardwell, 2011) this study did not explore the relationship of Lpn DsbA2 with DsbG. The major structural difference between DsbA and DsbC in *E. coli* is the presence of a dimerization domain on DsbC, which enables DsbC to exist as a homodimer and function as a protein disulfide isomerase.

Prior to this study, the *dsbA2* allele, previously known as Com1, was of unknown function and had not been studied in any bacteria, other than as a diagnostic antigen for *C. burnetii* (Hendrix *et al.*, 1993), and by Dr. Mann in *Francisella* where the DsbA2 is not essential for viability, but is for virulence (Qin *et al.*, 2011). In this study, we sought to characterize this novel DsbA-like oxidoreductase (DsbA2). We will demonstrate the following: i) DsbA exists as a homodimer and also functions as a protein disulfide isomerase, ii) deletion of DsbA2 dimerization domain results in a monomeric DsbA2 that exhibits oxidase activity, and is important for proper assembly and function of the T4SS by functional assays (invasion of HeLa cells, intracellular replication in amoeba, and contact-dependent lysis of RBC), iii) reconstitution of Lpn *dsb* genes in an *E. coli* null mutant reveal that DsbA2 is maintained as a mixture of SH/S-S, exhibiting

oxidase activity using soft agar motility assay, iv) and the DsbDs of Lpn show considerable divergence from *E. coli* DsbD, both structurally and functionally.

In addition to DsbA2, *Legionella* contains DsbA1, which is dispensable for intracellular multiplication. We sought to investigate the differences in conformation, and expression profile of DsbA1 and DsbA2 in *L. pneumophila,* and their ability to complement *E. coli* mutants. We also crystallized DsbA1 and then structurally and functionally compared it to *E. coli* DsbA. The results presented in this study will further broaden our understanding of the *dsb* genes in *Legionella* and reveal that the "DsbA2 system" differs fundamentally from the DsbA/DsbC system of *E. coli,* challenging the established paradigms.

Chapter 2. Materials and Methods

2.1 Bacteriological techniques

The bacterial strains and the cloning vectors used in this study are listed in Table 2. Bacterial stock cultures were maintained at -85°C in nutrient broth containing 10% dimethyl sulfoxide (DMSO) or glycerol.

2.1.1. *E. coli*

The *Escherichia coli* strains were grown on either Luria-Bertani (LB) agar or LB broth (per 1 liter of distilled water: 10 g yeast extract, 10 g tryptone, 5 g sodium chloride; for agar also add 1.5% bacto agar). *E. coli* strains RGP443, RGP663, RGP665, RGP665, RGP810, JCB570, and JCB571 were generously provided by Dr. James C. Bardwell and Dr. Ren Guoping (University of Michigan, Ann Habor, MI). Cells of these strains were grown at 37°C under aerobic conditions in appropriate media supplemented with the appropriate antibiotics. Proper antibiotic (Sigma-Aldrich) selection for *E. coli* strains was maintained when appropriate, as follows: streptomycin (100 μg/ml), kanamycin (40 μg/ml), gentamicin (10 μg/ml), chloramphenicol (20 μg/ml), metronidazole (20 μg/ml) and ampicillin (100 μg/ml). For transformation of pBlueScript-derived vectors, 40μl of a 20mg/ml solution of 5-bromo-4-chloro-3-indolyl-β-D-galactopyronidase (X-gal) or isopropyl-β-D-galactoside (IPTG) was added to a final concentration of 1 mM.

2.1.2. *Legionella pneumophila*

L. pneumophila strains were grown at 37°C on buffered charcoal yeast extract (BCYE) or in buffered yeast extract (BYE) broth: 10 g yeast extract, 1 g [2-(2-amino-2-oxoethyl)-amino] ethane sulfonic acid (ACES), and 1 g α-ketoglutaric acid, with pH adjusted to 6.6-6.7 with 6N potassium hydroxide. BCYE solid medium included all components of BYE broth as well as 16g/l agar and 1.5 g/l of activated charcoal. After autoclaving, all media received 0.4 g/l L-cysteine pH 6.6-6.7, 0.25 g/l ferric pyrophosphate and 100 μg/ml thymidine. Where appropriate, media were supplemented as follows: streptomycin (100 μg/ml), kanamycin (40 μg/ml), gentamicin (10 μg/ml), chloramphenicol (20 μg/ml), metronidazole (20 μg/ml) and ampicillin (100 μg/ml).

2.1.3. Culture conditions

Starter cultures from single colonies or diluted overnight cultures were prepared as previously described (LeBlanc *et al.*, 2006) and used to inoculate pre-warmed BYE or LB to an optical density at 600 nm (OD_{600}) of 0.2. For growth curve determinations, 1 ml aliquot samples were taken every 2 h (triplicate) and optical density was determined at 600 nm every 30 min until stationary phase was reached.

2.1.4. Determination of bacterial optical density

Bacterial optical density (OD) was determined at 600 nm (OD_{600}) using a Unico UV-2100 spectrophotometer. For agar plate grown bacteria, cells were resuspended in 1 ml of phosphate-buffered saline (PBS) [137mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 2 mM KH_2PO_4] or

appropriate culture medium, and vortexed until a homogenous suspension is obtained. Samples were diluted as required, transferred to a cuvette, and OD_{600} was determined. For broth cultures, one hundred microliters of this suspension were then removed and added to 900 μl of ddH_2O or appropriate media (1/100 dilution). The diluted sample was then mixed by inversion, transferred to a cuvette, and the optical density determined at 600 nm. An OD_{600} of 1.0 of *L. pneumophila* was found to be equivalent to 1×10^9 CFU.

2.2 Molecular biology techniques

2.2.1. Construction of P198T-His$_6$ mutants

To create mutant alleles of DsbA1 or DsbA2, proline at position 150 or 198 was changed to theorine using a PCR-based strategy with primer pairs P150T-F and P150T-R, or P198T-F and P198-R, respectively. The PCR amplicons were digested with EcoRI/ BamHI and cloned into pMMB206 to enable expression under a controllable promoter (*lacI^q*- and IPTG-inducible promoter) (Morales *et al.*, 1991). For both P150T and P198T constructs, a His$_6$-tag was also engineered by PCR. All constructs were confirmed by PCR, DNA sequencing and introduced into the Lp02 and AA100 strains by electroporation or by natural transformation.

Table 2. Bacterial strains and plasmids used in this study

Bacterial Strain or plasmid	Relevant properties	Reference or source
Legionella pneumophila		
AA100	Clinical isolate from Los Angeles, CA. Also called 130b	Yousef Abu Kwaik
AA100Δ*dsbA1*	AA100 *dsbA1* mutant	This study
LpO2	Philadelphia-1 derivative, *rpsL hsdR thyA* (Sm^R)	Berger & Isberg
AA100ΔdotA	AA100 *dotA* mutant	Yousef Abu Kwaik
Escherichia coli		
DH5α	F⁻Φ80d*lacZ*ΔM15Δ (*lacZYA-argF*) U169 *endA1 recA1 hsdR17 deoR thi-I supE44 guyrA96 relA1*	Clontech
BL21 Codon Plus	F⁻ *ompT hsdS* (rB⁻ mB⁻) *dcm*⁺ Tet^R *gal*λ (DE3) *endA* The [*argU ileY leuW*] (Cm^R)	Stratagene
JCB570	*E. coli* wild type strain	James Bardwell
JCB571	JCB570 Δ*dsbA*; Kan^R	James Bardwell
R P4 5	*E. coli* JP114 *dsbB-*	Ren & Bardwell
RGP438	*E. coli* JP114 Δ*dsbA* Δ*dsbB*	Ren & Bardwell
RGP209 JP114 *dsbC-*	*E. coli* JP114 *dsbC-*	Ren & Bardwell
RGP663:JP114 + PDI detector	JP114 + PDI detector, Tet and Amp resistance. Amp resistance goes to 3 g/L	Ren & Bardwell
RGP665: RGP209 PDI etector	Tet and Amp resistance (both from the plasmid of PDI detector). However, the Amp resistance is weak as 0.2 g/L.	Ren & Bardwell
RGP666	*E. coli* JP114 Δ*dsbD* + pPDI detector, Tet and Amp resistance	Ren & Bardwell,
R P8 0	*E. coli* JP114 Δ*dsbC* Δ*dsbD* + pPDI detector, Tet, Amp and Kan resistance	Ren & Bardwell,
RGP666: pBS:Lpn*dsbD1* + PDI detector	Tet, Amp (both from the plasmid of PDI detector) and Cm (from pBS).	This study
RGP666:pBS:Lpn*dsbD2* + PDI detector	Tet, Amp (bot from the plasmid of DI detector) and Cm (from pBS).	This study
RGP666: pBS:Lpn*dsbD1* + *dsbA2* + PDI detector	Tet, Amp (both from the plasmid of PDI detector) and Cm (from pBS). DsbD1 and DsbA2 are under the same promoter in the pBS vector.	This study
RGP666: pBS:Lpn*dsbD2* + *dsbA2* + PDI detector	Tet, Amp (both from the plasmid of PDI detector) and Cm (from pBS). DsbD2 and DsbA2 are under the same promoter in the	This study

	pBS vector.	
RGP810 JP114 *dsbC-dsbD-*	*E. coli* JP114 Δ*dsbC* Δ*dsbD* + pPDI detector, Tet, Amp and Kan resistance	R n & Bardwell
RGP810: pBS:Lpn*dsbD1* + PDI detector	Tet, Kan and Amp (from the plasmid of PDI detector) and Cm (from pBS).	This study
RGP810:pBS:Lpn*dsbD2* + PDI detector	Tet, Kan and Amp (from the p asmid of PDI detector) and Cm (from pBS).	This study
RGP810: pBS:Lpn*dsbD1* + *dsbA2* + PDI detector	Tet, Kan and Amp (from the plasmid of PDI detector) and Cm (from pBS). DsbD1 and DsbA2 are under the same promoter in the pBS vector.	This study
RGP810: pBS:Lpn*dsbD2* + *dsbA2* + PDI detector	Tet, Kan and Amp (from the plasmid of PDI detector) and Cm (from pBS). DsbD2 and DsbA2 are under the same promoter in the pBS vector.	This study
ZK01	RGP438 + Lpn*dsbD1*[c] (Gent[R], Kan[R], Amp[R])	This study
ZK02	RGP438 + Lpn*dsbD2*[c] (Gent[R], Kan[R], Amp[R])	This Study
ZK01::pA2B1	ZK01 + *dsbA2* and *dsbB1* domains in pMMB206 (Cm[R], Gent[R], Kan[R], Amp[R])	This Study
ZK01::pA2B2	ZK01 + *dsbA2* and *dsbB2* domains in pMMB206 (Cm[R], Gent[R], Kan[R], Amp[R])	This Study
ZK02::pA2B2	ZK02 + *dsbA2* and *dsbB2* domains in pMMB206 (Cm[R], Gent[R], Kan[R], Amp[R])	This Study
Plasmids		
pBlueScript II KS (+)	Cloning vector, Amp[R]	Stratagene
pBC	Cloning vector, Cm[R]	Stratagene
pBC::*dsbA1*	pBC containing *dsbA1* sequence	This study
pBC::*dsbA1 P150T*	pBC containing *dsbA1* P150T point mutation sequence using quick change site-directed mutagenesis strategy.	This study
pMMB206	Derivative of pRSF1010; *Ptac* promoter and IPTG-inducible *lacIQ* system; Cm[R]	Morales
pBH6119	Promoterless green fluorescent protein (GFP) vector; Amp[R], Thy[+]	Hammer & Swanson
pMMB206::*dsbA2*	pMMB206 containing *dsbA2* coding sequence	This study
pMMB206::*dsbA1*	pMMB206 containing *dsbA1*	This study

	coding sequence	
pMMB206::*dsbA1 P150T*	pMMB206 containing *dsbA1 P150T* coding sequence	This study
pMMB206::*dsbA1 P150T*-His6	pMMB206 containing *dsbA1 P150T* coding sequence with C-terminal His-tagged expression sequence.	This study
pMMB206::*dsbA2 P198T*	*dsbA2 P198T* mutant in pMMB206 cloning vector	This Study
pMMB206::*dsbA2ΔN*	*dsbA2* minus dimerization domain in pMMB206	This Study
pMMB206::*dsbA2ΔN P198T*	*dsbA2ΔN P198T* mutant in pMMB206	Jameson-Lee
pMMB206*dsbA2*::*dsbB1*	*dsbA2* and *dsbB1* domains in pMMB206	This Study
pMMB206*dsbA2*::*dsbB2*	*dsbA2* and *dsbB2* domains in pMMB206	This Study
pMMB206*dsbA2*::*dsbD1*	*dsbA2* and *dsbD1* domains in pMMB206; co-express under the same *Ptac* promoter and IPTG-inducible *lacIQ* system; Cm^R	This Study
pMMB206*dsbA2*::*dsbD2*	*dsbA2* and *dsbD2* domains in pMMB206; co-express under the same *Ptac* promoter and IPTG-inducible *lacIQ* system; Cm^R	This Study
pMMB206*dsbD1*	*dsbD1* domain in pMMB206	This Study
pMMB206*dsbD2*	*dsbD2* domain in pMMB206	This Study
pBC*dsbB1*	*dsbB1* in pBC cloning vector	This Study
pBC*dsbB2*	*dsbB2* in pBC cloning vector	This Study
pBC*dsbA2ΔN*	*dsbA2* minus dimerization domain in pBC	This Study
pMMB206:: Empty vector	AA100 containing pMMB206 empty vector	This study
pRDXA	Dual suicide vector pBOC20 with *B. subtilis sacB* (conferring sucrose sensitivity) and *H. pylori rdxA;* nitro-reductase, as counter selectable markers (conferring sensitivity to metronidazole); Cm^R	Ann Karen C. Brassinga
pRDXAΔ*dsbA1*::Gent	*dsbA1*::Gent suicide delivery vector	This study
pRDXAΔ*dsbA1:* pMMB206::*dsbA1 P150T*	Gent (from the knockout plasmid) and Cm from the pMMB206 plasmid.	This study
pBRDXΔ*dsbA1:* pMMB206::*dsbA1 P150T*-His$_6$	Gent (from the knockout plasmid) and Cm from the pMMB206 plasmid. Plus c-terminal his-tagged expressing sequence.	This study

PDI detector	Beta-lactamase with engineered non-consecutive disulfide bond (Ren & Bardwell, 2011). Requires disulfide isomerase for proper folding and Amp resistance.	Ren & Bardwell

Amp^R, Kan^R, Gent^R, Strep^R, and Cm^R indicate resistance markers to ampicillin, kanamycin, gentamicin, streptomycin, and chloramphenicol, respectively. Thy$^+$ indicates presence of the thymidylate synthase gene enabling the organism to grow in absence of added thymidine. (^{c}) Indicates chromosomally replaced gene.

2.2.2. Genomic plasmid DNA extraction

Genomic DNA was isolated from *L. pneumophila* using the Maxicell 16 DNA purification machine with paramagnetic particles (Promega, Madison, WI). 400 μl of overnight *L. pneumophila* culture was added directly to the first well, and the machine was run under the bacteria setting. Typically, 200 μl of purified DNA (~50 ng/μl − 130 ng/μl) was obtained that was suitable as PCR template (0.1μg) and stored at − 20 degrees.

2.2.3. Plasmid DNA extraction

Plasmid DNA (listed in Table 2) was isolated from *E. coli* and *L. pneumophila* using the QIAGEN Plasmid Miniprep Kit (QIAGEN Inc., Valencia, CA) following manufacturer's instructions. The only exception was *L. pneumophila* was incubated with lysis buffer P2 for 10 minutes on ice to increase bacterial lysis and plasmid DNA yield. For the miniprep kit, 5ml *E. coli* cultures were grown overnight, harvested by centrifugation (4000 x g, 6min, room temperature), and resuspended in 250 μl buffer P1. Following addition of 250 μl buffer P2, solutions were mixed by inversion until clarification. The only exception was *L. pneumophila* was incubated with lysis buffer P2 for 10 minutes on ice to increase bacterial lysis and plasmid DNA yield. Then, three hundred microliters of neutralization buffer N3 was added, solutions were mixed by inversion, and solutions were centrifuged at 13,000 rpm for 10 min at room temperature. The resulting supernatants were added to columns and subjected to centrifugation at 13,000 rpm for 1 min. After addition of 750 μl of PE wash buffer, columns were subjected to centrifugation again and the flow-through was discarded. The columns were centrifuged once more under the same conditions to remove residual ethanol. Plasmid DNA was eluted in EB buffer in a final volume of 50 μl.

2.2.4. Polymerase chain reaction

Polymerase chain reaction (PCR) amplifications were conducted with either *Taq* DNA polymerase (Life Technologies, Grand Isand, NY) or Expand High Fidelity Polymerase (Roche, Mannheim, Germany). All oligonucleotides (Table 3) were synthesized by Integrated DNA Technologies, Inc. (IDT) (Coralville, IA). Typically, 50 μl reactions (8 ul 1.25 mM dNTPs, 3 μl 25 mM $MgCl_2$, 5 μl of 10 x reaction buffer, 400 ng of each primer, 1 μl of polymerase, and 0.1 μg of template). Unless otherwise indicated, PCR conditions were as follows: an initial heating for 5 min at 94 degrees; then 35 cycles of denaturation at 94 degrees for 30 sec, annealing at 60 degrees for 30 sec, then elongation at 72 degrees (1 min per kb of amplicon). The final elongation step was 72 degrees for 7 min, then 4 degrees indefinitely. All PCR reactions were carried out in a Gene Amp PCR system 2400 (Perkin Elmer, Norwalk, CT USA). For colony PCR, one half of a colony was harvested using a sterile toothpick, placed in 100 μl of sterile ddH_2O, boiled for 10 min, and centrifuged at 13,000 rpm at room temperature to remove cell debris. 1 to 2 μl of this solution was used as template DNA in PCR reactions. Amplicons were stored at 4°C for use within 24 h, or at -20°C for long-term storage.

2.2.5. Agarose gel electrophoresis

Electrophoresis of DNA was performed in 1 x Tris acetate EDTA buffer (TAE) buffer, diluted from a 50 x stock, per liter: 242g Tris base, 57 ml glacial acetic acid, and 100 ml 0.5M EDTA pH 8.0, using 1% agarose gels containing 1 x TAE buffer and 1 μg/ml ethidium bromide. Loading buffer was added to DNA samples prior to loading into the wells of the gel. Agarose gels were subjected to electrophoresis at 125 V/cm for desired amounts of time. For visualization

of DNA, a UV transilluminator (Fotodyne, WI) was used and photographs were taken. DNA bands of interest were excised from gels using a scalpel blade.

2.2.6. Purification of DNA from agarose gels

Purification of DNA fragments from agarose gels for subcloning was carried out using a QIAquick gel purification kit (QIAGEN Inc., Valencia, CA) as described by the manufacturer using a microcentrifuge. For every 100 mg of agarose, 300 µl of QG solubilization buffer was added and agarose was placed at 50 degrees until the gel slice had completely dissolved. To ensure optimal DNA adsorption to the filter, 1 gel volume of isopropanol was added to the mixture before being loaded into the spin column. After centrifugation (13,300 rpm, 1min), flow through was discarded and DNA on the column was washed with 500 µl of buffer QG to remove all traces of agarose and centrifuged as before. A second wash with 750 µl of Buffer PE (ethanol added) was performed and centrifuged. After discarding flow through, the column was centrifuged dry to remove all traces of ethanol. DNA was eluted in 30 µl of elution buffer.

Table 3. Oligonucleotides used in this Study

Primer	Sequence
DsbA1- 1F	AGTACCGCCACCTAAGTCGACCTCAAGCTATAAAGGATATTC
DsbA1- 2R	AAACTCGAGCCCGATGTCGAGCCCTCTG
DsbA1- 3F	TCATGGATCCGCAAGGTATTAGTAC
DsbA1- 4R	ACAGGCTTATGTCAAGTCGACGTTGCCAAAGCAGTCATTGGC
c1P151T L v2 (P198T- F)	CATTTGATGGGTACAACAGCCTTTATAATTGG
c1P151T C v2 (P198T-R)	CCAATTATAAAGGCTGTTGTACCCATCAAATG
DsbA2LDNNdel	GGAATTCCATATGGCCGCAATTCAGGAAAAT
DsbA2expBamHI	TTAGATGGATCCTTAATTGCCAGCCGCC
DsbA2NF	TGGCAGCTGATCAGGAAAATGCTGAACAAG
DsbA2NR	GCATTTTCCTGATCAGCTGCCATTATTGCA
Com1FSD	GGGAATTCTAAGGGGAATTACGTGAAATTTAC
DsbA2 R	TAAGGATCCTTAATTGCCAGCCGCC
DsbA2 BamHIR	TAAGGATCCTTAATTGCCAGCCGCC
DsbB1 EcoRI F	ATCGAATTCAGCCGCAATACAATATATGTTGCCGAAATC
DsbB1 BamHI R	ACTGGATCCCGAAAGCACGCGTTTAATGCC
DsbB2 EcoRI F	CTATCGAATTCCAGCTTCAATTATCACAGCCATCTG
DsbB2 BamHI R	ACTGGATCCGCCTGTAAATATCCGCTGGC
DsbD1 XhoI F	ACTGACTCGAGACAATACCGATGACAGCGATATGG
DsbD1 XbaI R	ACTCTCTAGAAGTGTCCGTAGTTACTTTAGTTCTCATC
DsbD2 EcoRI F	CAGAATTCCTATGCAAGAGGTCCAGTGTAGGT
DsbD2 BamHI R	CCGGATCCTTCAACTCAAATGGCGCGCTTGCT

c13'BH16his	TAAGGATCCTTAGTGATGATGATGATGATGATTGCCAGCCGCC
P150T-F	AAATTAACGCCGTAACAGCCTTTGTCGTC
P150T- R	GACGACAAAGGCTGTTACGGCGTTAATTT
GentaSaI F XhoI	ATGCTACTCGAGGTCGACTTAGGTGGCGGTACTTG
Lp Com1 GentaSaIF	ATACTGCAGCTCGCAGATAACTTTATCATTAAT
Lp Com1 GentaSaIR	ATAAAGCTTGCCAAAGGGCTTGAGTTTCCGATTG
DsbA EcoRI	ATAATAGAATTCGAAGATATGGAAGCCGTTGC
DsbA BamHI	ATAATAGGATCCCTTTATAGCTTGAGTTAATTAGGCTG
DsbA 6His	TAAGGATCCTAAATTAGTGATGATGATGATGATGGGCTGATTTTCT
DsbA EcoRI F	GAATTCAAGGGTTATATTGAGGAATTGACTA
DsbD2 XO do Rc	CTTATTAATCACCACTGCAACCACC
DsbB 1kb c	ACTCGAGTGTGTTCAGCTTCATTCGC
DsbB XO upR C	ACAGGCTTATGTCTATTCATAGGTTC
DsbB XO upF	GCGAGCGGGCTCTTTTACCTG
DsbB XO doR C	CTGTGGGGGCTATTGCGGCG
DsbB XO doF L	AGTACCGCCACCTAGATAACCCGAGC
DsbD1 XO do Rc	GTCCACGCCATAAGCACTGGTATC
DsbD2 XO up F	GGAAGGAGCTGGGTTAAAGCAAC
DsbB 1kb L	AATCTAGAAGCGCAGTCAAAGCGCTC
DsbD2 XO up Rc	ACAGGCTTATGTCTATATGACCACGC
DsbD1 XO do F	AGTACCGCCACCTTTGAACGCCGATG
DsbD1 XO up F	CCTTAGGGCAAACAATACCGATGAC
DsbD2 XO do F	AGTACCGCCACCTAGCGCGCCATTTG

DsbD1 XO up Rc	ACAGGCTTATGTCCAAGAGGTGTTGC
DsbB XO upF	ATTACTCGAGGTTTGTATGAGACCAACGTTAGC
DsbB XO upR	ATTAGAGCTCGCATTCAACCCCTTGATTGCAG
DsbB XO doF	ATTAGAGCTCGCCGTTTCTGCGAATGAACAC
DsbB XO doR	ATTAGGATCCGATACCTTCCGCCACCAC
DsbD2 XO upF	AATTCTCGAGCGTGGAGTCAAGGAAACCAAAAC
DsbD2 XO upR	AATTCTCGAGCATATACCCGGCAAGCATCC
DsbD2 XO doF	AATTGAGCTCGCATGAATTTGATTCTTTGCTCTG
DsbD2 XO doR	AATTGGATCCCCAAACTGATTCATACCTATCGC

2.2.7. Purification of DNA from PCR

Purification of DNA fragments (15 kb – 200 bp) was performed using QIAGEN PCR clean-up kit (QIAGEN Inc., Valencia, CA) when agarose gel separation was deemed unnecessary. Manufacturer's instructions were followed using a microcentrifuge. Five volumes of buffer QC were added to each reaction before loading onto a spin column. After centrifugation, the column was washed with 750 μl of Buffer PE (ethanol added), and after decanting flow through a second dry spin removed all traces of ethanol. DNA fragments were eluted with 30 μl of EB buffer. All DNA was stored at -20°C.

2.2.8. Restriction endonuclease digestion

Restriction digestion was performed in 25-μl reactions according to instructions provided by the manufacturer (New England Biolabs [NEB], Ipswich, MA). Unless otherwise indicated, restriction digests were incubated at 37 degrees for 60 to 90 minutes; reactions consisted of 1 μg DNA, 1 x reaction buffer, 1 x bovine serum albumin (BSA), and appropriate units (U) of restriction enzyme. Often, 5'-phosphoryl groups were removed from the vector (to minimize vector self-ligation) by addition of 0.2 μl of calf intestinal phosphatase (CIP) (NEB) for the final 10 minutes of the reaction. Blunt ends were created by heat inactivation of the enzyme, followed by treatment with 1 μl of T4 DNA polymerase and 1 μl of 10 mM deoxynucleoside triphosphates (dNTPs) (Life Technologies, Burlington, ON) for 30 minutes at room temperature or overnight at 16°C. Reactions were terminated by addition of DNA loading buffer (200 mM Tris-HCl, pH 7.5, 200 μM ethylenediaminetetraacetic acid [EDTA] pH 8.0, 20% glycerol and 0.15% xylene cyanol)(Sambrook and Russel, 2001) and subjected to gel electrophoresis; alternatively, reactions

were terminated by addition of 5 volumes of buffer QG included in the PCR Cleanup Kit supplied by QIAGEN (QIAGEN) and purified as described above (PCR purification methodology).

2.2.9. DNA ligation

DNA ligations were performed using T4 DNA ligase (NEB). A 1:3 to 1:5 molar ratio of vector to insert was typically used with a vector concentration of 50 ng. Reactions consisted of appropriate volumes of vector and insert DNA, 1 x final concentration of T4 DNA Ligase Buffer (NEB), and 1 μl of T4 DNA ligase, with the final volume adjusted with sterile ddH$_2$O. Ligations were then left for 1.5 hours at room temperature, or overnight at 16°C.

2.2.10. Preparation of electrocompetent cells
2.2.10.1. *E. coli*

E. coli cultures were grown overnight at 37°C with aeration (200 rpm). LB broth was then inoculated 1:20 with the overnight culture, and allowed to grow for two hours until an OD$_{600}$ of ~0.6 was obtained. All harvests were performed at 3000 x g for 15 minutes at four degrees. Cells were then incubated for 5 minutes on ice. The pellet was then resuspended in 40 ml of 0.1M CaCl$_2$, followed by 20 min incubation on ice, and then harvested by centrifugation (4800 x g, 6 minutes, 4°C; 3x). The pelleted cells were then resuspended in 4 ml of 0.1 M CaCl$_2$. Cells were then aliquoted into 200 μl fractions and stored at -85°C until use.

2.2.10.2. *L. pneumophila*

All *L. pneumophila* strains were grown at 37°C for 48 h on BCYE medium with appropriate selection conditions, and then transferred to fresh media for additional 24 h incubation at 37°C as heavy lawns on BCYE. Bacterial lawns were harvested using sterile disposable loops, and resuspended in 20 ml of sterile ddH$_2$O by pipette. Cells were then centrifuged (3000 x g, 10 minutes, 4 degrees) and resuspended in 20 ml of cold ddH$_2$O, this process was repeated a total of three times. The final pellet was resuspended in 200 µl of cold 15% glycerol and stored at -85°C in 50 µl aliquots.

2.2.11. Electroporation of plasmids into *E. coli* or *L. pneumophila*

Electrocompetent *E. coli* or *L. pneumophila* cells were thawed on ice and then mixed with 1-5 µl of plasmid DNA. The mixture was then added to a 1-mm gapped electroporation cuvette and placed on ice for 5 min. Electroporation was performed at 1.1 kV, 200 Ω, 25 µFD. The cuvette was then flooded with 500 µl of pre-warmed media (for *E. coli*: LB without antibiotics; for *L. pneumophila*: BYE without antibiotics, ferric pyrophosphate, L-cysteine plus thymidine if appropriate), the mixture removed and incubated at 37°C for 1-2 hours with gentle agitation (100 rpm) in a micro-centrifuge tube. One hundred microliters of the transformation is then spread on medium (LB or BCYE) or the tube was spun down at 4000 rpm for 4 minutes, and the pellet resuspended and plated on LB or BCYE with appropriate antibiotics, and incubated at 37°C for 24 h (for *E. coli*) or 3-5 days (for *L. pneumophila*) for colonies to appear. Resulting colonies were replica plated onto fresh medium and incubated overnight at 37°C. PCR analysis and DNA sequencing were used to confirm the resulting transformants.

2.2.12. Phylogenetic analysis

DsbA1 and DsbA2 amino acid sequences used in phylogenetic analysis were obtained from the Legiolist web server (http://genolist.pasteur.fr/LegioList/genome.cgi) and orthologous genes from GenBank by BLASTP search using DsbA1 and DsbA2 of *L. pneumophila* Philadelphia-1 and DsbA, DsbC, and DsbG from *E. coli*. Phylogenetic trees were generated by multiple sequence alignment of unedited DsbA2 sequences using CLUSTALW. Analyses were further refined by BLASTP search using the highly conserved dimerization domain sequence (SLSDAQKKEIEKVIHDYLINNPEVLLEASQA). Protein prediction software Phyre (http://www.sbg.bio.ic.ac.uk/~phyre) was used to align DsbA2 onto the crystal structure of DsbA of *E. coli*.

2.3 Protein techniques

2.3.1. Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE analysis of proteins was performed by standard techniques described in Sambrook and Russell (2001). Briefly, protein samples were harvested as further described below in the next section or from bacterial cultures. Pellets were resuspended in SDS sample buffer (250 mM Tris-HCl pH 6.8, 20% glycerol, 2% SDS, 5% β-mercaptoethanol, 0.001% bromophenol blue) and then boiled for 10 min. After centrifugation to pellet cell debris, 15 μl of protein lysate were resolved on Invitrogen Nu-Page Bis/Tris 4–12% SDS-PAGE at 200 V for 45 min. Gels were then transferred to coomassie blue stain, silver stain, or western blot using specific antibody to targeted protein.

2.3.2 Diagonal gel electrophoresis of DsbA1 cellular extracts

Preparation of cellular extracts and diagonal gel electrophoresis were as described by Kadokura *et al.* (2004) and Jameson-Lee *et al* (2011). Briefly, 4 l of AA100 strain (with DsbA1 P150T construct) was induced at an OD_{600} of 1.0 with 1 mM IPTG for 15h. The cells were centrifuged and washed once with 1/10 volume ddH_2O, then resuspended in 1/10 volume cold ddH_2O. Dry TCA was added to ~8%, the cells were lysed on ice for 20 min, centrifuged for 10 min, and the pellet washed twice with acetone and dried. The pellet was alkylated in 100 mM Tris- HCl (pH 8.0) containing 0.5% SDS and 100 mM iodoacetamide. The alkylated lysate was then diluted four times with 50 mM Tris-HCl (pH 8.0) containing 300 mM NaCl and centrifuged at 10 000 *g* for 20 min at 4°C to pellet insoluble material. The cleared lysate was loaded onto four separate 1 ml Ni^{++} columns pre-equilibrated with buffer A [50 mM Tris- HCl (pH 8.0), 300 mM NaCl, 0.1% SDS]. The columns were washed with eight column volumes of buffer A containing 20 mM imidazole and protein fractions eluted with five column volumes of buffer A + 250 mM imidazole. Fractions containing DsbA1 P150T-His_6 were pooled and concentrated by spin column and centrifugation. Proteins were resolved on Invitrogen Nu-Page Bis/Tris 4–12% SDS-PAGE at 200 V for 45 min under non-reducing conditions. The lane was excised and incubated in warm 100 mM DTT/BME for 15 min, then placed in Laemmli loading buffer without bromophenol blue +100 mM iodoacetamide for 5 min. The excised lane was placed on top of another Invitrogen Nu-Page SDS Bis/Tris 4–12% gel that had been modified by removal of gel material separating lanes as previously described (Hoffman *et al.*, 1992). The gel slice was locked in place by addition of stacking gel, and electrophoresed at 200 V for desired amounts of time, followed by silver staining.

2.3.3. Western blot

Proteins were transferred onto nitrocellulose membranes with the IBlot Western blotting equipment from Invitrogen for 7 min at 20 V. The immune detection reaction was performed following these steps: blots were incubated 60 min in a l x PBS blocking solution (140 mM NaCl, 27 mM KCl, 43 mM Na_2HPO_4, 15 mM KH_2PO_4, pH 7.3), and 3% skim milk (Carnation). Membranes were then incubated overnight or for 60 min at room temperature in a 1x PBS supplemented with primary antiserum-antibody. Following a quick wash in l x PBS, blots were incubated for 60 min in 15 ml of 1x PBS with secondary antibody, membranes were washed with lx PBS for 15 min and then developed.

2.3.4. Isolation of native DsbA2 by osmotic shock

Stationary-phase bacteria (strain AA100 or expressed in *E. coli*) from overnight culture were collected by centrifugation at 13,000 x *g* for 15 min at 4°C, and the pellet (1 g [wet weight]) was suspended in 40 ml of sucrose buffer (0.5 M sucrose, 30 mM Tris-HCl plus 1 mM EDTA at pH 8) and gently shaken for 10 min at 25°C. Following centrifugation, the pellet was suspended in 20 ml of ice-cold 30 mM Tris-HCl plus 1 mM EDTA buffer, gently shaken for 10 min at 4°C, and centrifuged. The shockate was decanted and concentrated to 5 ml and loaded onto the calibrated HiLoad 16/60 Superdex-200 column, and fractions corresponding to the homodimer and monomer (by molecular masses) were subjected to SDS-PAGE. The HiLoad 16/60 Superdex-200 column was calibrated with RNase A (13.7 kDa), chymotrypsinogen A (25 kDa), ovalbumin (43 kDa), albumin (67 kDa), and blue dextran 2000 (void volume). The proteins were transferred to nitrocellulose and developed with DsbA2-specific antibody diluted 1:10,000 in phosphate-

buffered saline (PBS) with 0.1% Tween 20 as previously described (Helsel *et al*., 1987; Jameson-Lee *et al*., 2011).

2.3.5. Periplasmic redox status of DsbA2

The *in vivo* redox status of DsbA2 was determined by alkylation of free thiol groups by 4-acetamido-4'- maleimidylstilbene-2,2'-disulfonic acid (AMS) (Molecular Probes, Eugene, OR) essentially as described previously (Kadokura *et al*., 2004; Jameson-Lee *et al*., 2011). Briefly, *L. pneumophila* was grown to stationary phase (~24 h), and half of the culture was collected by centrifugation and washed once in sterile water and then suspended to an OD_{660} of 0.5 in sterile filtered tap water. The water- suspended bacteria were kept in the dark for 48h at 22°C, a period of time previously shown to promote differentiation into cyst-like dormant forms (Garduno *et al*., 2002). The stationary-phase bacteria were collected by centrifugation and were divided into aliquots, one of which was first treated with 10 mM DTT and then trichloroacetic acid (TCA) precipitated and alkylated with 100 mM AMS; one was TCA precipitated and alkylated with AMS, and one served as an untreated control. The water-treated bacteria were TCA precipitated and treated with 100 mM AMS. MalPEG 5000 (*N*-ethylmaleimide covalently bound to polyethylene glycol) was used to alkylate free thiols of DsbA2 and DsbA2N expressed in *E. coli*. Aliquots were treated as described for AMS except that 2 mM DTT was used to reduce DsbA2 prior to alkylation with 5 mM MalPEG 5000. In both treatments, samples were analyzed by SDS-PAGE and immunoblotting with anti-DsbA2 serum (Jameson-Lee *et al*., 2011). AMS increases protein mass by 490 Da and MalPEG by 5,000 Da, observed as band shifts.

2.4 Functional and enzymatic assays

2.4.1. Motility assays

E. coli wild type (JCB570), *dsbA* mutant (JCB571), *dsbB* mutant (RGP405), *dsbA dsbB* mutant (RGP438) strains were kindly provided by Dr. JC Bardwell (University of Michigan, Ann Harbor, MI). Plasmid pBC or pMMB206 containing either the coding sequence of Lpn*dsbA1*, Lpn*dsbA2*, Lpn*dsbB1*, Lpn*dsbB2*, or empty vector was heat-shocked into JCB571, RGP405, or RGP438. JCB570, JCB571, RGP405, and RGP438 (with different plasmids) were grown overnight in LB and appropriate antibiotics, diluted 1:20, and grown for 2-3 hours to an OD_{600} of 0.7 with 1 mM IPTG induction. Soft agar LB plates (0.4% agar) supplemented with 1 mM IPTG were prepared, and 2 μl of cell suspension was inserted into the middle of the plate. The LB plates were incubated overnight at 30°C, and motility was assessed by measuring the diameter of spreading bacterial growth and reported as a percentage of the WT control.

2.4.2. Insulin reduction assay

Reductase activity was assessed by an insulin precipitation assay with minor modifications (Bardwell *et al*., 1991; Jameson-Lee *et al*., 2011). Bovine insulin was dissolved in Tris-HCl to 10 mg/ml (1.67 mM) and titrated to pH 7.5, creating a clear solution. Reactions (triplicate) were carried out in 200 μl of 100 mM sodium phosphate buffer, pH 7.0, 150 μM insulin, 0.33 mM dithiothreitol (DTT), and 2 mM EDTA; reaction mixtures were incubated in a 96-well plate format at room temperature in a VersaMax (Molecular Devices) plate reader, and absorbance was measured at 650 nm. The insulin reduction assay was initiated by adding 5 μM DsbA2, DsbA2ΔN, and DsbA1 protein purified as His_6-tagged proteins following induction in *E. coli*

strain BL21. Both time to start of insulin reduction and specific activity were determined in triplicate using the enzyme kinetics program as previously described (Jameson-Lee *et al.*, 2011).

2.4.3. PDI detector assay

The PDI detector assay utilizes the TEM1 β-lactamase of pBR322 with an engineered nonconsecutive disulfide bond (cysteine residues added, S81C and T108C) that requires disulfide bond isomerase activity to be properly folded in the *E. coli* periplasm (Ren & Bardwell, 2011). Strains kindly provided by J. C. Bardwell and listed in Table 2 include RGP209 (*dsbC* mutant control), RGP663 plus pPDI detector plasmid (wild-type positive control), RGP665 (RGP209 plus pPDI detector plasmid), RGP666 (*dsbD* mutant plus pPDI detector plasmid), and RGP810 (*dsbC dsbD* mutant plus pPDI detector plasmid). Ampicillin resistance was evaluated at 0, 1, 2, or 3 g/liter. For complementation studies, *dsbA1*, *dsbA2*, *dsbA2LDN*, *dsbA2ΔN*, *dsbD1,* and *dsbD2* were cloned into pBC or pMMB206 with selection for chloramphenicol. Bacterial cells were grown overnight in the appropriate antibiotic, diluted 1:20, and grown for 2 to 3 h to an OD_{600} of 0.7 with 1 mM IPTG induction. Cells were then serially diluted and plated in triplicate on LB plates with 0, 1, 2, or 3 g/liter Amp, and CFU counts were recorded at 24 h.

2.4.4. Isomerization assay

TEM1 β-lactamase (WT and PDI detector mutant enzymes Bla and MBla) was obtained from spent culture supernatants of strains RGP663 and RGP665 following overnight growth in LB medium. Supernatants were concentrated by spin column (12 ml concentrated to 0.5 ml) (Amicon Ultracel 10k). Bla activity was tested by spotting 5 μl of concentrated supernatant onto

nitrocefin-impregnated paper disks (Becton, Dickinson). The relative concentration of Bla in each fraction was determined by SDS-PAGE following staining with Coomassie brilliant blue. For the PDI assay, either purified leaderless H_6-tagged DsbA1 (6.9 mg/ml) or H_6-DsbA2 (3 mg/ml) was added to concentrated culture supernatant from strain RGP665 in a ratio of 1:5 and incubated at 37°C for 30 min. Nitrocefin (Calbiochem, La Jolla, CA) was prepared according to manufacturer's instructions for a spectrophotometric assay, and a working dilution of 500 μg/ml was prepared in 0.1 M sodium phosphate buffer at pH 7.0. The refolding assay was configured in a 96-well microplate (Costar, Corning, NY) assay (100 μl/well) containing sodium phosphate buffer and 15 μl of MBla plus DsbA1 or MBla plus DsbA2, and the assay was started by the addition of 10 μl of nitrocefin stock. Controls contained no DsbA2 or DsbA1 protein or contained WT Bla as a positive control. The rates of hydrolysis of nitrocefin at 486 nm were obtained over the linear range of the reaction (30 min) at 30°C in a Molecular Dynamics plate reader; all reactions were run in triplicate.

2.4.5. Erythrocytes contact lysis

Human red blood cells (UVA Blood Bank) were diluted in PBS, washed by centrifugation until supernatant was colorless, and incubated with bacterial strains at an MOI of 25:1 to 50:1 in a final volume of ~1 ml as described (Kirby *et al.*, 1998; Charpentier et al., 2009; Jameson-Lee et al., 2011). *L. pneumophila* strains and red blood cells were mixed, pelleted for 3 min at 10 000 *g*, and after 1 to 2 h at 37°C followed by gentle vortexting, 100 ml aliquots of supernatants were transferred to microtitre plates, and absorbance measured at OD_{415} for hemoglobin release.

2.4.6. HeLa cell infection

HeLa cells were grown in Dulbecco's minimal essential medium (DMEM) with newborn calf serum (NCS) as described previously (Garduño *et al.*, 1998c; Jameson-Lee *et al.*, 2011). HeLa cells were resuspended in DMEM without antibiotics to a concentration of 10^6 cells per ml and left to adhere for 1–2 h. *L. pneumophila* was standardized to an OD_{600} of 1.0. One hundred microlitres of the bacterial suspension was added to triplicate wells to a final inoculum of 10^8 bacteria per 10^6 HeLa cells. Plates were centrifuged at 500 rcf for 5 min at room temperature in a clinical centrifuge to maximize contact of bacteria with the HeLa cell monolayer and then incubated for 3 h to facilitate infection. Following incubation, monolayers were washed six times with PBS, lysed with water and vigorous pipetting and the remaining bacteria (representing *L. pneumophila* which as attached or invaded the HeLa cells), was then serially diluted and plated in triplicate on BCYE agar for bacterial enumeration.

2.4.7. *A. castellanii* infection

Acanthamoeba castellanii (ATCC 30010) was maintained in ATCC medium 712 at 25°C. For infection, a 48 h culture of *A. castellanii* was washed and resuspended in Tris-buffered salt solution containing 2 mM KCl, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$ and 1 mM Tris, with the pH adjusted to 6.8–7.2 (Berk *et al.*, 1998). Approximately 10^5 *A. castellanii* per ml were infected with 10^3 cfu per ml of *L. pneumophila* from an overnight culture grown in the presence or absence of IPTG, as previously described (Morash *et al.*, 2009; Jameson-Lee *et al.*, 2011). All experiments were performed in triplicate, incubated at 30°C, and at each time point wells were scraped to remove adherent amoeba. Bacteria were enumerated via decimal dilution in sterile

water and plating on BCYE agar plates as previously described (Morash *et al.*, 2009). Data are presented as the mean and standard deviation.

2.4.8. L929 infectivity assay

Murine L929 cells were grown and maintained in 1 x DMEM supplemented with 10% bovine serum. For plaque assay, ~ 5 x 10^5 cells were added to each well of a 24-well tissue culture plate. After allowing the cells to adhere for 2-3 h, the confluent monolayers were washed once with PBS, and received 200 μl of 1 x DMEM (with 10% bovine serum). Stationary phase grown *L. pneumophila* cell were harvested and standardized to an OD_{600} of 0.5 in 1 ml of 1 x DMEM (with 10% bovine serum). Cells were then serially diluted 10-fold in 900 μl of 1 x DMEM (with 10% bovine serum). Immediately after removing the culture medium from the L929 cells, 400 μl of each dilution was added to duplicate wells. Plates were then subjected to centrifugation (1, 000 x *g*, 10 min) at room temperature and incubated for 3 h at 37°C. Following incubation, monolayers were washed six times with 1 ml DMEM before the addition of 1 ml of 0.6% agarose (SeaKem) in 1 x DMEM (with bovine serum) supplemented with appropriate antibiotic for selection. Cells were then incubated at 37°C for four days. After four days, 1 ml of PBS containing 10% formalin was added to each monolayer (gently placed under agarose plugs), and then incubated for 2 h to 24 h at room temperature. The agarose plugs were gently removed and the PBS/formalin solution was washed off the monoloyer with ddH_2O, and the monolayer were stained for 1 h with 1% crystal violet in 20% EtOH. Excess stain was then removed with ddH_2O and plates were left to air dry as described (LeBlanc, Dalhousie University thesis, 2006). Plaques in the monolayer were counted and plaquing efficiency was calculated as (number of plaques formed/ viable count of the inoculum) x 100.

2.5 Other methods

2.5.1. Complementation studies

Wild-type (WT) *E. coli* (JCB570) and a *dsbA* mutant (JCB571), *dsbB* mutant (RGP405), and a *dsbA dsbB* double mutant (RGP438) were kindly provided by J. C. Bardwell. The plasmid pBC or pMMB206 containing the coding sequence of *dsbA, dsbA2*, or *dsbA2ΔN* were transformed into JCB571, and p*dsbA1* (or with either p*dsbB1* or p*dsbB2*), p*dsbA2* (or with either p*dsbB1* or p*dsbB2*)*, p*dsbA2ΔN* (or with either p*dsbB1* or p*dsbB2*) were transformed into RGP438. Also, p*dsbB1,* p*dsbB2,* or empty vector was transformed into the (RGP405) *E. coli* strain. Appropriate antibiotic(s) resistance was used (see Table 2) for selection of all constructs.

2.5.2. Bioinformatics and analyses

Phylogenetic analyses of the various Dsb proteins of *L. pneumophila* (DsbB1, DsbB2, DsbD1 and DsbD2) was performed using CLUSTALW. Transmembrane topology of DsbB and DsbD proteins was determined using TMHMM Server 2 software and amino acid positioning by alignments. BLASTP was used to evaluate relatedness of DsbB and DsbD proteins.

2.5.3. Phylogenetic analysis of DsbA1, DsbA2 and EcDsbC

DsbA1 and DsbA2 amino acid sequences used in phylogenetic analysis were obtained from the LegioList web server (http://genolist.pasteur.fr/LegioList/genome.cgi) and orthologous genes from GenBank by BLASTP search using DsbA1 and DsbA2 of *L. pneumophila* Philadelphia-1 and DsbA, DsbC, and DsbG from *E. coli*. Phylogenetic trees were generated by multiple sequence alignment of unedited DsbA2 sequences using CLUSTALW. Analyses were

further refined by BLASTP search using the highly conserved dimerization domain sequence (SLSDAQKKEIEKVIHDYLINNPEVLLEASQA).

2.5.4. Construction of DsbA2ΔN

To construct an in-frame deletion of the dimerization domain of DsbA2 (DsbA2ΔN), a vector-free strategy was employed using overlapping primers (DsbA2NF & DsbA2NR) to join the 5' leader sequence with sequences downstream of the dimerization domain, and using flanking primers Com1FSD and DsbA2BamHIR to amplify the joined construct. The resulting amplicon was cloned into pBC and pMMB206 vectors.

2.5.5. Construction of ZK01 and ZK02

Utilizing RGP438 (*ΔdsbA dsbB*) mutant strain, we replaced the chromosomal copy of the *E. coli dsbD* with either LpnDsbD1 or LpnDsbD2 resulting to genetically altered strains ZK01 and ZK02, respectively. We utilized a vector free strategy using primers (DsbD1 XhoI F & DsbD1 Xbal R) and (DsbD2 EcoRI F & DsbD2 BamHI R) to amplify *dsbD1* and *dsbD2*, we then amplified a gentamicin cassessette using primer pairs (GentaSal F XhoI & Lp Com1 GentaSaIR) and (Lp Com1 GentaSaIF & Lp Com1 GentaSaIR). The amplicons were digested with appropriate enzymes and ligated creating *dsbD1*-gent and *dsbD2*-gent. The constucts were electroporated into RGP438 competent cells and plated on antibiotic plates for selection of chromosomally replaced *dsbD*. Colonies of the constructs were checked by PCR with primers (DsbD1 XhoI F & Lp Com1 GentaSaIR) and (DsbD2 EcoRI F & Lp Com1 GentaSaIR); PCR products were validated by visualization of gel electropheresis. Following validation, DsbA2 with

either DsbB1 or DsbB2 in pMMB206 inducible vector (pA2B1 or pA2B2) were added to ZK01 or ZK02, and then utilized in motility assay as described above (section 2.4.1) to assess DsbA2 oxidase activity.

Chapter 3. Results

3.1 Phylogenetic analysis

Previous phylogenetic analysis of the DsbA2 clade indicated that this group had diverged from the DsbA lineage of disulfide bond oxidoreductase (Jameson-Lee *et al*., 2011). Comparison of the structures of DsbA2, DsbA, and DsbC shows that DsbA2 contains an additional 56 amino acids (86 total) from the N-terminus to the CXXC motif compared with 31 for DsbA and 107 for DsbC. A refined BLASTP search using the dimerization domain region depicted in Fig. 6A revealed a 27-amino-acid (aa) sequence (bold in Fig. 6A) present in all the bacterial species expressing DsbA2 listed in Table 1. Moreover, this sequence was more discriminating in searches than the whole protein or the C-terminal DsbA-related sequences used previously (Jameson-Lee *et al*., 2011). Table 1 includes representative species based on a similarity cutoff of $\sim e^{-34}$ and is not meant to be comprehensive. We noticed that below this cutoff, the DsbA2 clade begins to merge into the DsbA clade. The DsbA2 clade includes a group of intracellular parasites that commonly cause disease in humans, animals, and plants and share a T4SS. Other genera in this clade that do not express T4SSs include the nitrogen-fixing endosymbionts associated with leguminous plants, including *Bradyrhizobium* and related genera (not listed), and many soil and aquatic free-living genera, including *Azospirillum Rhodopseudomonas*, *Rhodospirillum*, and *Caulobacter*. One exception is *Bordetella pertussis*, which has a T4SS (pertussis toxin [PtL] transporter) and expresses a DsbA/DsbC system similar to the *E. coli* system.

In addition, the spacing between the CXXC motif and the resolving *cis*-proline motif is conserved between DsbA2 and DsbA compared with DsbC. Finally, as highlighted in Fig. 5B, the first amino acid at the N-terminus of the *cis*-proline in DsbA2 (threonine) is similar to that of
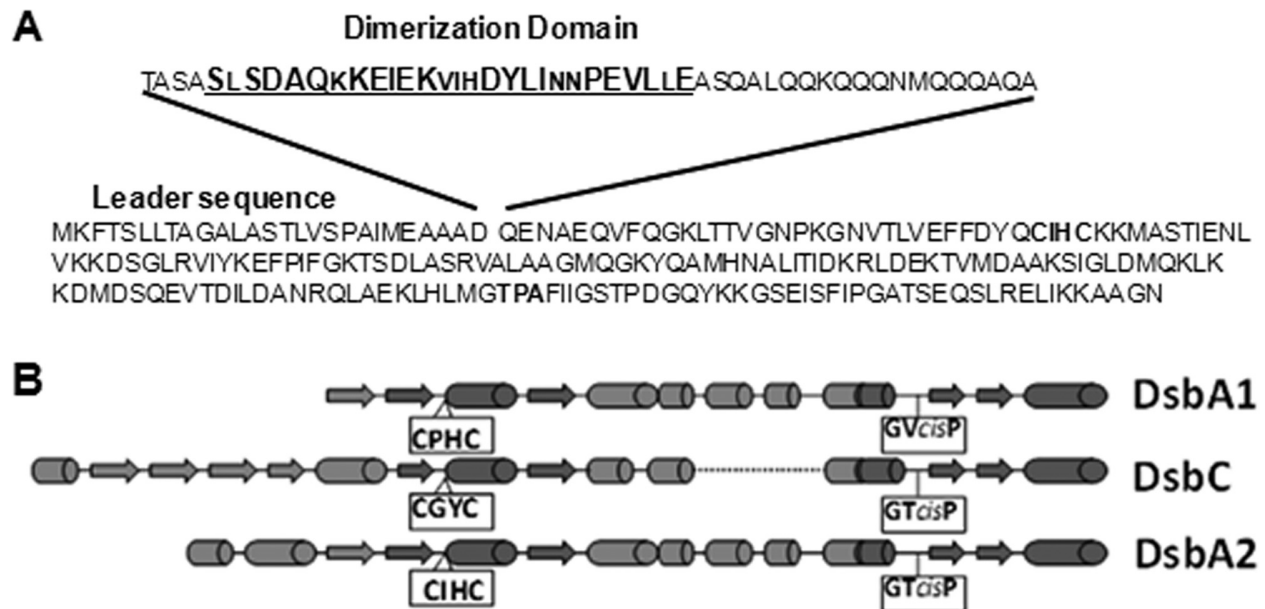
Figure 6. Dimerization domain and secondary structure comparison among DsbA proteins of Lpn and DsbC of *E. coli*. (**A**) DsbA2 amino acid sequence depicting the leader sequence and the detached dimerization domain sequence deleted to create DsbA2ΔN. The underlined sequences (27 amino acids) of the dimerization domain are conserved, and larger letters represent amino acids that are highly conserved among DsbA2 members listed in Table 1. The CXXC and *cis-proline* regions are in boldface. (**B**) Secondary structure predictions for *L. pneumophila* DsbA1, which is similar to DsbA of *E. coli, E. coli* DsbC, and *L. pneumophila* DsbA2 are depicted. The CXXC region and the *cis-proline* motifs are boxed. Note that the *cis-P* region of DsbA2 is similar to that of DsbC.

DsbC and not of DsbA which substitutes valine. Further genomic analysis using *E. coli* DsbC and DsbG amino acid sequences in the BLASTP searches revealed that the genera expressing DsbA2 listed in Table 1 also lacked orthologues of DsbC and DsbG. Our previous studies showed that *L. pneumophila ΔdsbA1* was indistinguishable from the wild-type strain for motility and infectivity for amoebae and HeLa cells, and in the close relative *Coxiella burnetii*, *dsbA1* is completely absent from sequenced genomes (Jameson-Lee *et al*., 2011). While there is plenty of precedent for interchangeability of DsbA and DsbC functions in *E. coli* (Bader *et al*., 2001; Segatori *et al*., 2004), we investigated whether DsbA2 represents a functional equivalent of DsbC or if equilibrium of monomers and homodimers is responsible for the apparent bifunctional phenotype and thus a variation of the *E. coli* DsbA/DsbC paradigm.

3.2 DsbA2 exists as a homodimer in *E. coli* and *L. pneumophila*

To test whether DsbA2 exists as a mixture of monomers and dimers, size exclusion chromatography was employed. For this study, His$_6$-tagged DsbA1 and DsbA2 were treated with iodoacetamide to limit formation of spurious disulfides prior to nickel interaction chromatography. Each purified protein was subsequently applied to a calibrated gel filtration column, and, as seen in Fig. 7A, DsbA1 eluted at 95 min, with an apparent mass of 27 kDa, consistent with a monomer. In Fig. 7B, DsbA2 eluted as a single peak at 75 min, with an apparent mass of 60 kDa, consistent with the size of the homodimer. Subsequent purifications of DsbA2 indicated that inclusion of iodoacetamide was unnecessary.

To ensure that the homodimeric form was not an artifact generated in *E. coli*, we applied osmotic shockates from *L. pneumophila* similarly treated with iodoacetamide over the same calibrated column, and DsbA2 eluted as the dimer (Fig. 7C).
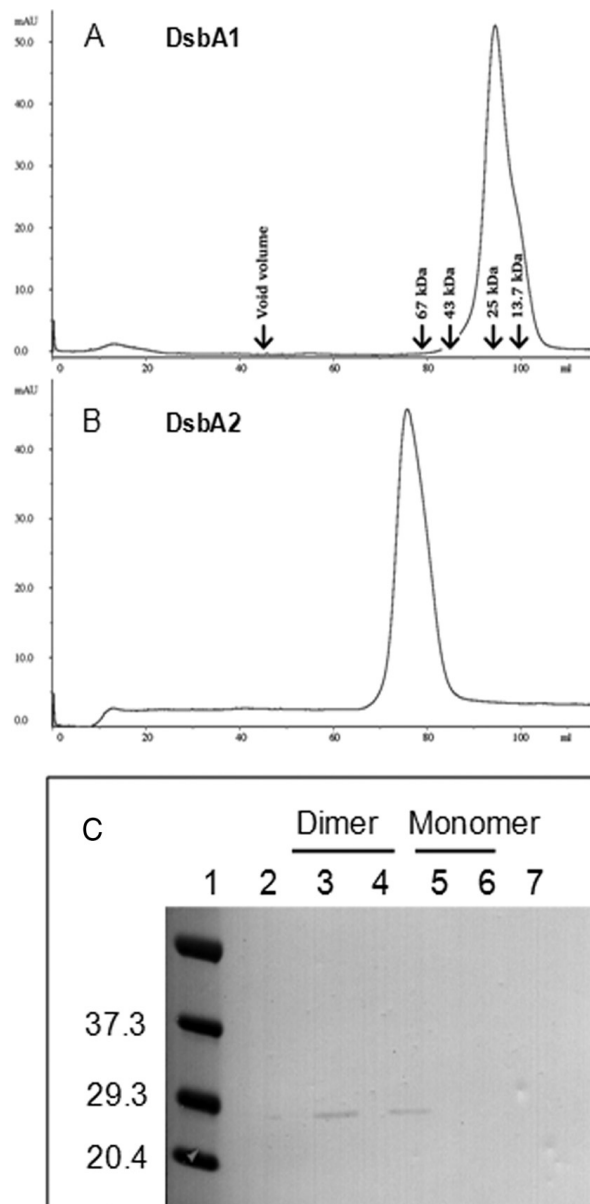
Figure 7. Size exclusion of native proteins from *L. pneumophila*.
(**A**) DsbA1 elutes as a single peak at 95 min, consistent with a monomer and an estimated

mass of 25 kDa. (**B**) DsbA2 elutes at 75 min, consistent with the homodimer and estimated

mass of 60 kDa. (**C**) Immunoblot of DsbA2 containing fractions prepared by osmotic shock from

L. pneumophila and collected from the calibrated column. The DsbA2 protein from *L. pneumophila* eluted at 75 min, consistent with the homodimer. The immunoblot was developed

with antibody specific for DsbA2.

The elution of DsbA2 as a single peak suggests that if the monomer form is present in *L. pneumophila*, it is below the limit of detection in our assay.

3.3 Lpn *dsbA1* and *dsbA2*ΔN, but not *dsbA2* restore motility to Ec *dsbA* mutant

In *E. coli*, DsbA is required for disulfide bond formation in the flagellar P-ring protein (FlgI), which is part of the flagella motor structure that is necessary for flagella driven motility in soft agar (Dailey & Berg, 1993). As seen in Fig. 9, wild-type *E. coli* strain JCB570 is motile in 0.4% soft agar, whereas Δ*dsbA* mutant strain JCB571 is not. We introduced Lpn DsbA1 and DsbA2 expressed from pBC plasmids into JCB571 to test whether expression of these proteins could restore motility. As seen in Fig. 9, *dsbA1* complemented motility to ~60% that of wild type (diameter of spreading growth), whereas *dsbA2* did not, consistent with the general view that dimeric proteins, such as DsbC, are poor substrates of the *E. coli* DsbB (Bader *et al*., 2001; Segatori *et al*., 2004; Shouldice *et al*., 2011). We next removed the dimerization domain of DsbA2 (DsbA2ΔN) by an in-frame deletion that retained the signal sequence as depicted in Fig. 6A to create the monomer. The DsbA2ΔN monomer was confirmed by gel filtration (Fig. 8). Expression of *dsbA2*ΔN in JCB571 restored motility to nearly the wild-type level (~70%) of the control (Fig. 9). While growth rate differences might account for the partial complementation in our studies, these were not apparent. It is more likely that these differences reflect different efficiencies of DsbA1 and DsbA2ΔN oxidation by *E. coli* DsbB compared with DsbA. These results indicate that it is the dimerization domain of DsbA2 that prevents interaction of the homodimer with DsbB.
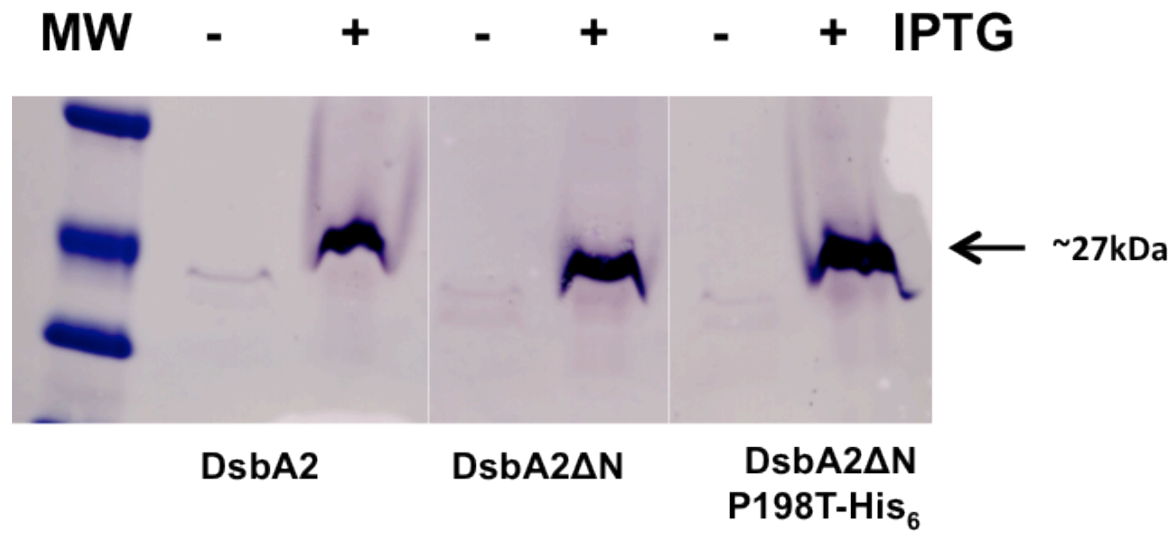
Figure 8. Western blot of Dsb2ΔN mutant constructs. Protein expression of all constructs using 1mM IPTG at stationary phase. The immunoblot was developed with antibody specific for DsbA2.
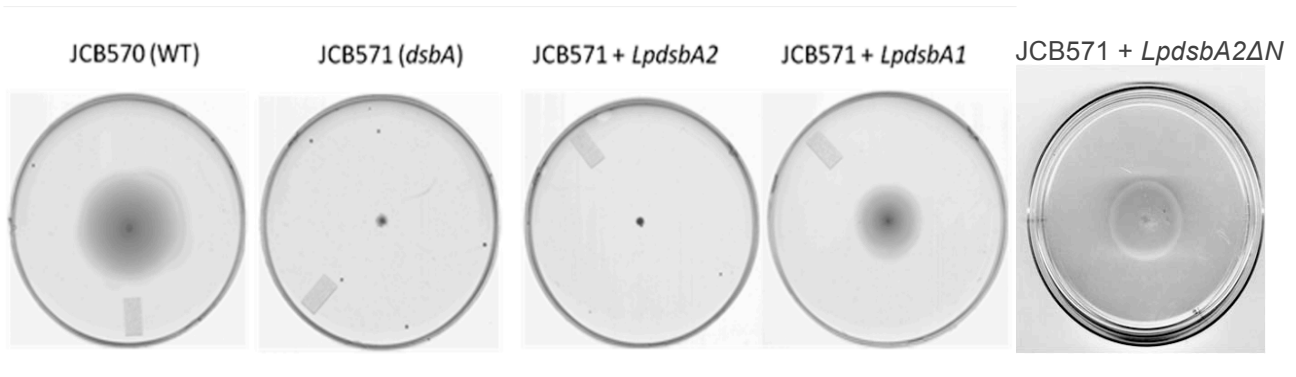
Figure 9. Complementation of motility defect in soft agar. Spreading motility by *E. coli* in soft agar is dependent on functional flagella and chemotaxis. (A) Wild-type *E. coli* (JCB570) is motile in soft agar at 30°C. Strain JCB571(*dsbA*) has a *dsbA* mutation and is nonmotile in this medium. Strain JCB571(pBCLpn*dsbA2*) did not complement the motility defect, whereas JCB571(pBCLpn*dsbA1*) complemented (~60%) this strain for motility. Also, expression of JCB571 (pBCLpn*dsbA2ΔN*), which contains an in-frame deletion of the dimerization domain depicted in Fig. 6A, restored motility to ~70% of wild-type strain JCB570 activity. The results presented in both panels are representative of typical results obtained from triplicate platings. Lp=Lpn in data.

3.4 Periplasmic redox status of monomeric and homodimer DsbA2 in *E. coli*

In Gram-negative bacteria, DsbA is maintained fully oxidized by DsbB, while DsbC is maintained fully reduced by the DsbD system. We previously reported that DsbA2 existed as a mixture of oxidized and reduced forms in *L. pneumophila* during stationary phase (Jameson-Lee, UVa thesis, 2012). To evaluate the redox status of DsbA2 and DsbA2ΔN in *E. coli*, we used the thiol alkylating agent MalPEG5000 to alkylate free thiols, and those thiols reactive with MalPEG resulted in an increased mass of 5,000 Da per cysteine residue. As seen in Fig. 10, whole *E. coli* bacteria expressing DsbA2 were first treated with DTT before alkylation with MalPEG (lane 1) or in the absence of prior DTT treatment (lane 3) and in both cases showed shifted high-molecular-weight bands resulting from complete alkylation by MalPEG relative to untreated control (lane 2). In contrast, when DsbA2ΔN was treated with reducing agent and then MalPEG, multiple shifted bands were observed (Fig. 10, lane 6), whereas treatment with MalPEG without reducing agent (lane 8) produced no shifted bands and showed no differences from that of the untreated control (lane 7), indicating that DsbA2ΔN is maintained as the disulfide in *E. coli*.

These results indicate that DsbA2 but not DsbA2ΔN is fully reduced to the free thiol in the periplasm of *E. coli*.

3.5 Insulin reduction assay

An insulin reduction assay is often used to demonstrate thioredoxin-like activity of DSBs (Holmgren, 1979; Bardwell *et al*., 1991). This assay can distinguish between strong oxidases like DsbA and reductases such as thioredoxin by both the time to reduction and the rate of insulin precipitation resulting from the reduction of its disulfide bond (Bessette *et al*., 1999). In this regard, we previously demonstrated that DsbA2 was similar to thioredoxin in this assay

A. DsbA2　　　　　　　　　**B. DsbA2ΔN**
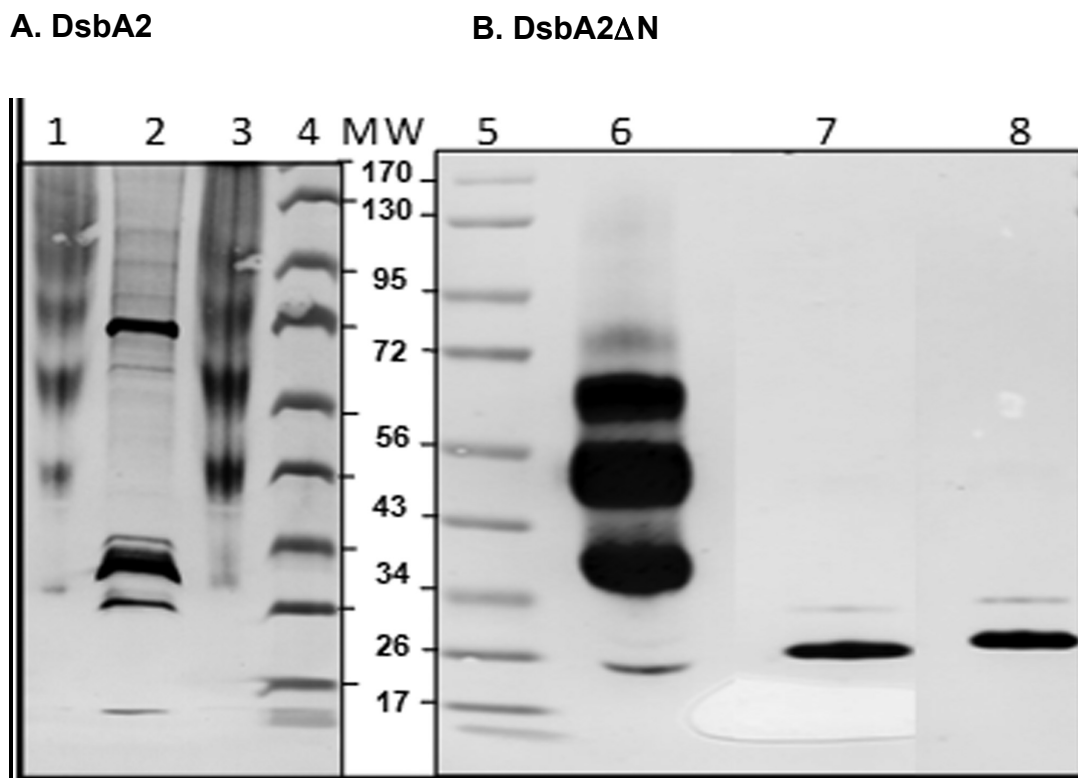
Figure 10. DsbA2 and DsbA2ΔN redox status in *E.coli*. (**A**) Redox status of DsbA2 in *E. coli*. Lane 1, whole cells reduced with DTT for 30 min and alkylated with MalPEG5000 (reduced control); lane 2, untreated control; lane 3, whole cells treated with MalPEG5000. Lanes 4 and 5 contain the molecular weight standards. (**B**) Redox status of DsbA2ΔN in *E. coli*. Lane 6, whole cells treated with DTT for 30 min and then alkylated with MalPEG5000; lane 7, untreated control; lane 8, whole cells treated with MalPEG5000. The molecular weight of DsbA2 is ~28,000, and that of DsbA2ΔN is ~26,000. Anti-DsbA2 mouse serum was used to identify the DsbA2 proteins. The multiply shifted bands are indicative of DsbA2 or DsbA2ΔN covalent complexes with MalPEG5000, which adds 5,000 Da per cysteine. In *E. coli*, DsbA2 is maintained as the free thiol and DsbA2ΔN as the disulfide.

(Jameson-Lee *et al*., 2011). As seen in Fig. 11, DsbA1 of *L. pneumophila* has a lag time of 20 min compared to DsbA2, which has a lag time of 5 min, more typical of reductases like DsbC and thioredoxin. However, as a monomer, DsbA2LDN is much more active in this assay than DsbA1, though the time to reduction is doubled for the monomer compared with the dimer. These results suggest that monomeric DsbA2LDN, while structurally more similar to DsbA than DsbC, favors the reductase function (Jameson-Lee *et al*., 2011).

3.6 *Legionella pneumophila* DsbA2, but not DsbA1 or DsbA2ΔN, exhibits PDI activity

In *E. coli*, DsbA catalyzes disulfide bonds between consecutive cysteine residues as the nascent polypeptide enters the periplasm (Kadokura *et al*., 2004). For those proteins for which nonconsecutive disulfide bonding is required for proper folding, consecutive disulfide bonds must be reduced and then reformed between nonconsecutive cysteine residues. In *E. coli*, the protein disulfide isomerase DsbC catalyzes this reaction (Ren & Bardwell, 2011). In our previous studies, we noted that some of the proteins captured by DsbA2 contained an odd number of cysteine residues, and prediction software analyses suggested that some of these might be nonconsecutive (Jameson-Lee *et al*., 2011). Since DsbA2 exists as a homodimer and fully reduced in *E. coli*, we tested the possibility that DsbA2 might complement a *dsbC* mutant of *E. coli* by restoring PDI activity. To test this hypothesis, *dsbA2*, *dsbA2N*, and *dsbA1* were cloned into an *E. coli* strain carrying a *dsbC* deletion and containing a pPDI detector system (Ren & Bardwell, 2011). In this system, a TEM β-lactamase (Bla) which naturally contains two cysteine residues at positions 52 and 98 that are not required for biological activity was engineered to contain an additional two cysteine residues (81 and 108) positioned so that DsbA
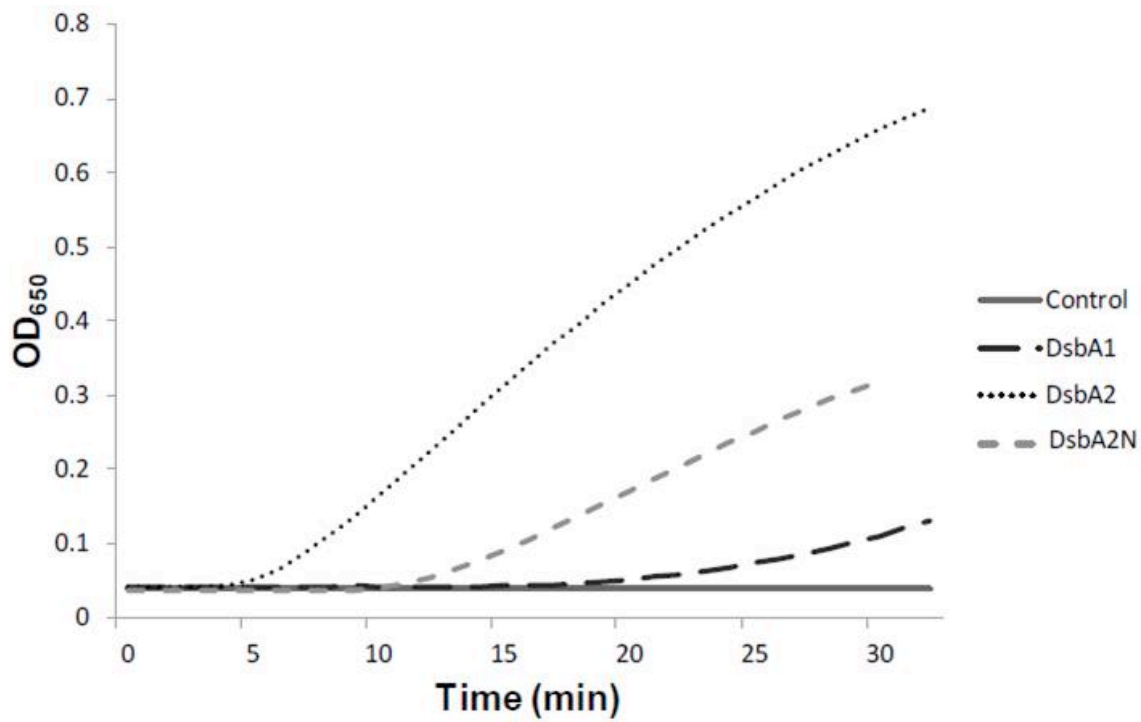
Figure 11. Insulin reduction assay. Reductase activities of equimolar concentrations of DsbA1, DsbA2, and DsbA2N (DsbA2ΔN) were followed spectrophotometrically at 650 nm in a microplate assay as described above. The assays were performed in triplicate, and a representative assay is presented. The time to reduction indicates that DsbA2ΔN is intermediate between DsbA2 and DsbA1 (more reducing than oxidizing).

will introduce consecutive disulfide bonds and produce an inactive enzyme (Ren & Bardwell, 2011). In the presence of DsbC, biological activity is restored by PDI activity (Ren & Bardwell, 2011). In this PDI detector assay, only *dsbA2* expression restored ampicillin resistance to nearly wild-type DsbC control levels at 2 or 3 g/liter of drug (Fig. 12A and B). In contrast, DsbA1 (depicted in Fig. 12A and B), DsbA2LDN (Fig. 12A), and DsbA2N (DsbA2ΔN) (Fig. 12B) failed to complement in the PDI detector assay. The potent PDI activity displayed by DsbA2 in *E. coli* also suggests that DsbA2 is an efficient substrate of the DsbD reductase system, perhaps tipping the balance in favor of PDI/reductase activity over oxidase activity.

*3.*7 DsbA2 exhibits PDI activity *in vitro*

To confirm *in vivo* PDI activity for DsbA2, we developed an assay in which the PDI detector TEM β-lactamase serves as the substrate. In this assay, gain of Bla activity, as measured with the chromogenic substrate nitrocefin, is dependent on PDI enzyme reducing and shuffling disulfide bonds until correct nonconsecutive disulfides are formed (Hailu *et al*., 2013). As seen in Fig. 13, the Bla activity of MBla in concentrated spent culture supernatant is below the level of detection over the course of the assay. Incubation of DsbA2, but not DsbA1 with MBla efficiently corrected disulfide bonding and catalyzed protein folding, as noted by a gain in enzyme activity with nitrocefin. Compared with the WT Bla similarly concentrated from culture supernatants, near-equivalent activity was obtained. These studies were repeated several times, and results of a representative experiment are presented. These studies confirm the biological assay for PDI activity for DsbA2.

While *L. pneumophila* expresses a canonical DsbA (LpnDsbA1), the gene is neither essential for growth nor for virulence (will be discussed in detail from page 97) leaving
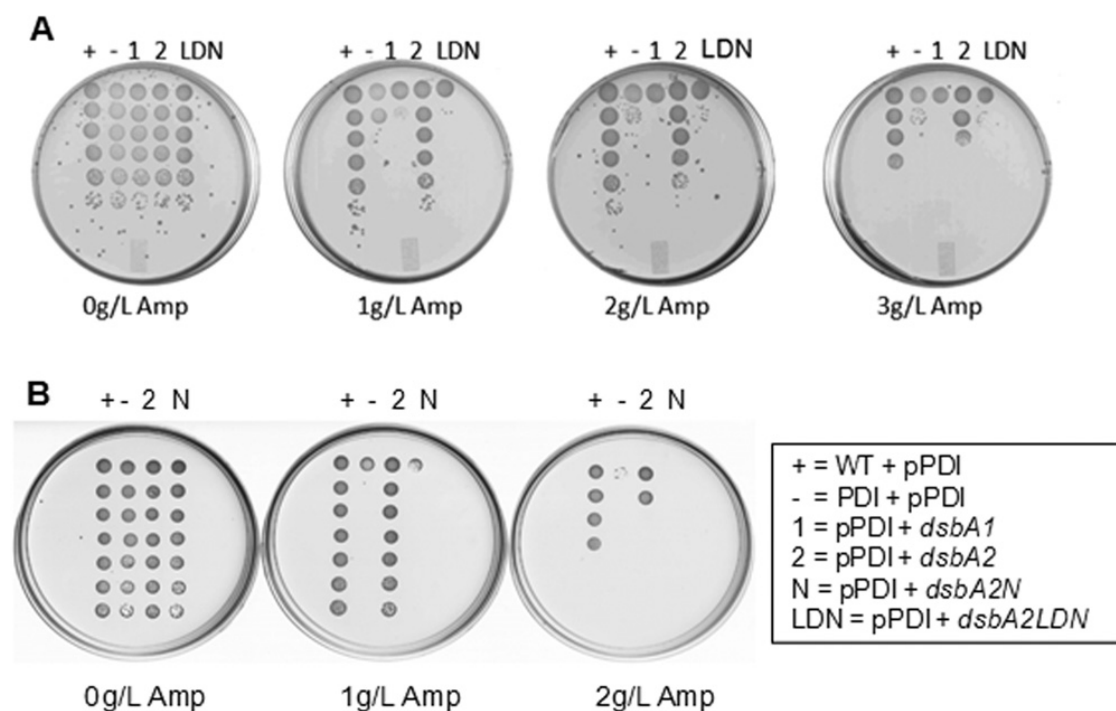
Figure 12. Complementation tests in PDI detector system. The PDI detector system uses a β-lactamase engineered to form consecutive disulfide bonds, which require DsbC PDI activity to correct to nonconsecutive to facilitate protein folding and gain of enzyme activity (Ren & Bardwell, 2011). Growth on ampicillin indicates that β-lactamase is properly folded. Ampicillin concentrations are 0, 1, 2, or 3 g/liter. Bacterial dilutions are plated from top to bottom as detailed in the text. (A) + , wild-type *E. coli* strain RGP663 plus pPDI detector plasmid; - , strain RGP665*dsbC* plus pPDI detector plasmid; 1, strain RGP665 plus pPDI detector plasmid plus pBC*dsbA1*; 2, strain RGP665 plus pPDI detector plasmid plus pBC*dsbA2*; LDN; strain RGP665 plus pPDI detector plasmid plus pBC*dsbA2LDN* (LDN, deleted leader and dimerization domain sequences). (B) Replicate PDI detector assay that includes RPG665 plus pPDI detector plasmid plus pBC*dsbA2N* (pBC*dsbA2ΔN*) containing the leader sequence and an in-frame deletion of the dimerization domain sequences.
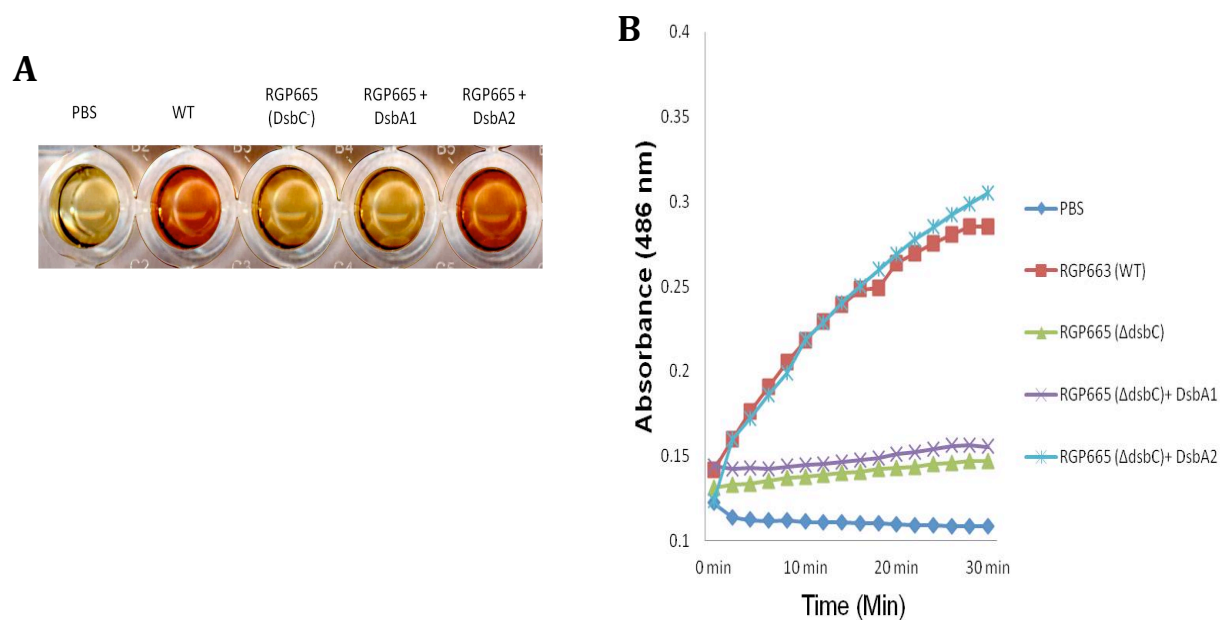
Figure 13. Nitrocefin PDI folding assay. TEM1 β-lactamase (Bla) and pPDI mutant TEM1 β-lactamase (MBla) were obtained by concentrating culture supernatants from strains RGP663 and RGP665, respectively. MBla contains consecutive disufide bonds and is inactive (nitrocefin negative). DsbA1 or DsbA2 was added to aliquots of MBla for 30 min at 37°C, and 15 μl aliquots were tested in triplicate for Bla activity in microplates with nitrocefin as described in the text. **A**) Cleavage of the β-lactam of nitrocefin is monitored as a change in color over 30 min, **B**) rates of hydrolysis are plotted by the increase in absorbance at 486 nm over time in micro-titer plate. DsbA2, but not DsbA1, exhibited PDI activity.

homodimeric DsbA2 to catalyze both sequential disulfide bond formation in nascent polypeptides and conversion of consecutive or inappropriate disulfide bonds into non-consecutive disulfide bonds through protein disulfide isomerase (PDI) activity to promote correct protein folding (Jameson-Lee *et al*., 2011; Kpadeh *et al*., 2013). Evaluation of the redox status of DsbA2 in *L. pneumophila* revealed an equilibrium between the free thiol (SH) and the disulfide (S-S) forms while in *E. coli*, DsbA2 was exclusively maintained in the reduced thiol form (Kpadeh *et al*., 2013). This suggests that in *L. pneumophila* the activities of partner proteins DsbB that typically re-oxidize DsbA and DsbD that typically reduces DsbC, according to the *E. coli* paradigm, must cooperate to maintain equilibrium of redox forms.

3.8 Identification and nomenclature of DsbB and DsbD proteins

Analysis of the complete sequenced genomes of *L. pneumophila* and related species reveals multiple alleles of DsbB and DsbD. In the Philadelphia 1 strain, one DsbB is annotated as LidJ (lethal in Dot) as it was selected as non-essential in a *dotA* mutant background (Conover *et al*., 2003). Comparative amino acid sequence analysis of LidJ with DsbB of *E. coli* reveals both similarity and divergence (see Figure 14). In our studies LidJ is referred to as DsbB2. One of the unique features of DsbB2 is a unique periplasmic domain containing a thioredoxin fold (see Figure 14). A second DsbB in the Philadelphia-1 strain is mis-annotated as DsbH. In all other sequenced strains, a canonical DsbB is present and shows a high degree of similarity with the DsbB of *E. coli* (see Figure 14 alignment). A re-examination of the *dsbH* locus in the Philadelphia-1 strain reveals the complete gene is present, which we verified by functional complementation studies in *E. coli* and named this allele DsbB1 (figure 16).

```
                                                             S---S
LpnDsbB2    --------MNKQMQWHLFGNTSGLLIICCLLILAFLDQFIFYELPCPICILQRICFIAVG   52
LpnDsbB1    MKKLTYR-----KIQSFQAII-----TVLVIFASFYLEYAVGLQPCPLCLMQRVCVFILL   50
EcDsbB      MLRFLNQCSQGRGAWLLMAFT-----ALALELTALWFQHVMLLKPCVLCIYERCALFGVL   55
                       :  .              :  :  ::    :.  .     **  :*:  :*  ..:  :

LpnDsbB2    LCFVMNLMLGIRASHYGL---MLLATLLGLSISVRQMYIHLTPGDPGY---GGLI-----  101
LpnDsbB1    GLMGLSLGTVKKA-HIVSLM-QFLVACAGLYFSLRQLWLQSLPSDQAPACMPGLDVLIQY  108
EcDsbB      GAALIGAIAPKTPLRYVAMVIWLYSAFRGVQLTYEHTMLQLYPS-----PFATCDFMVRF  110
                 :               :            :   :   *:  ::  .:    ::    *.

LpnDsbB2    -------------------------FGLHLYTWSAIIFLVIILLIAGALIL-DKGFMP  133
LpnDsbB1    F---PWQTVAKALFWGAGDCAEVTWTMFGISMPGWAAMYFLFMAIM--GLFLFFRTR---  160
EcDsbB      PEWLPLDKWVPQVFVASGDCAERQWDFLGLEMPQWLLGIFIAYLIV--AVLVVISQPFKA  168
                                   :*:  :   *       *:     ::   .  :: .

LpnDsbB2    DYKVRSKSAKVMIWVFFILILANGISTFLECGPHECPDNPSKYYLLNNN      182
LpnDsbB1    ---------T--------------IN------------------------      163
EcDsbB      ----K----K-------------RDLFGR--------------------      176
```

Figure 14. Clustal O Alignment of DsbB proteins. The cysteine residues are bold red and the grey highlighted regions represent regions extending into the periplasm. The S-S identifies cysteine residues that form a disulfide bond.

Similarly, *L. pneumophila* expresses two alleles of *dsbD, dsbD1,* as depicted in Fig. 15, which is also annotated as *lidC* and located just upstream of the *groELS* operon. The second DsbD (Lpg1680) is referred to as DsbD2 and is located elsewhere. While Conover *et al.* (2003) were able to obtain *lidJ* (*dsbB2*) and *lidC* (*dsbD1*) mutants, our attempts to generate these mutants in *L. pneumophila* Lp02 and in the AA100 backgrounds have not been successful. In our studies we used either Km or Gm cassettes inserted in-frame within the truncated open reading frame (orf) such that mutations were non polar on downstream genes. In these studies, we were routinely able to produce *dsbA1* mutants, which we had previously reported to be non-essential (Jameson-Lee *et al.*, 2011). In all studies, we were not successful in obtaining *dsbA2* mutants as previously reported (Jameson-Lee *et al.*, 2011). While a TraSH mutant screen performed in rich medium with the Lp02 strain confirms the essentiality of *dsbA2*, *dsbB1* could not be evaluated (*dsbH* orf incorrect) and both *dsbD1* and *dsbD2* were borderline and *lidJ* (*dsbB2*) was called as non-essential (Conover *et al.*, 2003). Of interest though is the apparent lack of functional redundancy, suggesting that the DsbB and DsbD alleles might interact with different periplasmic proteins whose functions are distinct. To begin testing function for each of the *dsb* alleles, we used a complementation approach in a set of defined *E. coli dsb* mutants. In this strategy, DsbA function was evaluated by gain of motility in a soft agar motility assay. DsbA is required for formation of a disulfide bond in FlgI, a motor protein required for flagellar function. To assess protein disulfide isomerase function, we employed an *in vivo* folding assay developed by Ren and Bardwell and as described by us (Ren and Bardwell, 2011; Kpadeh *et al.*, 2013).

```
EcDsbD    ----------MAQRIFTLILLLCSTSVFAGLFDAPGRSQFVPADQAFAFDF-QQNQHDLN    49
DsbD2     ------------------------------------------------------------    0
DsbD1     MFISNSKRYRMKKWLLFSLLFLTSLCVH---------AEPLPASEVFKVSVKKIDPNTFA    51


EcDsbD    LTWQIKDGYYLYRKQIRITPEHAKIAD---VQLPQGVWHEDEFYGKSEIYRDRLTLPVTI   106
DsbD2     ------------------------------------------------------------    0
DsbD1     IQWNILPKYFLYSDRIQLNSDNDDIAQLGTLRFPTPLTKTDKQGRTFKVYRNQLNLPVGV   111

                      Nα disulfide bond region
EcDsbD    NQASAGAT-LTVTYQGCADAGFCYPPETKTVPLS-------EVVANN-----EASQPVS   152
DsbD2     -----------MLFITY--------WDIK-MRKLIDSFMILLLIFFTQAAWSYTPGFESS    40
DsbD1     LGITPGETIVNLRFQGCADDGFCYPPEVKQIKLAIDDKLALSQV--DLETFHAPEATPLE   169
                        : :            : * :           :        .   . .

EcDsbD    VPQQEQPTAQLPFSALWA------LLIGIGIAFTPCVLPMYPLISGIVLGGKQRLSTARA   206
DsbD2     NPD-TVM--HFITGNSAFVYLSAFFGLGILLAFTPCVLPMVPILSGIIVGQNSI-STGKA    96
DsbD1     KPQQDIA--DIFANHNWIMILLIFYGFGLLLSFTPCILPMVPVLSGIIVGHGKTATTKKA   227
           *:       .:               :*: ::****:*** *::***::*   .  :* :*

EcDsbD    LLLTFIYVQGMALTYTALGLVVAAAGLQFQAALQHPYVLIGLAIVFTLLAMSMFGLFTLQ   266
DsbD2     FKLSLSYVTGMAITYAGAGMLAGYMGSTIQTVMQRPVVITLFSVVFVAMALSMFGFFELK   156
DsbD1     FFLSLSYVLSMSVTYAVVGAVVALLGANLQISMQSPWAISLFSLIFVLLALSMFGFYEFK   287
           : *:: ** .*::**:  *  :..  *   :*   :* * .:  :::*. :*:****:: ::

EcDsbD    LPSSLQTRLTLMSNRQQGGSPGGVFIMGAIAGLICSPCTTAPLSAILLYIAQSGNMWLGG   326
DsbD2     FPTSLGNRLNKNNQLGKQRSYLSVGIMGVLSTLIVSPCVTAPLIGVLSYIGQNGQVIMGG   216
DsbD1     LPDAWQSKIVGSSREQRGGHYLGAAIMGCLSTLILSPCVTAPLIGVLTYIAQTGNVLLGS   347
           :* :   .:      ..   :      .. *** :: ** ***.****  .:* **.*.*:: :*.

EcDsbD    GTLYLYALGMGLPLMLITVFGNRLLPKSGPWMEQVKTAFGFVILALPVFLLERVIGDIWG   386
DsbD2     LILFVMALGMGVPLLIVGAGYGTLLPKAGSWMLKIKQLFGFIMLGIAIWMLSRILNQTIT   276
DsbD1     VTLFTLSLGMGTPLLLIGTSAGKWLPETGSWMNAVKAFFGILLLAVAIYLMARILPAGLV   407
           *:  :**** **::: .       **::* **   :*   **:::*.: :::: *::

EcDsbD    LRLWSALGVAFFGWAFITSLQAKRGWMRV------------VQIILLAAALVSVRPLQDW   434
DsbD2     HILWAGLLM--VASISLGALKSQNNWRGYLAQGLGILALISGGIIFYNTAIPSVK-----   329
DsbD1     MGLWACLLI--FSGIYSGALTKSNTNQEKLCQGIGIILLTYGLLILIGASMGSSNPLQPL   465
           **: * :   ..      :*   ..            :*:   ::: * .
                                                         Cγ disulfide bond region
EcDsbD    AFGATHTAQTQTHLNFTQIKT--VDELNQALVEAKGKPVMLDLYADWCVACKEFEKYTFS   492
DsbD2     --IKQA---SVENSPFIKINTLNALQLQLEQAKKVRKAVFLDFSADWCGDCQEMDTKVFN   384
DsbD1     ANLQAA---PTVSNAFESAKAQSVKSVELAIKQAFGKPVMLDFYADWCASCKVMENTTFK   522
                   * . .: . . .::  :   * *:*** ****  *: ::. .*.

EcDsbD    DPQVQKALADTVLLQANVTANDAQDMALLKHLNVLGLPTILFFDGQGQEHPQARVTGFMD   552
DsbD2     QPEIQKAMSDWVSIKVDISDKNAEVDSIKKAFGIYGTPTMIFFNTSGEPITQMTSVGLIS   444
DsbD1     DPRVQKALSHFIVIKVDVTANNKNDKALMQHFRVVAPPTFIFFNADGVQLNNLKRVGELN   582
           :*.:***:. : ::.:: :: :  :: : : : . **::**: .*     :   .* ..

EcDsbD    AETFSAHLRDRQP--        565
DsbD2     LSKMLQLLEQARHLS        459
DsbD1     ADEFMQTIKSIDEN-        596
           . :   :..
```

Figure 15. Clustal O Alignment of DsbD proteins. The cysteine residues are bold red, depicting the N-terminal α-disulfide bond region and C-terminal γ-disulfide bond region with conserved amino acids, shaded regions.

3.9 Evaluation of DsbB1 and DsbB2 in *E. coli dsbB* mutants

We first determined whether *dsbB1* and *dsbB2* could functionally complement a *dsbB* mutant of *E. coli* by evaluating strains for motility in soft in soft agar. Our results reveal that both Lpn DsbB1 and DsbB2 expressed in RGP405 strain were able to partially restore motility compared to wild type (JP114), as shown in (Fig. 16). This indicates that Lpn DsbBs are capable of re-oxidizing DsbA leading to partial gain of motility.

3.10 Both Lpn DsbB1 and DsbB2 interact with monomeric DsbA2 (DsbA2ΔN) in *E. coli*

DsbA2 exhibits both oxidase activity (Jameson-Lee *et al*., 2011) and isomerase activity (Kpadeh *et al*., 2013), but until now its interacting partners have been unidentified. We sought to elucidate the ability of either DsbB1 or DsbB2 in oxidizing DsbA2 using an *E. coli dsbA dsbB* double mutant (RGP438), and then assess motility. We overexpressed Lpn DsbA2 and Lpn DsbB1 or Lpn DsbA2 and Lpn DsbB2 in an *E. coli* double mutant (*ΔdsbA dsbB*) background. Interestingly, neither DsbB1 nor DsbB2 were able to restore motility in a DsbA2 producing strain (Figure 17B). Most importantly, *dsbA2::dsbB2* construct was toxic and unable to show any measurable growth in broth upon induction after many trials. The toxicity may indicate the functional difference of Lpn DsbB2 due to its structural difference from Lpn DsbB1 and EcDsbB, as shown in Figure 14. To further investigate the importance of Lpn DsbBs existence in *L. pneumophila*, we decided to investigate the interaction of monomeric DsbA2 (DsbA2ΔN), which has been reported to complement the function of Ec*dsbA* in a motility assay (Kpadeh *et al*., 2013), suggesting it is a substrate of DsbB for re-oxidation. As seen in Figure 17A, wild-type *E. coli* strain (JP114) is motile in 0.4% soft agar, whereas *ΔdsbA dsbB* double mutant strain
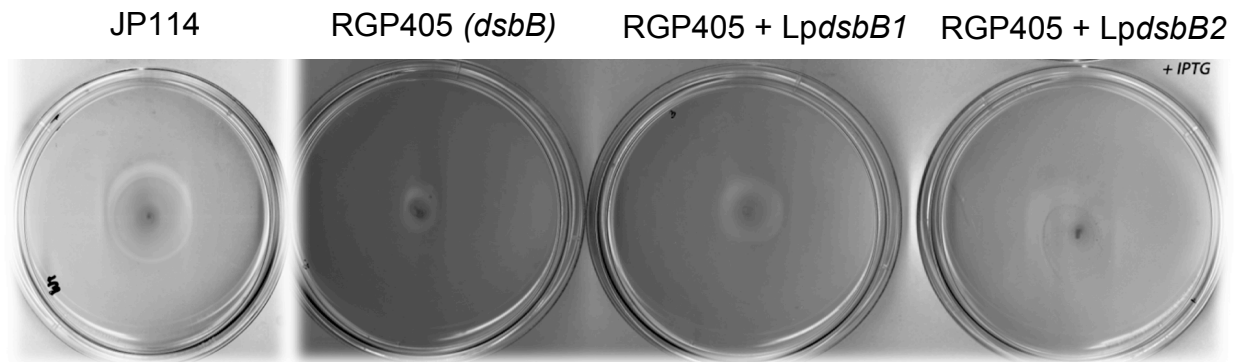
Figure 16. Lpn DsbB1 and Lpn DsbB2 complement motility in an *E. coli dsbB* mutant strain. Spreading motility by *E. coli* is the measured of chemotaxis, which is dependent on the proper assembly and function of flagella motor protein. Left: Wild type *E. coli* (JP114) is motile in soft agar and forms a large zone of growth, while *dsbB* mutant strain RGP405 is non-motile and grows only where it is stabbed into the center of the plate. Addition of either Lpn DsbB1 or Lpn DsbB2 in a plasmid is able to functionally replace *E. coli dsbB,* ~30% and ~45%, respectively, as demonstrated by the large zone of growth. The results are representative of typical results obtained from triplicate platings.

RGP438 is not. We introduced DsbA2ΔN with either *L. pneumophila dsbB1* or *dsbB2*, and co-expressed from pBC plasmids into RGP438 to test whether expression of these proteins could restore motility. As depicted in Figure 17A, *dsbA2ΔN::dsbB1* and *dsbA2ΔN::dsbB2*; and also as depicted in Figure 17B, *dsbA1::dsbB1* and *dsbA1::dsbB2* complemented motility to ~30 and 50% to that of wild-type, respectively. Growth/motility is measured by the diameter of spreading. Note that the differences in growth rate of bacterial containing *E. coli* proteins to bacterial containing a mixture of *L. pneumophila* and *E. coli* proteins might account for the partial complementation noted in our studies. These results further validate the consistency of the general view that dimeric proteins, such as DsbC or DsbA2 (containing the dimerization domain), are poor substrates of DsbB (Bader *et al*., 2001; Segatori *et al*., 2004; Shouldice *et al*., 2011; Kpadeh *et al*., 2013). Therefore, we suggest that it is the dimerization domain of DsbA2 that prevents or restricts interaction of the homodimer with DsbB as previously indicated in (Kpadeh *et al.,* 2013). Therefore, we decided to investigate the interaction of Lpn DsbA2 with the reductants (DsbD1 and DsbD2).

3.11 Lpn DsbD1 and Lpn DsbD2 are unique for DsbA2 bifunctional activity

In *E. coli*, the DsbA-DsbB pathway introduces disulfide bonds *de novo*, while the DsbC-DsbD pathway functions to isomerize disulfides. In order to investigate the DSB activity of the different DsbDs in *L. pneumophila*, we sought to establish the role of Lpn DsbD1 and Lpn DsbD2 as reductants by complementing *E. coli dsbD* mutant (RGP666) using a PDI detector assay developed by (Ren and Bardwell, 2011; Kpadeh *et al*., 2013). In this assay, the reductant role of DsbD is important for the isomerase activity of DsbC. In order to test Lpn DsbD1 or
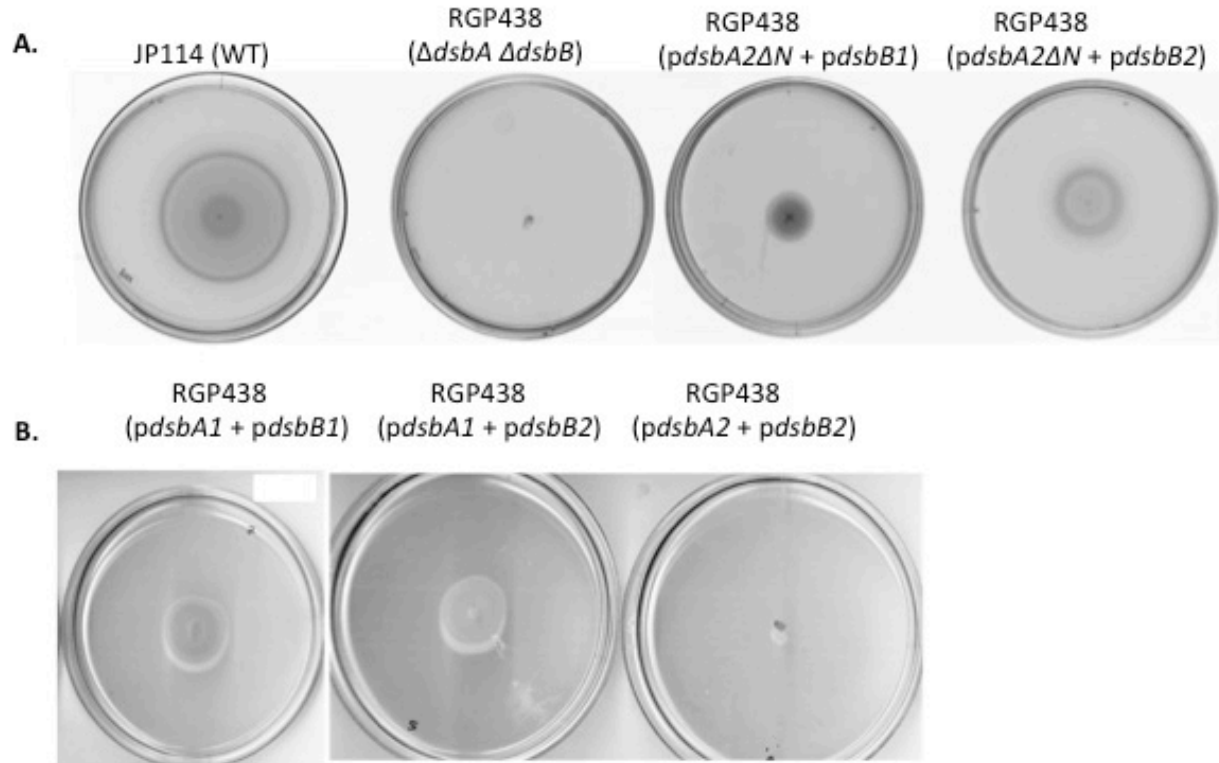
Figure 17. Both Lpn DsbB1 and DsbB2 interact with monomeric DsbA2 (DsbA2ΔN) in *E. coli.* Lpn DsbA1 and monomeric DsbA2 (DsbA2ΔN), but not homodimeric DsbA2, interact with both Lpn DsbB1 and Lpn DsbB2 to complement motility in an *E. coli dsbA dsbB* mutant strain. We assessed the spreading motility by *E. coli* as in Fig. 3. Wild type *E. coli* (JP114) are motile in soft agar and form a large zone of growth, while *dsbA dsbB* mutant strain RGP438 is non-motile and grows only where it was stabbed into the center of the plate. Addition of plasmids encoded (**A**) Lpn DsbA2ΔN and (**B**) Lpn DsbA1 with either Lpn DsbB1 or Lpn DsbB2 added to RGP438 are able to functionally replace *E. coli dsbA dsbB*, but (**B**) Lpn DsbA2 with either Lpn DsbB1 or Lpn DsbB2 on a plasmid did not restore motility in RGP438 as demonstrated in this figure. Results shown here are representation of a single experiment obtained from triplicate platings.

Lpn DsbD2 role in contributing to EcDsbC function, we expressed either Lpn DsbD1 or Lpn DsbD2 from inducible plasmid constructs in RGP666 and then tested their ability to maintain EcDsbC in the reduced form. Both Lpn DsbDs were unable to interact with EcDsbC to complement Ec *dsbD* mutant for isomerase activity as shown in Figure 18A (lanes 3 & 4). However, co-expression of *dsbA2::dsbD1* or *dsbA2::dsbD2* in RGP666 restored PDI activity as measured by growth on Amp plates (Fig. 18A, lanes 5 & 6), suggesting a unique affinity of both Lpn DsbD1 and Lpn DsbD2 for DsbA2, and not for EcDsbC. In order to validate the interaction of DsbD1 and DsbD2 with DsbA2 was specific we utilized a *dsbC dsbD* double mutant (RGP810) of *E. coli*. As shown in Figure 18B expression of either *dsbA2::dsbD1* or *dsbA2::dsbD2* constructs in RGP810 (lane 5 & 6) restored TEM-1 β-lactamase function, indicating that both DsbD1 and DsbD2 control the reduction of DsbA2 in *L. pneumophila*, revealing a novel interaction of DsbA-like protein interaction with a reductant; contrary to the *E. coli* DsbC/DsbD "classical" pathway. In addition, as depicted in Figure 15, *E. coli* DsbD differs from both Lpn DsbD1 and Lpn DsbD2. EcDsbD α-disulfide bond region, which is located in the periplasmic region appears earlier (around aa position 123-129) while in Lpn DsbD1 it begins at aa position 128-134, and this conserved region seems to be missing in Lpn DsbD2 (Fig. 35). While the conserved γ-disulfide bond region seems to be conserved amongst these DsbDs, Lpn DsbDs seem to show some diversity and or differences in the β-disulfide region compared to EcDsbD; thereby contributing to their inability to reduce EcDsbC as observed in the isomerase assay. All together, these results indicate that both DsbD1 and DsbD2 are structurally diverse from EcDsbD; suggesting a moderate interaction of Lpn DsbDs with DsbA2 is needed for DsbA2 to exist in a bifuntional state *in vivo*, and the fully reduced status of DsbA2 as reported in (Kpadeh *et al*., 2013)
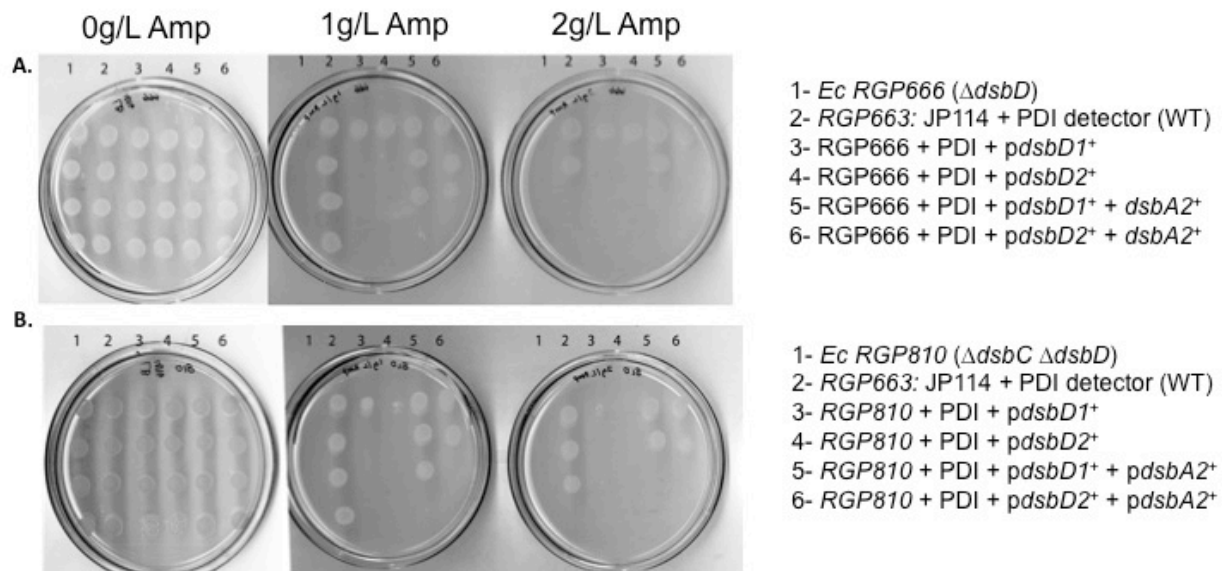
Figure 18. Isomerization activity in a PDI detector assay. Protein isomerase activity is required for the proper folding and function of those proteins that require nonconsecutive disulfide folding. We utilized β-lactamase engineered to form consecutive disulfide bonds, which require DsbC (reductase) and/or DsbD (reductant; maintains DsbC in the reduced form) PDI activities to correct the mis-disulfides to nonconsecutive to facilitate protein folding resulting to an active enzyme (Ren et al., 2011; Kpadeh et al., 2013). Growth on ampicillin demonstrates β-lactamase is properly folded and active. Ampicillin concentrations are 0, 1, or 2 g/liter. Bacteria were serially diluted with concentrations ranging from top (10^9) to bottom (10^3). (A) Lpn DsbD1 and Lpn DsbD2 do not interact with *E. coli* DsbC (lanes 3 & 4). (A & B) Both Lpn DsbD1 and Lpn DsbD2 interact with Lpn DsbA2 (lanes 5 & 6). Experiments were performed three times independently. Reported here is a representative of the results obtained from single experiment performed in triplicate.

is due to the EcDsbD efficiently reducing DsbA2 while preventing Lpn DsbBs interaction with DsbA2.

3.12 DsbA2 exhibits oxidase activity in the presence of Lpn *dsbBs* and Lpn *dsbDs*

So far, we have identified the DSB interacting partners of the different forms of Lpn DsbA2. Hence, the genetically constructed monomer form of DsbA2 seems to be active and functional. However, this form does not exist in nature. We sought to evaluate the hypothesis that Lpn DsbBs and DsbDs moderately interact with DsbA2 to produce the mixture of oxidized and reduced redox forms, with the oxidized form contributing to a DsbA-like activity in *L. pneumophila*, as reported in (Kpadeh *et al*., 2013). Utilizing *ΔdsbA ΔdsbB* double mutant strain RGP438, we chromosomally replaced *E. coli dsbD* with Lpn *dsbD1* or Lpn *dsbD2* creating ZK01 and ZK02 by PCR as described in the method section. Expression of pMMB206*dsbA2:dsbB1* or pMMB206*dsbA2*:*dsbB2* in either ZK01 or ZK02 was able to restore motility as shown in Figure 19. We then evaluated the redox status of DsbA2 using the above genetically altered constructs that were motile. As seen in Figure 20, DsbA2 with *E. coli dsbB* and *dsbD* genes was fully reduced (lane 3), while in the presence of both Lpn *dsbB* and Lpn *dsbD* (lane 6), DsbA2 is shown to exhibits a mixture of oxidized and reduced redox forms in *E. coli*; similar to what is biological reported in *L. pneumophila* (Kpadeh *et al*., 2013). These results indicate that when both Lpn DsbD proteins are absent, Lpn DsbA2 accumulates mostly in the reduced form in the presence of Ec DsbD (Fig. 20; lane 3), suggesting Lpn DsbDs role are essential for DsbA2 to exist as oxidized in the periplasm. In addition, these results established that DsbA2 is a bifunctional protein; indicating oxidative and reductive pathways. This research challenges the *E. coli* DsbA/DsbC paradigm established in the field and introduces a novel single
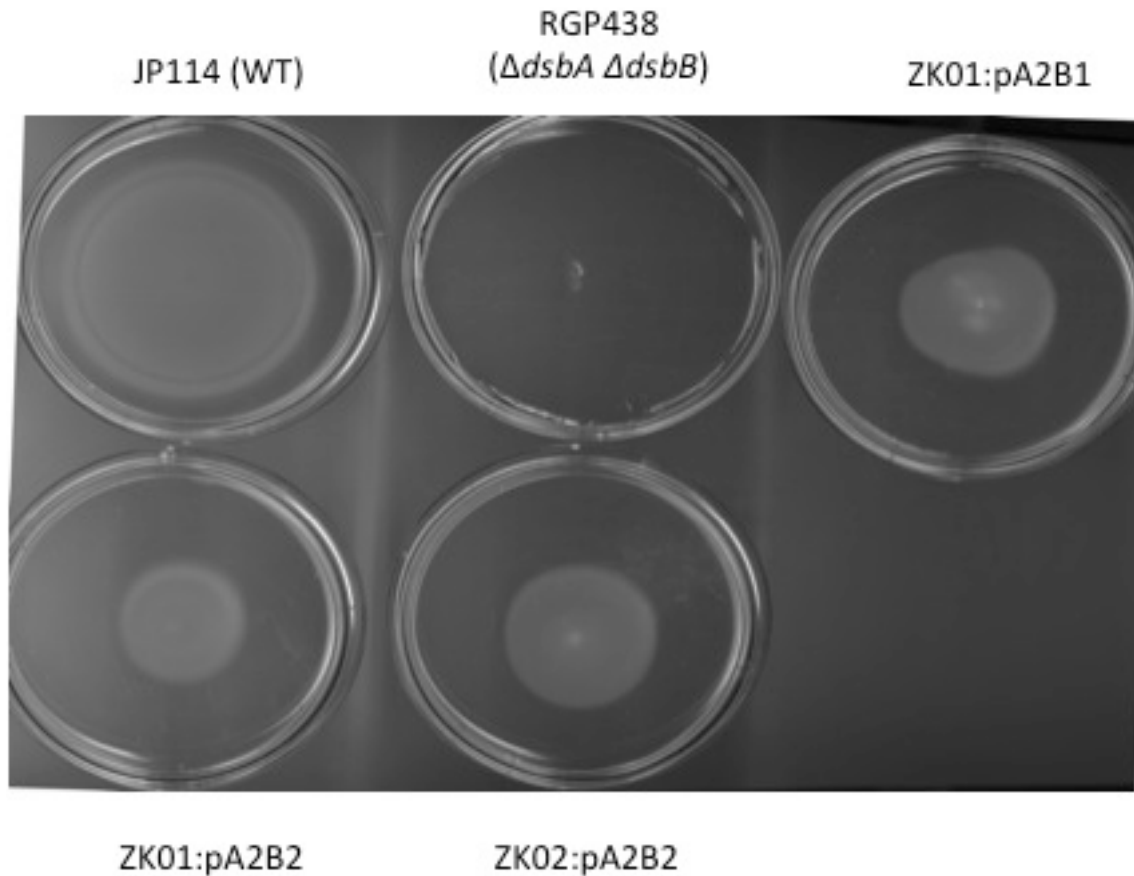
Figure 19. Lpn DsbA2 exhibits oxidase activity when both Lpn DsbB and Lpn DsbD are present. In order to investigate the oxidase activity of DsbA2 in the presence of Lpn DSB proteins, we chromosomally replaced *E. coli dsbD* with either Lpn *dsbD1* or Lpn *dsbD2* resulting to genetically altered strains ZK01 and ZK02, respectively. We then complemented *E. coli dsbA dsbB* with Lpn DsbA2, and then added either Lpn DsbB1 or Lpn DsbB2 (constructs including *dsbA2*, *dsbB1* or *dsbB2* require IPTG). We assessed the spreading motility by *E. coli* as in Fig. 3. Addition of plasmids encoding Lpn *dsbA2* and Lpn *dsbB1* or Lpn *dsbB2* with either chromosomally-replaced Lpn *dsbD1* or Lpn *dsbD2* in RGP438 were able to functionally complement an *E. coli dsbA dsbB* double mutant to restore motility as demonstrated.
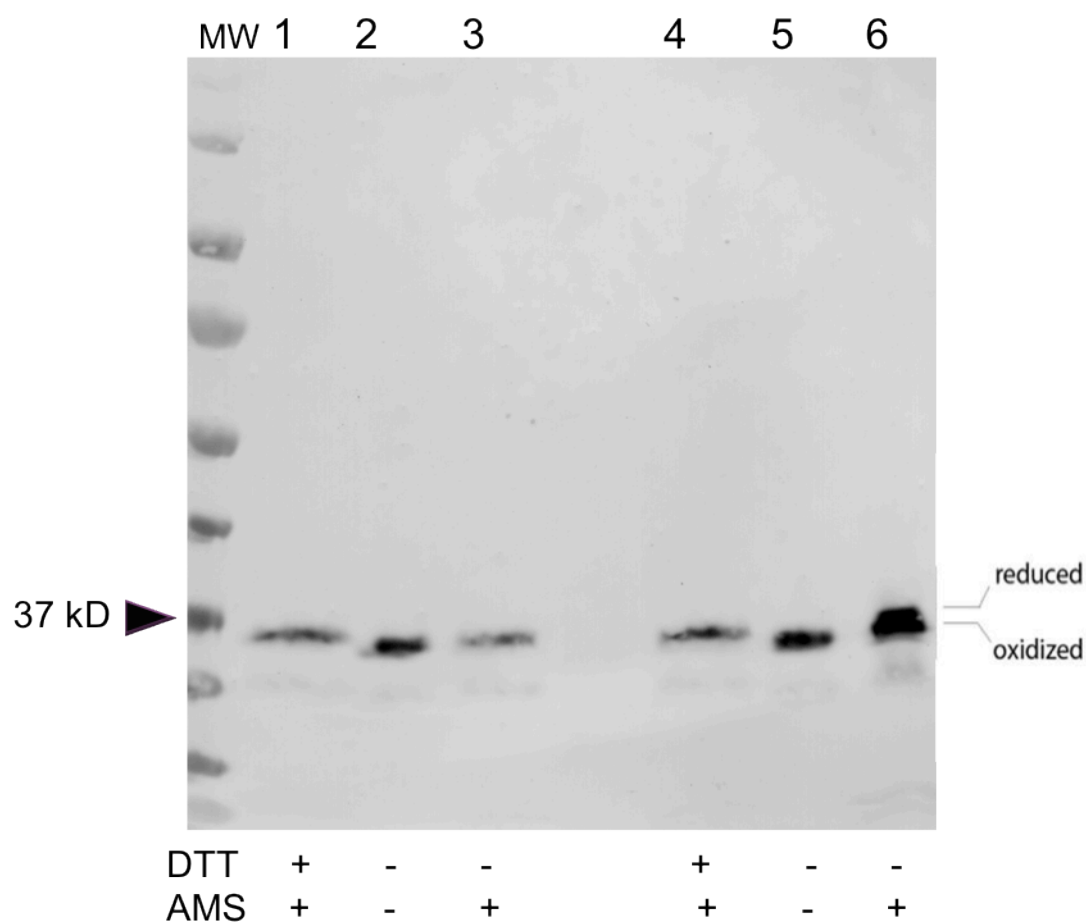
Figure 20. Redox status of Lpn DsbA2 in *E. coli* as determined by AMS-alkylation. After TCA precipitation of whole cells, AMS alkylation adds ~495 Da to free cysteine residues, which can then be visualized as a shift in apparent molecular weight. Lanes 1 & 4 correspond to the fully reduced and alkylated migration of Lpn DsbA2. Lanes 2 & 5 correspond to the oxidized control, DsbA2 without AMS alkylation. Lanes 3 & 6 AMS-alkylated whole cells, and in (lane 6), DsbA2 redox status in *E. coli* showed that DsbA2 exists as a mixture of oxidized and reduced forms in the presence of Lpn *dsb* genes during stationary phase.
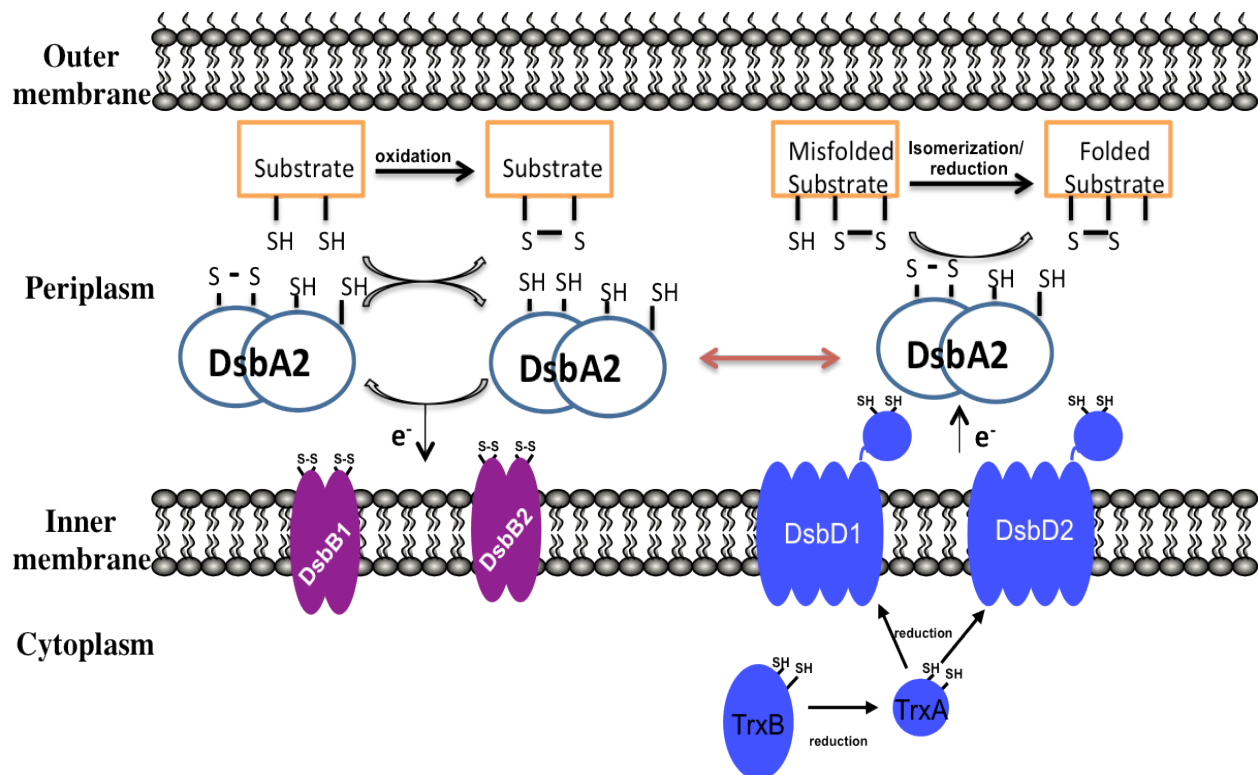
Figure 21. Disulfide bond trafficking in *L. pneumophila*. DsbA2 catalyzes the formation of consecutive disulfide bonds in reduce substrates in the periplasmic. Either DsbB1 or DsbB2 to repeat the oxidation cycle reoxidizes DsbA2. However, if misoxidation occurs in substrates requiring non-consecutive disulfide bonds, DsbA2 also contributes the isomerase activity; reduces and correctly oxidizes disulfide bridges and is kept in a reduced state by either DsbD1 or DsbD2.

player DSB pathway that appears most often in slow-growing, pathogenic bacteria such as Lpn (Fig. 21).

3.13 DsbA2 mutant constructs have no growth or expression defect *in vitro*

The pathogenicity of *L. pneumophila* is highly dependent on the production of virulence factors such as type IV secretion system (T4SS). In addition to *L. pneumophila*, many slow-growing water-borne and soil bacteria as well as vector-borne pathogenic bacteria containing the T4SS also contain DsbA2-like protein as described in (Kpadeh *et al*., 2013). Here, we show for the first time DsbA2 is a bifunctional enzyme in Lpn. In order to investigate if this system has an advantage in slow-growing bacteria, we utilized *E. coli* (WT) and RGP438 constructs containing pMMB206 empty vector, ZK01::pA2B1, ZK01::pA2B2, and ZK02::pA2B2 in *in vitro* growth assay for any defect due to the reconstitution of *dsb* genes in *E. coli*. *In vitro* growth characteristic in LB broth with 0 and 1 mM IPTG were monitored overtime. As seen in Fig. 22, expression of these mutant proteins resulted in a distinguishable defect of bacterial growth compared to the empty vector control and uninduced controls. These studies are consistent with the hypothesis that expression of *L. pneumophila dsb* genes in *E. coli* has a measurable effect on *in vitro* growth, suggesting that the DsbA2 system might be favored in slow growing soil and aquatic bacteria.

3.14 Monomeric DsbA2 mutant exhibits a defect in attachment, invasion and intracellular replication

For intracellular pathogens, such as *L. pneumophila*, the ability to attach, invade, and multiply within host cells is pivotal for virulence. We recently reported that the extracytoplasmic assembly of the T4SS of *L. pneumophila* is dependent on correct disulfide bond formation
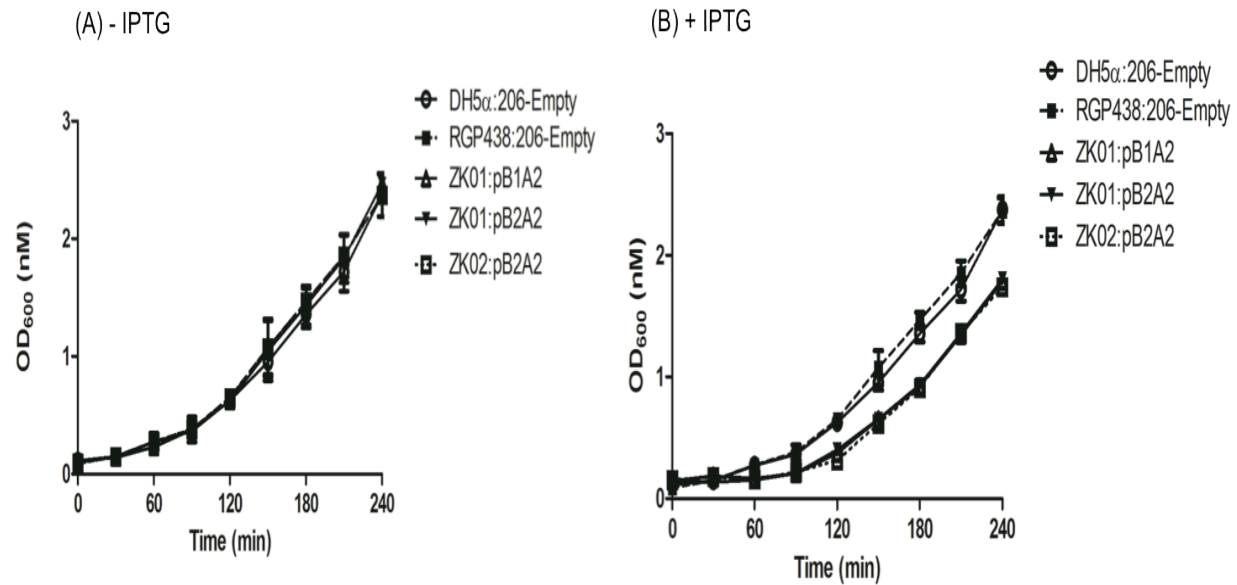
Figure 22. Growth assay. *In vitro* growth assessment of *E. coli* containing *L. pneumophila dsb* genes before and after induction with 1mM IPTG. Overnight grown bacteria were resuspended in fresh medium to an OD ~0.05, incubated for 30 min, and then induced at OD ~0.2 with 1mM IPTG. Bacteria growths were assessed at each time point as indicated.

catalyzed by a novel and essential disulfide bond oxidoreductase DsbA2 and not by DsbA1, a second dispensable DSB (Jameson-Lee *et al.*, 2011).

To assess whether expression of monomeric DsbA2 is important for virulence as compared to reduced DsbA2, DsbA2ΔN was used as a tool to assess the effect of oxidized DsbA2 activity *in vivo*. We created a P198T mutant in both monomeric and dimeric proteins in *L. pneumophila* strain AA100, a strategy adapted from (Jameson-Lee *et al.*, 2011) to test whether expression of mutant proteins might affect periplasmic funtions related to virulence. Expression of constructs was pre-induced with 1 mM IPTG. We first assessed the role of the mutant DsbA2 proteins in replication inside amoeba, a natural host of Lpn. Expression of DsbA2ΔN P198T exhibited a defect in intracellular replication in amoeba as compared to AA100 WT (Fig. 23). However, since amoebae are naturally phagocytic, it is difficult to conclude whether the dominant-negative effect on intracellular growth by DsbA2ΔN P198T was due to defects in cellular attachment, invasion, and/or replication. In order to examine these possibilities we utilized a model of Lpn invasion of non-phagocytic HeLa cells (Garduno *et al.*, 1998c; McCusker *et al.*, 1991; Newton et al., 2008; Jameson-Lee *et al.*, 2011). As depicted in Figure 24, *L. pneumophila* strain AA100 pMMB206:empty vector (positive control) under inducing conditions readily attached and invaded HeLa cells, while DsbA2ΔN P198T expressing mutant exhibited a three fold decrease in internalization nearly, similar to *dotA* mutant (negative control) and the DsbA2 P198T mutant previously shown to be dominant negative (Jameson-Lee *et al.*, 2011). Note that *dotA*, which has a defect in Dot/Icm activity is able to attach to HeLa cells but unable to invade after PBS wash. These results indicate that overexpression of monomeric DsbA2 P198T mutant protein interferes with DsbA2 function in the periplasm; thereby resulting
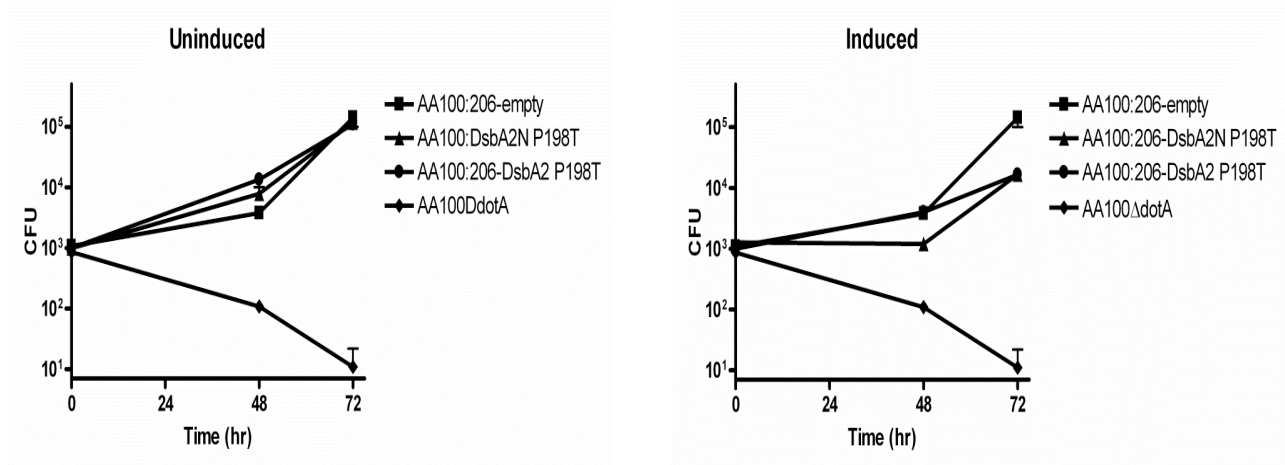
Figure 23. Intracellular replication in amoeba. *Acanthamoeba castellanii* were infected with *L. pneumophila* AA100 constructs (uninduced) in the absence of IPTG induction, (induced) induction at 1 mM IPTG. Colony-forming units (cfu) were determined in triplicate at the indicated times, and a representative assay is presented. Expressions of P198T mutant proteins inhibit intracellular growth in amoeba. DsbA2N represents DsbA2ΔN. Statistical significance of ≤ 0.043 by Student's *t-test* at 72h. Experiment performed in conjunction with Shandra Day, MD.
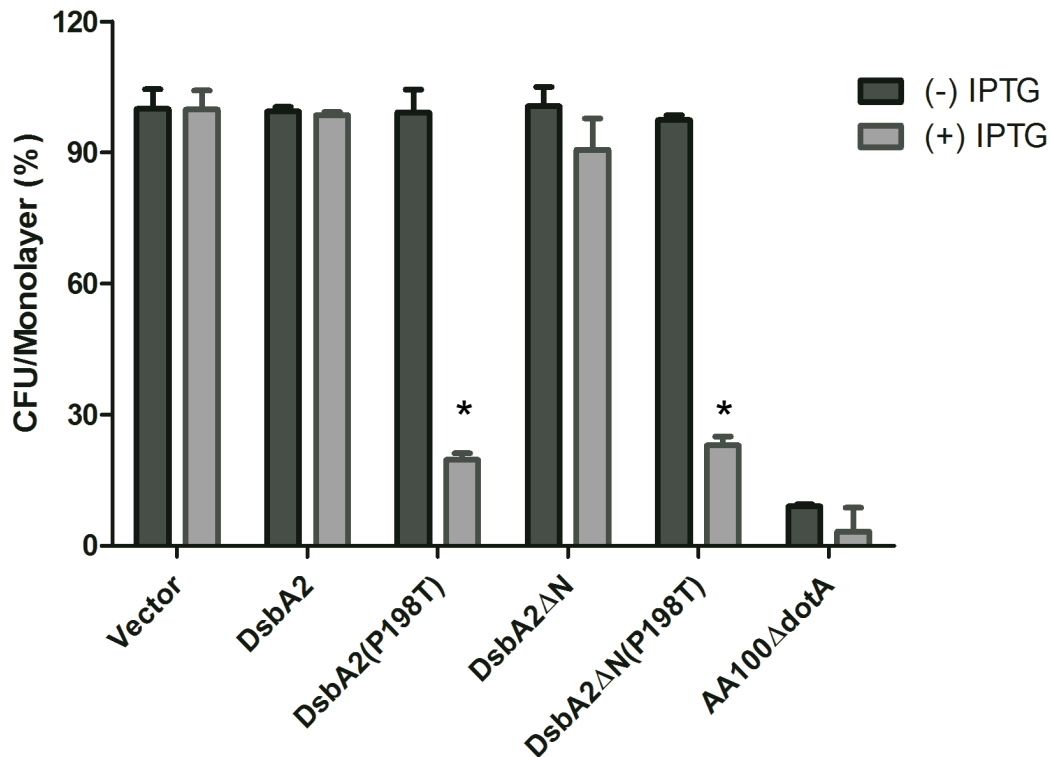
Figure 24. Attachment and invasion of non-phagocytic HeLa cells by Lpn expressing wild-type and mutant DsbA2 constructs. HeLa cells were infected with *L. pneumophila* AA100 constructs: AA100 WT pMMB206-empty vector (positive control), pMMB:*dsbA2* P198T and *dotA* (negative controls), pMMB:*dsbA2*, pMMB:DsbA2ΔN and pMMB:*dsbA2*ΔN P198T in the absence (dark gray) or presence of 1mM IPTG (gray). Colony-forming units (cfu) were determined in triplicate at the indicated times for a single experiment; experiment was repeated multiple times. Over expression of DsbA2ΔN P198T protein in *L. pneumophila* exhibits a defect in attaching and invading HeLa cells. Asterisk (*) denotes statistical significance of ≤0.005 by Student's *t*-test. Experiment performed in conjunction with Shandra Day, MD.

in a dominant-negative effect on attachment, invasion, and intracellular multiplication, indicating a role for DsbA2 function in *L. pneumophila* pathogenesis.

3.15 Functional Dot/Icm T4SS assessment of monomeric DsbA2 activity

Since a functional Dot/Icm T4SS (T4SS) is essential for invasion and intracellular multiplication of *L. pneumophila* in host cells (Berger and Isberg, 1993; Brand *et al.*, 1994; Franco *et al.*, 2009; Jameson-Lee *et al.*, 2011), we considered it likely that the dominant-negative effect of the DsbA2ΔN P198T mutant protein was due to interference on DsbA2 bifunctional activity, thus affecting with either assembly or function of the T4SS secretion apparatus. To directly address this possibility, we employed a well-established contact hemolytic assay that was previously validated with specific Dot/Icm T4SS (Δ*dotA*) and dimeric DsbA2 P198T mutants (Kirby *et al.*, 1998; Charpentier *et al.*, 2009; Jameson-Lee *et al.*, 2011). As seen in Figure 25, AA100 pMMB206:empty vector mediated lysis of erythrocytes in the presence or absence of IPTG. In contrast, bacteria expressing the dominant-negative DsbA2ΔN P198T mutant protein did not exhibit contact-dependent hemolysis similar to DsbA2 P198T (induced), and nearly equivalent to that of a *dotA* mutant (negative control), respectively. Here, expressed DsbA2ΔN construct also show a slight defect in hemolysis but was not significant compared to wild-type AA100 strain. Longer incubations also showed no change in erythrocyte lysis by bacteria expressing either DsbA2ΔN P198T or DsbA2N P198T. These results further validate the correlation of defects seen in HeLa cells infectivity assay, suggesting that the dominant-negative avirulence phenotype of the DsbA2ΔN P198T mutant protein most likely results from interference on DsbA2 optimal function in the periplasm, leading to the disruption of assembly or function of the Dot/Icm T4SS.
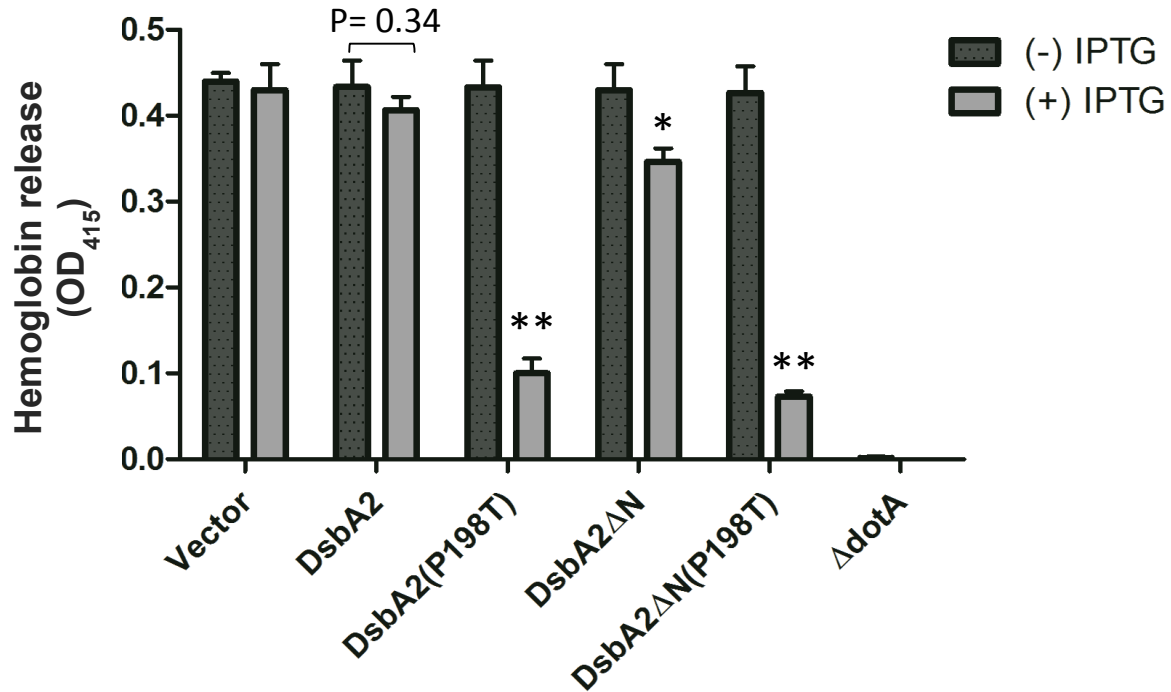
Figure 25. Effects of DsbA2 wild-type and mutant constructs on Dot/Icm T4SS-mediated erythrocyte lysis. Red blood cells were infected with *L. pneumophila* AA100 constructs in the absence of IPTG induction (dark gray), and induction at 1mM IPTG (gray). Hemoglobin release was measured after 2h incubation of erythrocytes with AA100 WT pMMB206-empty vector (positive control), pMMB:*dsbA2* P198T and *dotA* (negative controls), pMMB:*dsbA2*, pMMB:*dsbA2*ΔN and pMMB:*dsbA2*ΔN P198T of post-induced stationary phase grown bacteria. Asterisks (*), (**) denote statistical significance of ≤0.0432 and ≤0.001 by Student's *t*-test, respectively. Experiment performed in conjunction with Shandra Day, MD.

3.16 The importance of DsbA1 for *L. pneumophila* pathogenesis

Proteins that contain disulfide bonds are often slow to fold *in vitro* because the oxidation and correct pairing of the cysteine residues is rate limiting. The folding of such proteins is greatly accelerated by a periplasmic oxidoreductase DsbA, but the mechanism of this rate enhancement is not well understood. *L. pneumophila* contains two oxidoreductase proteins; DsbA1 and DsbA2. DsbA1 has been shown to be non-essential and without phenotype (Jameson-Lee, UVa thesis 2012) when compared to DsbA2, which has been characterized in detailed above. In addition to further characterizing the role of DsbA2 for Lpn pathogenesis, our study here shows that a *dsbA1* knockout mutant is defective in pigment production (Fig. 28) and demonstrates a hypervirulent phenotype in an L929 cell infectivity assay (Fig. 29) and enhanced lysis of erythrocytes (Fig. 30A). The mutant also retained the ability to replicate in amoeba (data not shown), and to attach and invade HeLa cells (Fig. 30B). We also determined the crystal structure of Lpn DsbA1, and found that its structure closely resembles the ubiquitous redox protein thioredoxin and *E. coli* DsbA, despite very low sequence similarity. An important similarity to *E. coli* DsbA is the presence of another domain that forms a cap over the thioredoxin-like active site of DsbA1; however, this differs when compared to *E. coli* DsbC which is a dimer. These features suggest that DsbA1 may compete for substrates with DsbA2 or might act by binding to partially folded polypeptide chains before oxidation of cysteine residues like *E.coli* DsbA. Also, its hypervirulent phenotype is indicative of its modulating role on Lpn virulence. We hypothesized that DsbA1 functional and structural similarities to *E. coli* DsbA suggest a contributing role to the virulence of *L. pneumophila* in its host.
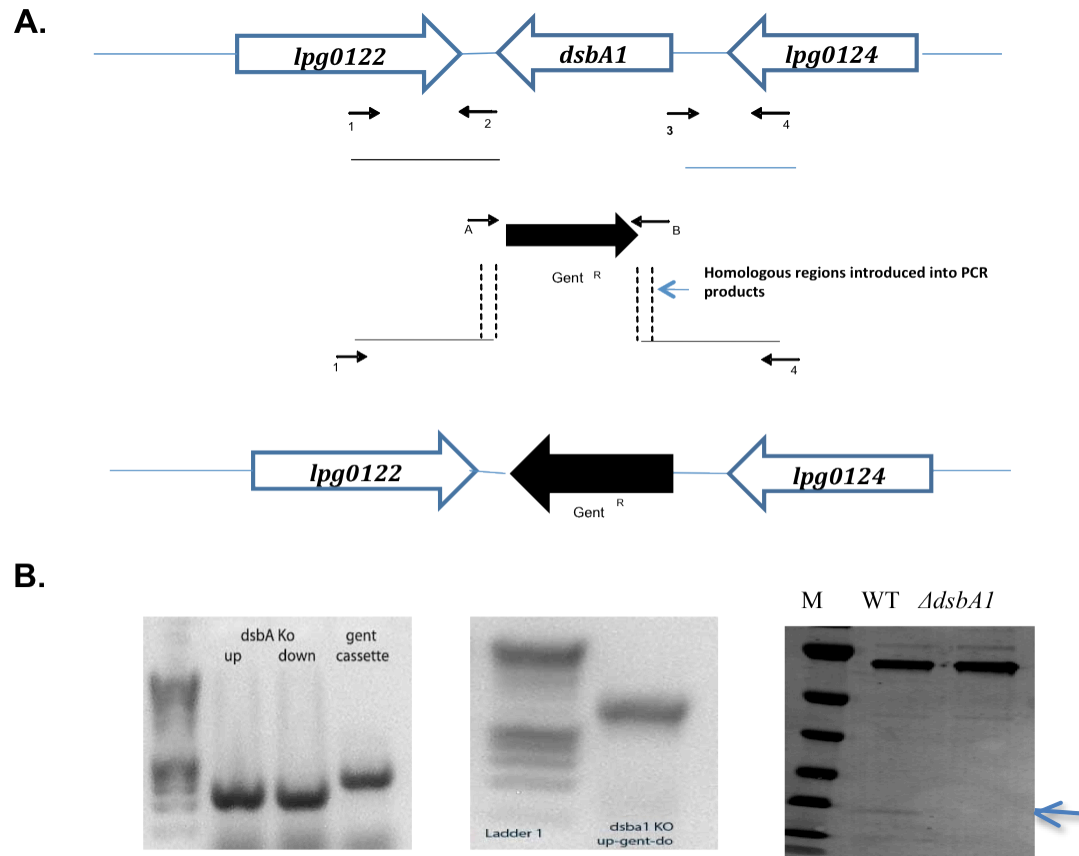
Figure 26. *dsbA1* **knockout construction. A**) Genetic organization of the *dsbA1* locus and allelic replacement strategy to introduce a gentamicin cassette downstream of *dsbA1*. Vector-free allelic replacement. Primers (arrows) amplify ~500bp chromosomal regions (lines) surrounding Lpn *dsbA1*. Primers were used to amplify an antibiotic resistance cassette (Gent^R). In a separate reaction, the two flanking regions and cassette were amplified with the two outside primers (1&4). The dashed lines denote homologous regions introduced into the PCR products where they can anneal to form one large template (**B**; middle diagram). The final PCR product contains a resistance cassette flanked by chromosomal regions to facilitate homologous recombination. **B**) Last diagram - α-DsbA1 antibody was used to confirm *dsbA1* knockout. The blue arrow points to the 24 kDa DsbA1 protiein.

A. α-DsbA1

| Marker | AA100: | ΔdsbA1 | ΔdsbA1: DsbA1 | ΔdsbA1: DsbA1 P150T |

← ~24kDa
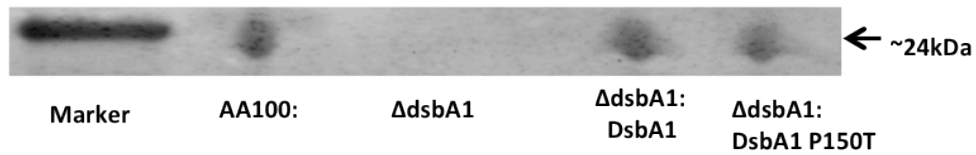
B. Extracellular growth of *Legionella pneumophila*
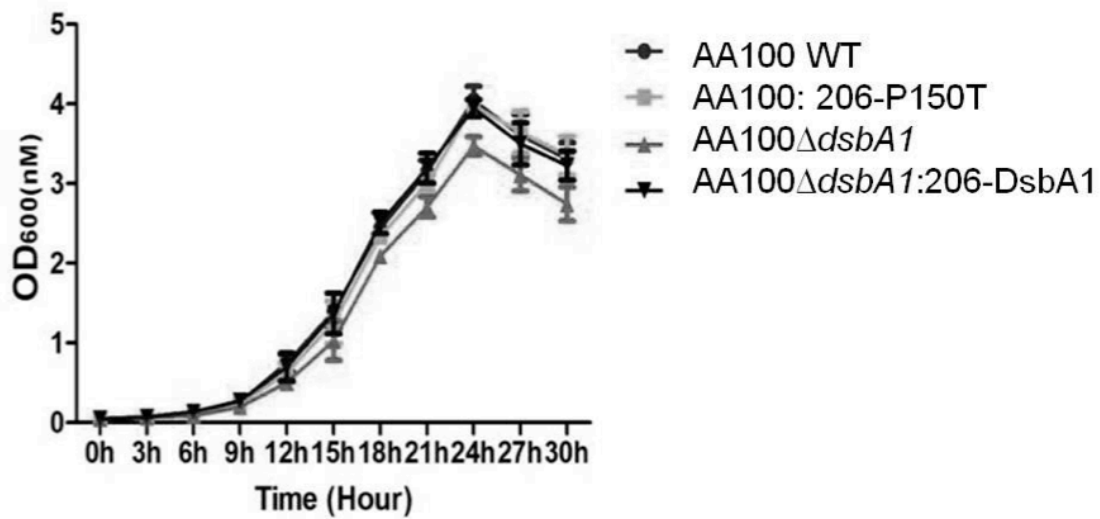
Figure 27. Protein expression and *in vitro* growth assessments of WT and *dsbA1* mutant Lpn. (A) Proteins expressions of all construct using 1mM IPTG at stationary phase. Western blot analysis with DsbA1-specific antibody validated a successful deletion of *dsbA1.* (B) Growth curve of *L. pneumophila* constructs show no significant difference
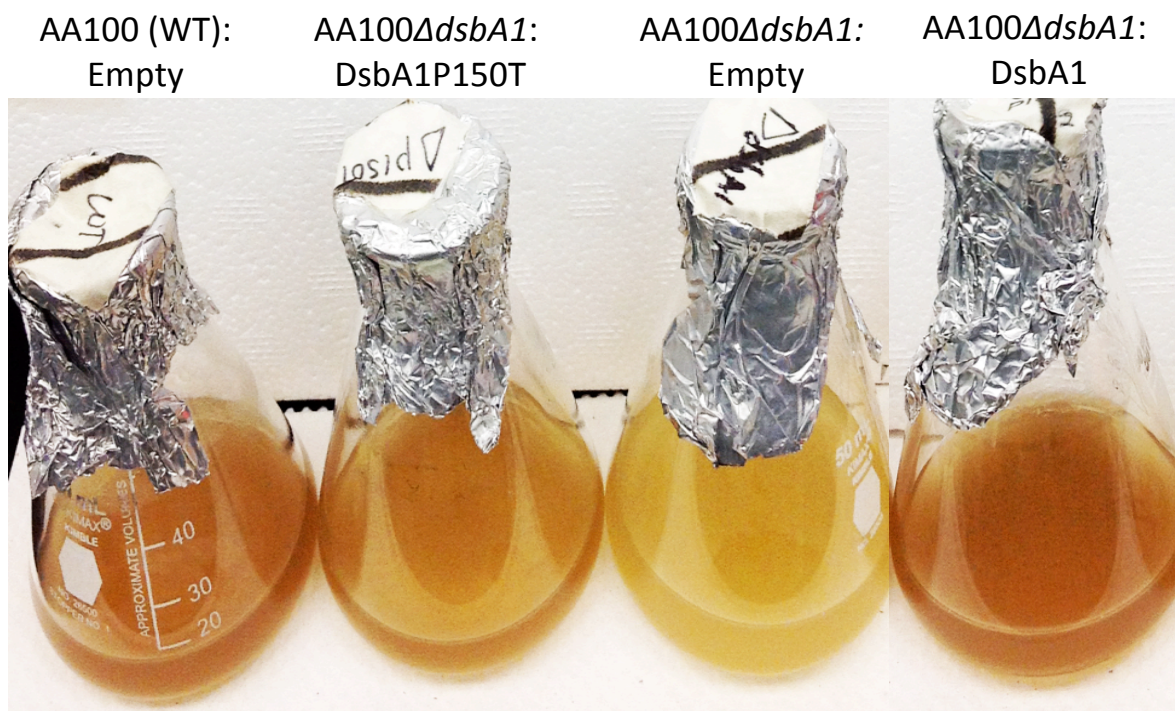
Figure 28. *L. pneumophila* **DsbA1 has a defect in pigment production.** Overnight culture

of *L. pneumophila* WT strain AA100 (pMMB206::empty vector), AA100Δ*dsbA1*

(pMMB206::empty vector), pMMB206::*dsbA1* P150T, and pMMB206::*dsbA1* were grown to

OD$_{600}$ ~0.6, then induced with 1mM IPTG and grown till 30h (stationary phase). AA100Δ*dsbA1*

exhibited a defect in pigment production when compared to AA100 WT and Δ*dsbA1*

complemented with expression of DsbA1 in an inducible vector (pMMB206::*dsbA1*).

3.17 Novel phenotype: *dsbA1* knockout exhibits a defect in pigment production

Prior to this study, DsbA1 has been reported to be dispensable for virulence in *L. pneumophila* (Jameson-Lee, UVa thesis, 2012). We sought to closely assess the growth differences of Lpn AA100 strains with or without the *dsbA1* gene. Our results of overnight grown constructs in BYE broth reveal that DsbA1 knockout exhibits a loss in pigment production, as depicted in Fig. 28. However, the defect was restored upon expression of pMMB206:*dsbA1* in a *dsbA1* knockout background. This result suggests that the reason *dsbA1* is not selected against in Lpn may be due to its ability to modulate virulence both during exponential growth where DsbA1 and DsbA2 levels are low and to a lesser extent during stationary phase when *dsbA2* expression is four fold higher than that for DsbA1. In head to head growth studies *in vitro*, there was no significant difference between the WT AA100 and the *dsbA1* mutant (Fig. 27).

3.18 *dsbA1* knockout exhibits a hypervirulent phenotype in plaque assay

Plaque assay is a widely used approach for determining the quantity of infectious bacteria/virus. This technique was first developed to calculate the titers of bacteriophage stocks. Renato Dulbecco modified this procedure in 1952 for use in animal virology, and it has since been used for reliable determination of the titers of many different viruses, which has been adopted by bacteriologists.

We constructed a *ΔdsbA1* mutant using a vector-free allelic replacement mutagenesis strategy (Fig. 26). We performed plaque assay using *Legionella* AA100 strain containing our *ΔdsbA1* mutant and complements containing (pMMB206:empty vector, pMMB206:*dsbA1*-Pl50T, and pMMB206:*dsbA1*), 10-fold dilutions of overnight grown bacterial stocks were prepared, and

A.

| Control 1.5 x 10⁵ L929 Cells only | AA100:pMMB206-Empty | AA100ΔdsbA1: pMMB206-Empty | AA100ΔdsbA1: pMMB206-DsbA1 |

B.
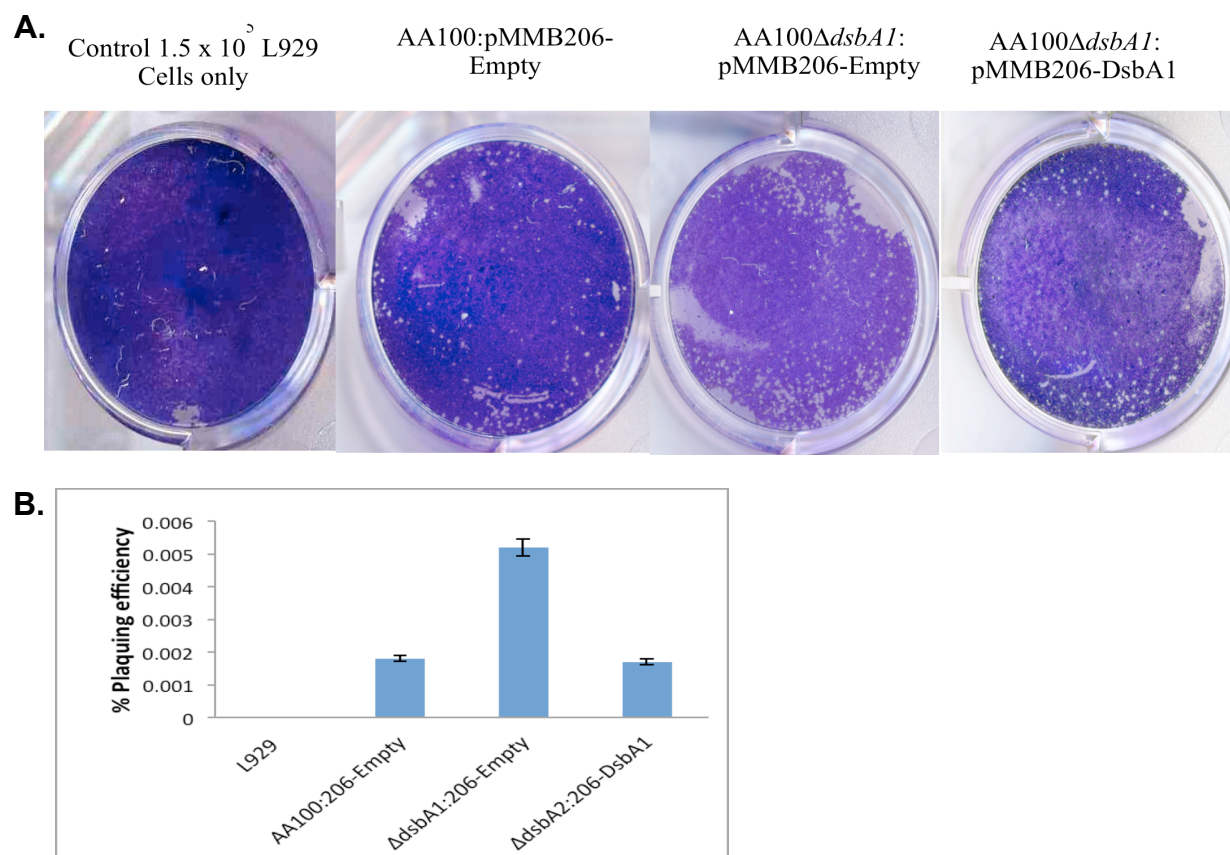
Figure 29. Lpn DsbA1 knockout exhibits a hypervirulent phenotype in L929 infectivity assay. (**A**) Crystal violet which stains live cells. Zone of clearance is indication of plaques forms due to the lysis of L929 cells by virulent bacteria. (**B**) Plaquing efficiency of Lpn DsbA1 knockout exhibits a hypervirulent phenotype compared to *L. pneumophila* WT AA100 strain (positive control), and L929 cells without any bacteria (negative control). *ΔdotA* mutant displayed no plaquing efficiency similar to L929 negative control (data not shown). Experiment performed in conjunction with Shandra Day, MD.

0.2 ml aliquots are inoculated onto susceptible L929 cell monolayers in 1.8ml DMEM in a 24 well plate, as described in methods . After an hour incubation period, to allow bacteria to attach to cells, DMEM containing the unattached bacteria are removed, and the monolayers were covered with DMEM plus 0.6% agarose (SeaKem) gel pad. The plates were incubated for four days. The original infected cells released progeny bacteria, and the spread of the new bacteria was restricted to neighboring cells by the gel. Consequently, each infectious particle produces a circular zone of infected cells called a plaque. Eventually the plaque becomes large enough to be visible to the naked eye. Dyes that stain living cells, such as cystal violet, which is used here, are often used to enhance the contrast between the living cells and the plaques. Only bacteria/virues that cause visible damage of cells can be assayed in this way. As depicted in Fig. 29, our results revealed L929 cells infected with DsbA1 knockout strain showed an increase in plaquing compare to AA100 WT strain. Also, overexpression of DsbA1 from an inducible vector was able to restore the plaquing efficiency to WT levels. These results suggest the novel role of DsbA1 as a modulator in the system for *L. pneumophila* pathogenesis.

3.19 DsbA1 is a modulator of virulence in *Legionella pneumophila*

In order to validate the modulating role of DsbA1 for pathogenesis in *Legionella*, we sought to utilize *in vivo* infectivity assays to demonstrate the contributing role of DsbA in the DSB system using *ΔdsbA1* mutant. As reported in Fig. 30A, *ΔdsbA1* mutant bacteria were very efficient in lysing RBC as depicted by the increased in hemoglobin release as compared to AA100 WT. Overexpression of WT DsbA1 in a *ΔdsbA1* mutant background reversed the hypervirulent phenotype to wild type level. Next, we tested the ability of *ΔdsbA1* mutant to attach and invade non-phagocytic HeLa cells. HeLa cells as previously described are commonly used
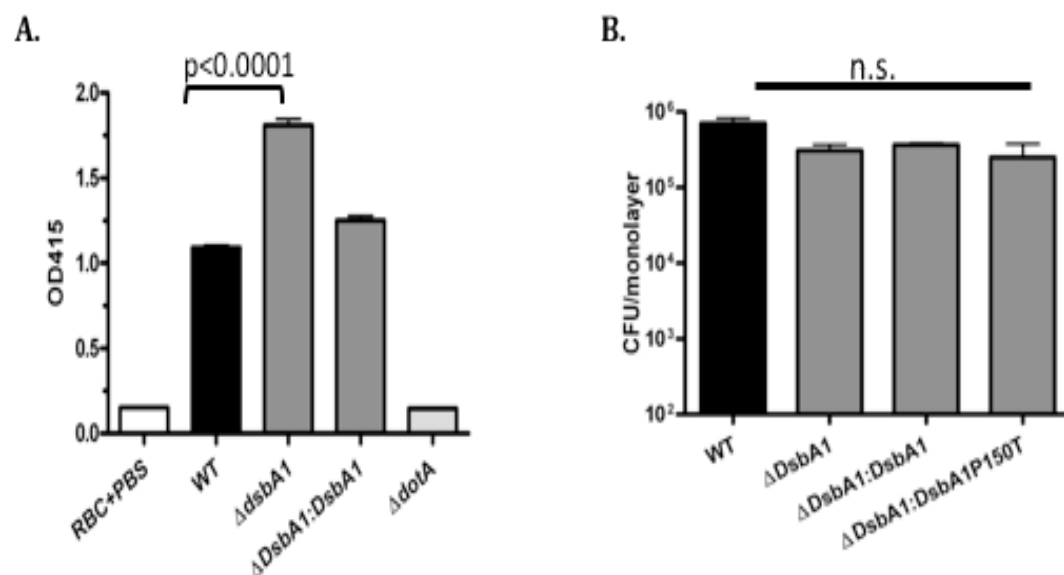
Figure 30. *In vivo* assays demonstrating the role of DsbA1 in *L. pneumophila*. (**A**) Functional Dot/Icm T4SS assessment was examined utilizing contact-dependent lysis of human RBC. Hemoglobin release was measured after 2 h incubation of erythrocytes with AA100 WT (positive control), *dsbA1* KO, *ΔdsbA1*:pMMB206-*dsbA1*, *ΔdsbA1*:pMMB206-*dsbA1* P150T, PBS and *dotA* (negative controls) of post-induced stationary phase grown bacteria. (**B**) Attachment and invasion of non-phagocytic cell. DsbA1 KO has no defect in attaching and invading HeLa cells. Experiment performed in conjunction with Shandra Day, MD.

to test the invasiveness of many bacterial species. The studies below aim to address the infectivity- attachment and invasion capabilities of DsbA1 mutants (knockout and P150T) as compared to AA100 WT for the assay. Using a stationary phase grown *ΔdsbA1* mutant expressing DsbA1 P150T mutant protein, which also has a defect in resolving its substrates (as shown below) and DsbA1 WT in a *ΔdsbA1* background, we showed that there was no measureable defect in attachment and invasion of HeLa cells (as seen in Fig. 30B). These results reveal that while DsbA1 has an attenuating effect on virulence, it is not sufficient to have a dominant negative effect on DsbA2 funciton.

3.20 DsbA1 interacts with substrates

DsbA, a member of the thioredoxin superfamily, introduces disulfide bonds into newly translocated proteins. We sought to build on previous studies in which a His_6-DsbA2 P198T mutant protein was used to capture and identify its substrate repertoire by using a *cis*-proline mutant of His_6-DsbA1 P150T constructed by site directed mutagenesis strategy, then SDS-PAGE, immunoblot/silver stain, and 2-dimensional (2D) diagonal gel were performed to examine DsbA1 substrate repertoire. Fig. 31A, shows the silver stained gel of DsbA1 interacting proteins separated by reducing and non-reducing SDS-PAGE. Remarkably, we saw DsbA1 P150T mutant accumulated bands of different apparent molecular masses, suggesting that these bands represent DsbA1-substrate complexes; these bands disappeared when samples were treated with reducing agent before electrophoresis (Fig. 31A, lane 3).
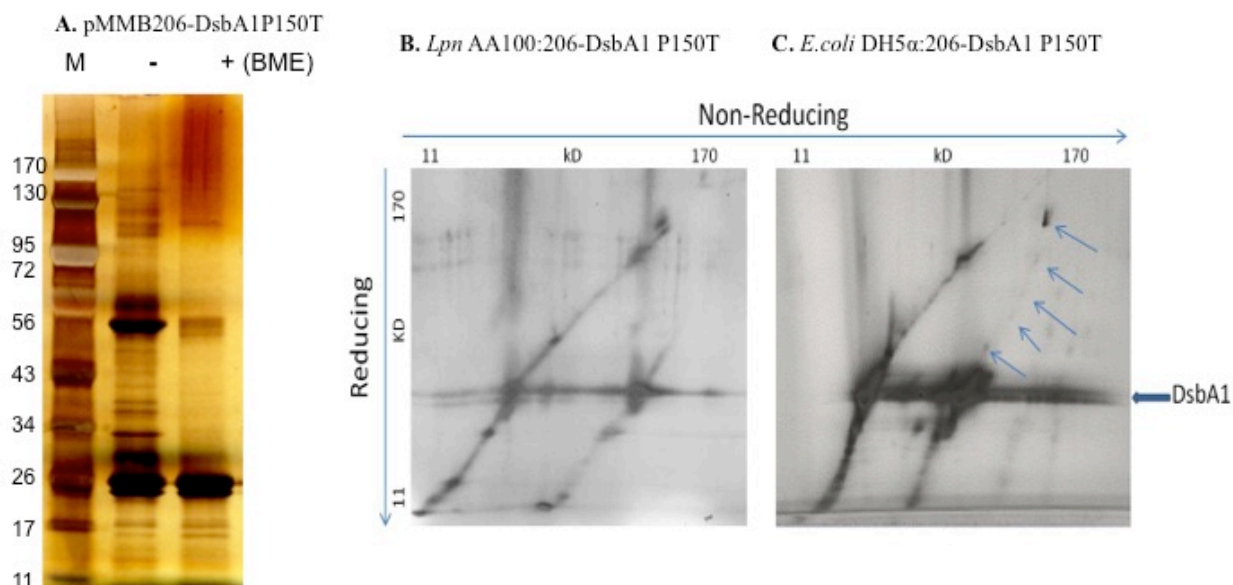
Figure 31. Substrate proteins capture by DsbA1 P150T mutant protein in *L. pneumophila* and *E. coli*. *Legionella* and *E. coli* strains containing C-terminal His-tagged DsbA1 P150T were induced *in vitro*. Substrate complexes were purified by nickel chromatography and diagonal gels of captured proteins were run under non-reducing (direction of electrophoresis indicate by arrows), then reducing conditions. Protein spots (silver stained) off the diagonal are DsbA1 interacting proteins whose disulfide bridge had been reduced in the second dimension. These proteins are yet to be identified by Mass-spectrometry (MS). (**A**) His$_6$ DsbA1-substrate complexes enriched for by Ni^{++} column chromatography and separated by SDS-PAGE and then silver stained: lane 1 (MW marker), lane 2 (non-reducing conditions), lane 3 (under reducing conditions; note disappearance of high MW complexes), (**B**) In *Legionella* - DsbA1 P150T captured less periplasmic protein as compared to (**C**) *E. coli*, where the thinner arrows point to the difference in spots of substrate proteins that were captured. Thicker arrow points to DsbA1 that has dissociated from interacting proteins in the second dimension.

To establish that the proteins were indeed captured, 2D diagonal gels with first dimension non-reducing and second dimension under reducing conditions was used (Fig. 31, **B**. *Legionella*, **C**. *E. coli*). Silver stained proteins spots off the diagonal represent DsbA1 captured proteins, which suggest that DsbA1 is an oxidase and interacts with substrates in the periplasm, and thus has a functional role in both *L. pneumophila.* Interestingly, overexpression of DsbA1 in *E. coli* shows an increased interaction with substrates than was noted for *L. pneumophila.* This finding suggests a moderate role/effect of DsbA1 for the proper folding of substrates in the periplasm of Lpn.

3.21 Crystallization of *L. pneumophila* DsbA1

The crystallization of DsbA1 was initiated by my predecessor (Jameson-Lee, UVA 2012). Lpn DsbA1 is predicted to have signal sequence peptides that cleaved between residues 20 and 21 (Signal P 4.0 online). We utilized the protocol of Missiakas *et al*. (1994); wherein periplasmic shockate of DsbA1 followed by anion exchange chromatography was used to generate a full-length, un-tagged protein, and then sent to CSGID consortium for crystallization. Figures 32, 33, and 34 illustrate the similarities and differences between the crystal structures of the thioredoxin folds of Lpn DsbA1, EcDsbA, and EcDsbC, with the active site and N-terminus domain of Lpn DsbA1 being nearly similar to EcDsbA, but different from EcDsbC. Also, a crystallization study of Lpn DsbA2 using the same strategy is currently in progress.
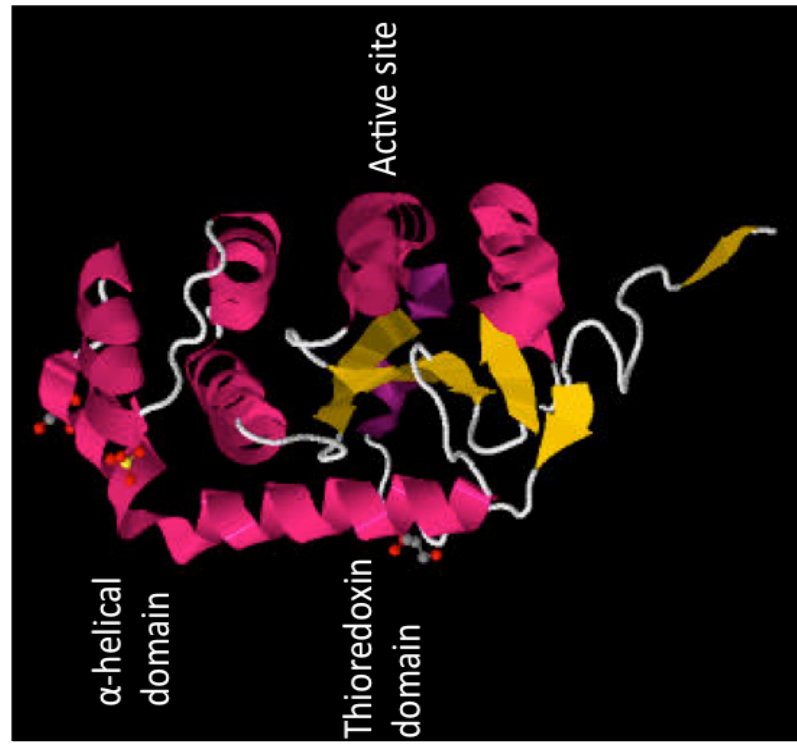
Figure 32. Crystal structures of EcDsbA and Lpn DsbA1. (**A**) EcDsbA (PDB 1a2L) and (**B**) Lpn DsbA1 (PDB 4jrr) depicting the active site consisting of the CXXC motif and *cis*-proline region. *E. coli* DsbA and Lpn DsbA1 thioredoxin folds have similar helices and conformations.

Figure 33. Superimposed. DsbA1 thioredoxin fold homologue crystallized from *L. pneumophila* is nearly identical to *E. coli* DsbA. Lpn DsbA1 (PDB 4jrr, orange) aligns well in space with EcDsbA (PDB 1a2j, blue).
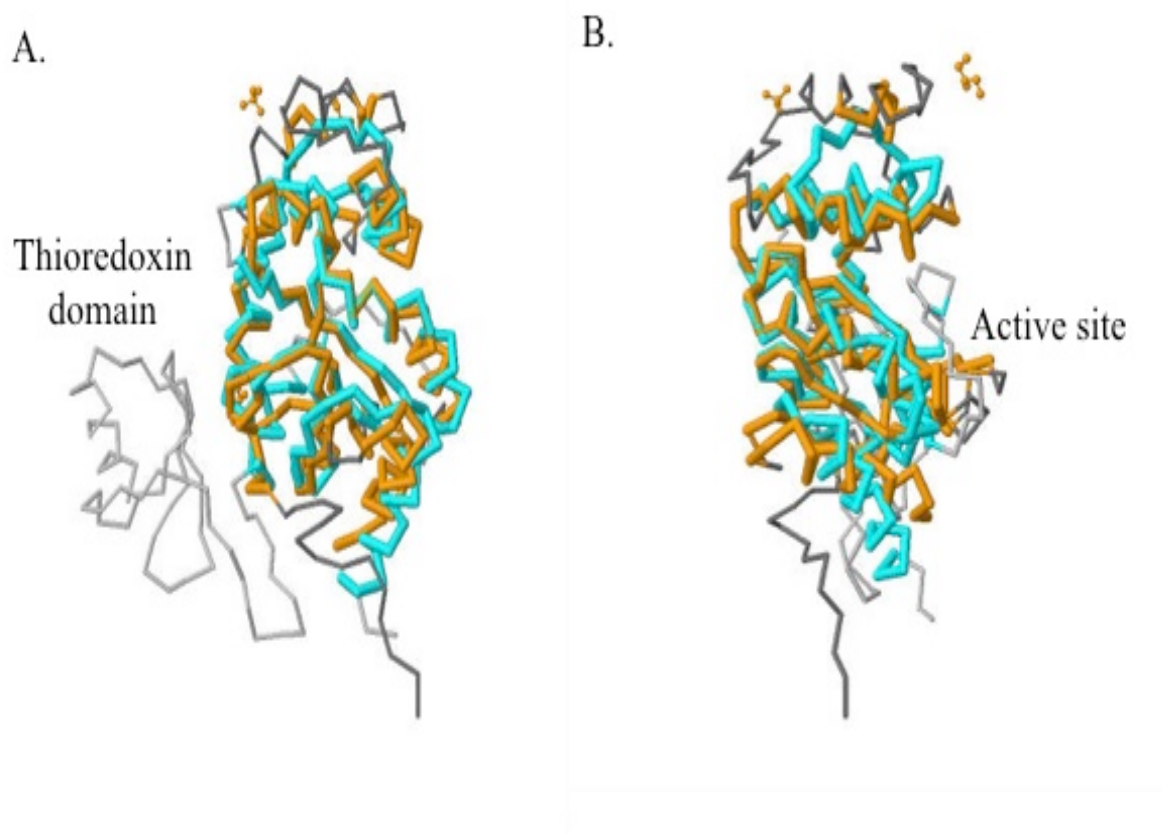
Figure 34. The thioredoxin fold and the active site of EcDsbC do not match up well with Lpn DsbA1. (**A**) *E. coli* DsbC (PDB 1eej, blue) contains extra alpha helices at its N-terminus that do not line up well with *L. pneumophila* DsbA1 (PDB 4jrr, orange) crystal structure, (**B**) the active site of Lpn DsbA1 is pointed downward while EcDsbC active site is pointed upward. Both the thioredoxin fold and active site of Lpn DsbA1 are more similar to EcDsbA than EcDsbC.

Chapter 4. Discussion

Over the past 20 years, *E. coli* has served as the primary model system for the study of disulfide bond formation in Gram-negative bacteria. However, this picture changed with the discovery of sets of enzymes that utilize the chemical properties of cysteines to form or reduce disulfide bonds in the periplasm of other bacterial species (Kadokura *et al*., 2003; Jameson-Lee *et al*, 2011; Kpadeh *et al*., 2013). The findings of this study build on the seminal work of Dr. Max Jameson-Lee who discovered DsbA2 and speculated that DsbA2 likely formed a novel system that similarly managed disulfide bond formation and protein folding in *L. pneumophila*. The studies presented herein provide solid evidence to support the hypothesis that the DsbA2 system differs fundamentally from the DsbA/DsbC system of *E. coli*. In the DsbA2 system, a single bifunctional homdimeric protein (DsbA2) catalyzes both the formation of and reductive isomerization of disulfides to promote protein folding. Extensive phylogenetic analysis reveals that the bifunctional DsbA2 system, first discovered in Lpn, is common to much of the microbial world that includes diverse species of Gram-negative soil and aquatic bacteria and all intracellular pathogens that express a type IV secretion system. Perhaps the most significant finding of these studies is that the DSB system of *E. coli* can be functionally replaced by the DSB system of Lpn. Our work raises many new questions about the advantages and disadvantages of a one player (DsbA2) system versus two-player (DsbA/DsbC) system in the management of disulfide bond formation and rearrangement in microbial systems.

4.1 Importance of DsbA2

Remarkably, the newly discovered DsbA2 system appears to be quite common and includes many soil and aquatic species such as *Caulobacter*, *Bradyrhizobium*, *Aeromonas*, and

Azospirillum and most human, animal, and plant pathogens that express T4SSs as major virulence factors (Christine *et al.*, 2005). Interestingly, the DsbA2 homologue we first reported for *C. crescentus* (Jameson-Lee *et al.*, 2011) has recently been studied in more detail by the Beckwith group (Cho *et al.*, 2012). They named the protein ScsC as it was found in an operon containing a novel DsbD that had been named ScsB. Their studies confirmed that ScsC was a homodimer and exhibited protein disulfide isomerase activity. However, we were surprised that they reported ScsC was an orthologue of DsbC based on phylogenetic criteria (Cho *et al.*, 2012). This contrasts with our conclusion based on phylogenetic criteria, including the conserved 27 amino acid dimerization domain sequence, as well as structural biology (crystal structure) considerations (Jameson-Lee *et al.*, 2011; Kpadeh *et al.*, 2013). First, superimpositions of the crystal structure of a DsbA2 orthologue from *Silicibacter* (PBD 3gyk) onto DsbA revealed structural identity that was not observed with the DsbC structure. Using Phyre simulations, DsbA2 from *L. pneumophila* also precisely fit onto the structure of DsbA and not onto DsbC (Jameson-Lee *et al.*, 2011). The similarities between DsbA2 and DsbA and differences with DsbC are obvious from Figure 6. Finally, the NCBI site now shows that DsbA2/Com1 is a distinct clade that evolved from DsbA and not from DsbC. The Beckwith group did however note that like the DsbDs of *L. pneumophila*, the DsbD of *C. cresentus* was also essential.

DsbA2 is found in bacteria containing type IVb secretion systems including *Rickettsia*, *Legionella*, *Bartonella*, *Anaplasma*, *Brucella*, *Ehrlichia*, *Coxiella*, and *Agrobacterium*. Most of these genera have doubling times of 2 h or more, which raises the possibility that in fast-growing bacteria, the DsbA/DsbC system is required to accelerate correct disulfide bond formation to enable protein folding to keep pace with cell division. This possibility seems to be supported by growth rate differences noted with *E. coli* strains carrying single mutations in *dsbA* and double

mutations (*dsbA dsbC* and *dsbA dsbD*) (Vertommen *et al*., 2008). In this regard, DsbA accelerates disulfide bond formation between consecutive cysteines as most nascent polypeptides enter the periplasm (Zapun *et al*., 1993; Kadokura & Beckwith, 2009; Kadokura & Beckwith, 2010). However, in other proteins, disulfide bonds may form after the protein has entered the periplasm (Kadokura & Beckwith, 2009) or require correction of mispaired disulfides by DsbC (Denoncin *et al*., 2010). We suspect that in those slow-growing bacteria expressing DsbA2, such as *L. pneumophila*, disulfide bonds might be introduced after proteins enter the periplasm, which is supported by the efficiency and diversity of proteins captured by the DsbA2 P198T mutant protein (Jameson-Lee *et al*., 2011). It follows that the apparent essentiality of *dsbA2* is due to both its bifunctional nature and an absence of any backup system to repair mis-oxidized proteins that would accumulate to toxic levels in the periplasm. We also noted that overexpression of the DsbA2P198T mutant protein rendered *L. pneumophila* noninfectious by interfering with assembly and function of the T4SSs (Jameson-Lee *et al*., 2011). Since *dsbA2* was also essential in strains with mutations in *dot/icm* structural genes, we concluded that DsbA2 must be associated with proper folding of other proteins whose functions might also be essential, including OmpS porin and periplasmic Hsp60 (Butler *et al*., 1985; Fernandez *et al*., 1996; Garduno *et al*., 1998; Jameson-Lee *et al*., 2011).

The cell envelope of the prokaryotic cell is a major line of defense against environmental challenges (Hatahet *et al*., 2014). It is also a major site for the maturation of proteins exported from the cytoplasm to the periplasm, outer membrane and to the external environment. In many bacteria, the enzymes that catalyze disulfide bond formation form a distinct pathway from those enzymes that catalyze disulfide bond isomerization. The major argument for support of separate pathways has been that it prevents futile cycling of reducing equivalents from the cytoplasm.

However, it seems clear from our work that the two processes can be carried out by a single protein, without apparent drain on reducing equivilents. A single system is also supported by examples of the dual activities ascribed to eukaryotic PDI, a four domain oxidoreductase capable of performing thiol oxidation as well as disulfide isomeration (Tian *et al.*, 2006), and rational genetic design experiments in prokaryotes (Segatori *et al.*, 2004). These studies raise the possibility that some bacteria may catalyze oxidation and isomerization by a single protein in the same compartment without any interference, hence supporting the existence of the alternative DsbA2 system discovered in our laboratory.

4.2 DsbA2 of *L. pneumophila* differs from canonical DsbA of *E. coli*

The results of our studies show that DsbA2 of *L. pneumophila*, which contains a 56-amino-acid N-terminal extension relative to DsbA of *E. coli*, forms a homodimer in *L. pneumophila* and displays protein disulfide isomerase activity in both *in vivo* and *in vitro* assays. Our lab has previously reported that DsbA2 behaved similarly to DsbA by catalyzing the formation of disulfide bonds in secreted proteins of *L. pneumophila* (Jameson-Lee *et al.*, 2011). Consistent with DsbA-like activity, a DsbA2 *cis*-proline (P198T) mutant protein formed stable disulfide cross-linked complexes with substrate proteins (Jameson-Lee *et al.*, 2011). Only the disulfide is capable of capturing substrate proteins (Kadokura *et al.*, 2004). In addition, DsbA2 exhibits protein disulfide isomerase activity similar to Ec DsbC. While the measured reduction potentials of DsbA and DsbC are similar (Hatahet *et al.*, 2014), a stark structural difference between the two is found in domain organization of DsbC. Taken together; it appears that structural and kinetic barriers maintain the segregation of reducing and oxidizing pathways. For instance, the homodimeric arrangement of DsbC exerts steric constraints that preclude its

interaction with and subsequent oxidation by DsbB (Segatori *et al*., 2006). Interestingly, DsbA2 is a homodimer, which interacts with either DsbB of *L. pneumophila* when either Lpn DsbD is present, suggesting its structural and functional distinctiveness from the canonical DsbA.

4.3 How are the redox forms maintained?

In support of the above suggestion, we determined that DsbA2 was present as a mixture of reduced thiol and disulfide forms *in vivo* (Jameson-Lee, UVa thesis 2012, Kpadeh *et al*., 2013), which contrasts with DsbA and DsbC, which are mostly oxidized and reduced, respectively (Shouldice *et al*., 2011). We determined by gel filtration that the mixture of oxidized and reduced forms was not due to equilibrium of monomer and dimer forms. Our studies do not rule out the possibility that each arm of the DsbA2 dimer might contribute to the mixture, one as the disulfide and the other as the free thiol. Our studies suggest that structural differences within the thioredoxin folds of DsbA2 might render them more accessible to the DsbB oxidases of *L. pneumophila*. In this regard, *L. pneumophila* expresses two alleles, DsbB1 and DsbB2 (also known as LidJ) (Conover *et al*., 2003) that show sequence divergence from *E. coli* DsbB. As mentioned above, the N-terminal domain of DsbA2 consists of 57 additional amino acids which may contribute to steric hindrance, thus preventing interaction with DsbB in the absence of either Lpn DsbDs. Specifically, deletion of the dimerization domain, which resulted in a monomeric DsbA2 (DsbA2ΔN) was structurally different and interacted with Ec DsbB, and both DsbBs of *L. pneumophila*. Due to the distinct biology of Lpn DsbA2 reported thus far, we suggest that Lpn DsbBs can accommodate interaction with DsbA2 at a certain phase of the growth cycle.

As previously reported, *L. pneumophila* also expresses two alleles of DsbD (Jameson-Lee *et al*., 2011). We suggest that these competing oxidases and reductases interact with DsbA2 at

differing efficiencies to maintain equilibrium between oxidized and reduced forms. Interestingly, Segatori *et al*. (2004) showed that a chimera composed of the dimerization domain of DsbC fused to the catalytic domain of DsbA also existed as a mixture in *E. coli* and displayed bifunctional activity, including partial complementation of a *dsbA* mutant for motility (Segatori *et al*., 2004). Since DsbA2 did not restore motility to a *dsbA* mutant, DsbA2 is either a poor substrate of DsbB or a good substrate of DsbD. Our studies found that DsbA2 is maintained as the free thiol in *E. coli* by DsbD and efficiently complemented a DsbC mutant in the PDI detector assay. By demonstrating that DsbA2ΔN was able to complement a *dsbA* mutant of *E. coli*, we conclude that the lack of interaction between DsbA2 and DsbB is most likely the result of steric hindrance by the homodimer as well as efficient interaction with EcDsbD. Interestingly, reconstitution of Lpn *dsb* genes in *E. coli* null mutant (RGP438) validated the redox status of DsbA2 as seen in *Legionella* (mixture of oxidized and reduced forms), thus restoring motility in *E. coli*. This finding supports our hypothesis that the reduced redox status observed by DsbA2 in *E. coli* is due to the efficient interaction of EcDsbD with DsbA2; thereby supporting the structurally differences from Lpn DsbDs.

Also, growth assessment of these genetically altered *E. coli* strains showed attenuation in growth overtime when expressed (Figure 22). Although we did not include data for *E. coli* strain JP114, we observed no difference in *in vitro* growth compared to RGP438. This prompted us to use the virulent DH5α strain as a positive control in the growth assessment assay. It is important to indicate that the growth defect seen by the reconstituted strains may not be solely due to the effect of Lpn DsbDs, but also due to the contributing effect of the necessary antibiotics required for retention of the plasmids or to over expression of recombinant proteins.

In addition, our findings thus raise a lot of questions about localization and structural differences, as other bacteria containing DsbA orthologues, such as pathogenic bacterium *N. meningitidis*, has three DsbA proteins, which differ in terms of localization, redox properties, and surface characteristics (Tinsley *et al*., 2004; Lafaye *et al*., 2008 & 2009). Particularly, in certain bacteria, such as *M. tuberculosis*, DsbA is not re-oxidized by DsbB, but by a membrane protein homologous to eukaryotic vitamin K epoxide reductase (VKOR) (Dutton *et al*., 2008), which support our view that there are many variations and strategies to maintain extracytoplasmic redox associated with management of disulfide bond formation and protein folding.

4.4 Functional activity of homodimeric DsbA2

Our studies suggest that DsbA2 is not a homodimeric DsbA as demonstrated with the *E. coli* DsbC-DsbA chimera. We suggest that key amino acid differences in the *cis*-proline domain (TPA versus VPA in DsbA) similar to DsbC may favor PDI reductase activity. In the insulin reductase assay, DsbA2 and even the DsbA2ΔN monomer exhibited reductase characteristics more similar to those of DsbC than to those of DsbA (Fig. 11). Thus, the DsbA2 lineage appears to be a unique adaptation that combines both oxidase and reductase functions into a single protein *in situ*. To explore the basis for this adaptation, we have replaced the *E. coli dsbB* and *dsbD* genes with alleles from *L. pneumophila*, and we observed that DsbA2 did not interact with either Lpn DsbB1 or Lpn DsbB2, but did interact with both Lpn DsbD1 and Lpn DsbD2 to restore PDI activity. These results suggested that the combined activity of both Lpn DsbB and Lpn DsbD is required for DsbA2 to exist in the mixture of oxidized and reduced forms.

A number of refolding assays have been developed to demonstrate *in vitro* PDI activity (Berkmen *et al*., 2005; Kadokura & Beckwith, 2009; Kadokura & Beckwith, 2010). These assays

often rely on refolding of scrambled enzymes like RNase I that contains consecutive as well as nonconsecutive disulfide bonds to restore RNase activity (Messens *et al*., 2007; Kadokura & Beckwith, 2010). We can speculate that most DSB enzymes are technically bifunctional (oxidase and reductase). For example, EcDsbA is mostly oxidized but contains some reduced forms that do not exhibit PDI ativity. This may suggest that the bifunctional nature of a DSB enzyme depends on the oxidant (s) and reductant (s), respectively. In addition, several mutational analyses and structural studies (Bader *et al.*, 2001; Arredondo *et al.*, 2009) have shown that formal oxidases can be made into isomerases and vice versa, through small point mutations, over-production of an enzyme, or by subtly changing the redox environment. These experiments suggest that while a strict division of oxidase and reductase function is necessary to prevent a futile cycle, in reality there exists a gradient between oxidase and reductases and that enzymes can be coaxed toward one end or the other.

On the other hand, Lpn DsbA2, which is proposed in this study as a naturally bifunctional enzyme, is unique in that while a P198T mutation trapped interacting substrates similar to the *E. coli* DsbA monomer, it is equally reduced and oxidized *in vivo* and phylogentically distinct from DsbA and DsbC. This could suggest that DsbA2 may be a natural scaffold that has been adapted through evoulution to contain both oxidase and PDI activity.

PDI assays can yield mixed results, such as DsbA refolding RNase I (Messens *et al*., 2007). One advantage to the use of the PDI detector MBla for these assays is that the protein does not require denaturation prior to assay and the consecutive disulfide bonds produced are the result of DsbA activity (Ren & Bardwell, 2011). In this folding assay, gain of enzyme activity is readily detected with the chromogenic penicillin substrate nitrocefin. DsbA2, but not DsbA1 efficiently restored the correct nonconsecutive disulfide bond pairing required for protein folding and Bla

activity. The adaptation of this assay to a microplate format should enable development of rapid screens of proteins for PDI activity. In our hands the deep red color of the cleaved β-lactam is easily distinguished by visual observation. We have shown that the DsbA2 protein of *L. pneumophila* is a homodimer and is capable of delivering disulfide bonds to native unfolded proteins or through PDI activity to repair inappropriate disulfide bonds. While the DsbA2 lineage represents a subset of the DsbA family, the dimerization domain and threonine substitution for valine in the *cis*-proline domain are more similar to DsbC and represent unique adaptations that may facilitate or enhance the bifunctional activity of the enzyme.

4.5 Reconstitution of *L. pneumophila dsb* genes in *E. coli* background

We described above that expression of DsbA2 complemented a Δ*dsbC* mutant in a PDI/ reductase detector assay in *E. coli* (Ren and Bardwell, 2011, Kpadeh *et al*., 2013). On the other hand, DsbA2ΔN complemented a *dsbA* mutant in soft agar motility assay in an *E. coli* background (Kpadeh *et al*., 2013). Knowing these two distinct pathways (oxidative and reductive) are independent in *E. coli*, and any cross talk may result in a futile cycle, we suggested that the DsbA2 paradigm in *L. pneumophila* is completely novel. Most importantly, none of the *dsb* genes studied to date in other bacteria have been found to be essential (Heras *et al*., 2009); however, all of the *dsb* genes in *L. pneumophila* seem to be essential except for *dsbA1*. In order to address whether there is any difference in the efficiency of protein interactions between the oxidized DsbA2 and *L. pneumophila* oxidases (DsbB1 and DsbB2) in contributing to the maintenance of disulfide bond folding in the periplasm, we used a surrogate system to address the roles of two DsbB alleles (DsbB1 and DsbB2) in the maintenance of DsbA2 oxidase activity. An interaction assay of *L. pneumophila dsb* genes; *dsbA2ΔN, dsbA1* or *dsbA2* with either *dsbB1* or *dsbB2* in an

E. coli dsb double mutant (Δ*dsbA dsbB*) obtained from James Bardwell showed DsbA1 and DsbA2ΔN, but not DsbA2 interact with both *L. pneumophila* DsbBs to complement motility in the *E. coli* double mutant (Figure 17). Findings from these experiments validated our previous *dsbA* complementation result with DsbA2ΔN (Kpadeh *et al*., 2013), suggesting that DsbA2ΔN partners with DsbB1 and DsbB2 for its oxidative activity; DsbB2 (LidJ) seens to be slightly more efficient. Since DsbA2ΔN does not exist in nature and DsbA2 is oxidized *in vivo* in *L. pneumophila*; we speculated that DsbA2 must interact with *L. pneumophila* DsbBs. However, in the *E. coli* background, EcDsbD efficiently maintained DsbA2 as the free thiol, thus preventing oxidation by DsbBs. We found that by expressing the Lpn DsbDs in *E. coli* that their poor efficiency in reducing DsbA2 permitted interaction and oxidation by Lpn DsbBs in this background. Future studies might examine the structural interactions between DsbBs and DsbA2 to confirm these interactions.

Although we did not investigate all of the possibilities stated above, knowing that DsbA2 contains a N-terminal extension, which is important for DsbA2 to form a homodimer in Lpn that behaves similarly to DsbC (dimer) by displaying protein disulfide isomerase activity in both *in vivo* and *in vitro* assays (Kpadeh *et al*., 2013). We sought to reveal how DsbA2 interactions with the reductants (DsbDs) could contribute to or moderate its interaction with DsbBs *in vivo*. Like DsbC, DsbA2 is a disulfide isomerase that is thought to correct wrongly formed disulfide bonds to produce a functional protein. DsbC is part of the reductive pathway that is linked, via DsbD, to the thioredoxin system of the cytosol (Rietsch *et al*., 1997). DsbC is noted to have an active-site cysteine pair with a redox potential almost as low as that of DsbA (Zapun *et al*., 1993; Wunderlich *et al*., 1993; Zapun *et al*., 1995), and should at least be sufficient to catalyze both disulfide-bond formation and rearrangement in the cell without the need for a specialized oxidant.

However, this was not the case with DsbC as described by (Segatori *et al.*, 2004); even though it contains high oxidation activity *per se*, in the periplasmic space it is maintained exclusively in the reduced state, and therefore can only catalyze disulfide-bond rearrangement. In contrast, we have demonstrated in the lab that periplasmic DsbA2 exists as a mixture of oxidized and reduced redox forms in *L. pneumophila* (Kpadeh *et al.*, 2013), suggesting DsbA2 is able to associate with both Lpn DsbBs and Lpn DsbDs. We think this interaction is solely due to its moderate interaction with Lpn DsbDs, which are divergent to *E. coli* DsbD, which fully reduced DsbA2 when *E. coli* DsbB is present and not Lpn DsbBs.

DsbD consists of three domains: a N-terminal periplasmic domain (DsbDα), a C-terminal periplasmic domain (DsbDγ), and an intervening hydrophobic membrane-embedded domain (DsbDβ) (see Figs. 35 & 36). Each of the three domains contains two redox-active cysteines, essential for the protein's function (Stewart *et al.*, 1999). DsbD homologues are found in many other bacteria (Katzen et al., 2002). In *E. coli*, enzymes including DsbC, a protein that is involved in disulfide bond isomerization, DsbG, which restores the reduced form of cysteines that have been oxidized to sulfenic acid (Depuydt *et al.*, 2009), and CcmG, which reduces the oxidized cysteines of apocytochrome *c* so as to allow heme binding (Metheringham *et al.*, 1995; Fabianek *et al.*, 1998) derive their reducing power from cytoplasmic thioredoxin via the cytoplasmic membrane protein DsbD (Fig. 36). However, many bacteria including *L. pneumophila*, express protein homologues that comprise the cytoplasmic, intervening hydrophobic membrane-embedded and a N-terminal periplasmic domains, or only the cytoplasmic domain of DsbD as reported for CcdA of Rhodobacter capsulatus which reduces proteins that are homologues of CcmG (Deshmukh *et al.*, 2000; Katzen *et al.*, 2002). It seems like electrons transfer from DsbDα is enough to reduce its substrates (Gupta *et al.*, 1997; Katzen & Beckwith, 2000; Collet *et al*.

2002; Haebel *et al*., 2002; Stirnimann *et al*, 2005). Bioinformatic analyses of Lpn DsbDs, in this study reveal that Lpn DsbD1 N-terminal periplasmic domain is more similar to the *E. coli* DsbD (Fig. 35) than to the *Salmonella Typhimurium* encoded homologue of DsbD, *scsB* (Gupta *et al*., 1997; Cho *et al*., 2012), a newly identified distinct class which comprises DsbD homologues with quite different α domains. However, Lpn DsbD2 lacks the N-termial Nα domain (Fig. 35, bottom right) found in DsbD1 that in *E. coli* delivers the reducing equivalents to DsbC. DsbD2 does share domain organization with the β and γ domains as EcDsbD. A further analysis of Lpn DsbD1 reveals the protein to contain several additional cysteine residues, which compared to EcDsbD could conceivably alter disulfide bonding or other structural changes that would likely influence catalytic efficiency with DsbA2. In summary, these results indicate DsbDs of *L. pneumophila* are divergent from EcDsbD structurally and functional, and DsbA2 has to moderately interact with both Lpn DsbBs and Lpn DsbDs in order to exhibit a bifunctional activity *in vivo*.
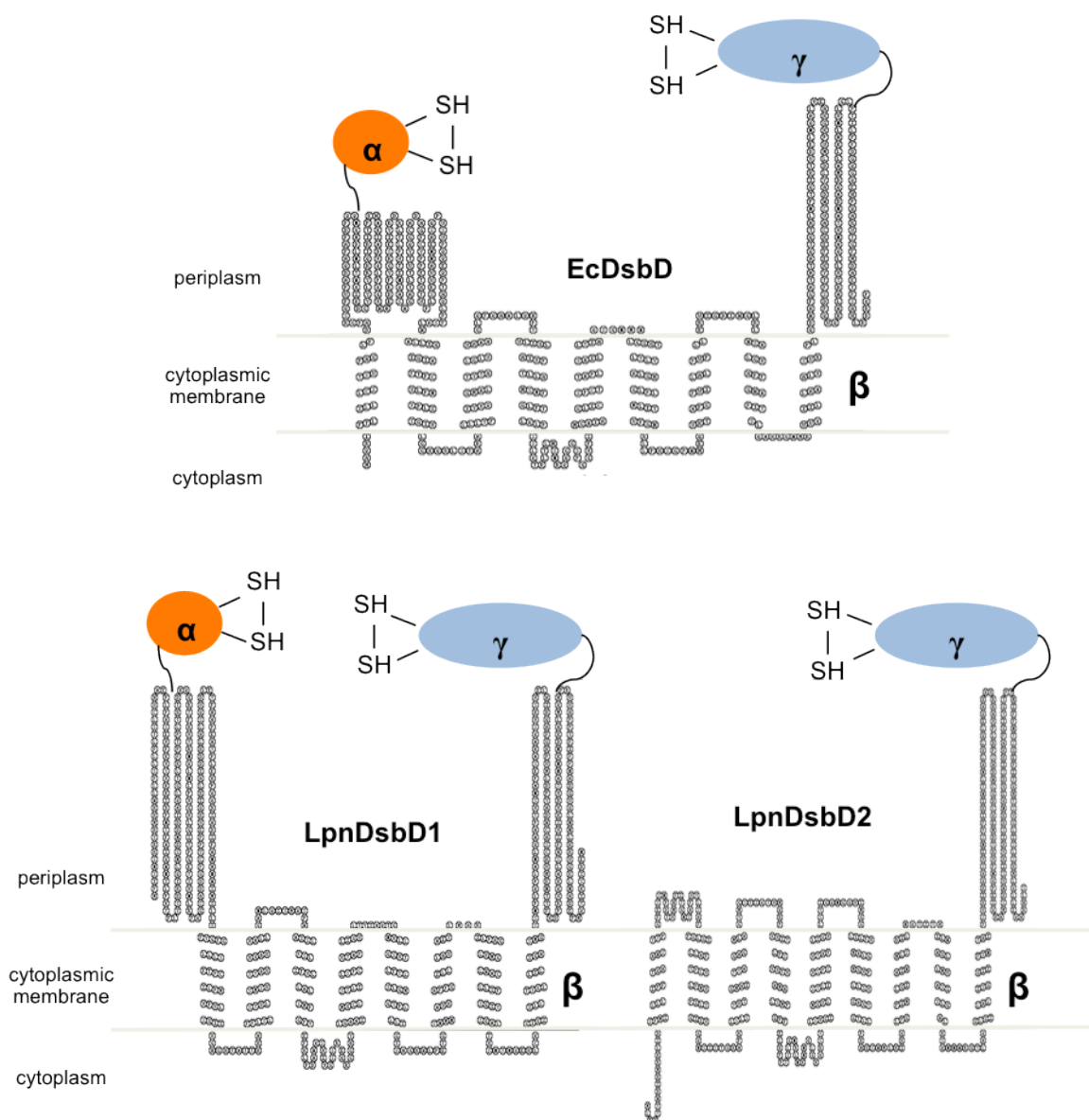
Figure 35. DsbD topological models of *E. coli* DsbD and Lpn DsbDs. Comparisons between Ec DsbD and Lpn DsbDs depicting the α, β, and γ domains. Lpn DsbD2 lacks the Nα domain as compared to EcDsbD and Lpn DsbD1.
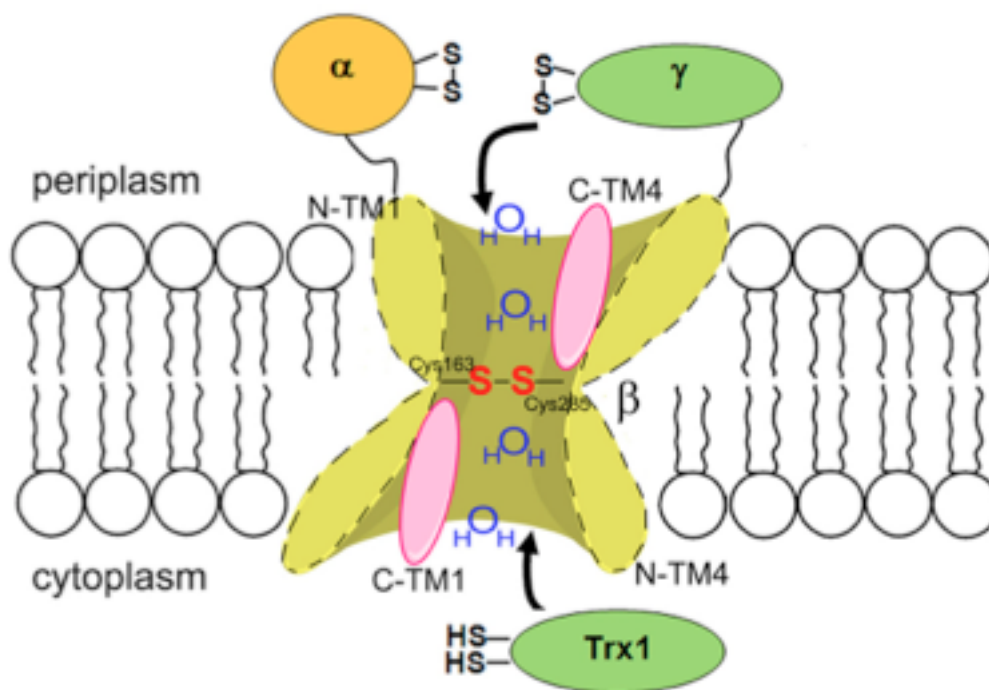
Figure 36. Structural model of *E. coli* DsbD. The model above shows that DsbDβ adopts an hourglass-like structure where Cys163 and Cys285 are in the middle of the two water-exposed cavities. Halves of C-terminal TM1 and TM4 (C-TM1 and C-TM4) are water exposed (pink), while those of N-terminal ones (N-TM1 and N-TM4) are not. Two thioredoxin modules (thioredoxin-1 and DsbDγ) may interact with Cys163 and Cys285 through these two water-exposed cavities. S, sulfur of thiol in a cysteine residue. It is noteworthy that Lpn DsbD2 is missing the α redox arm believed to directly reduce DsbA2. Adapted from Cho *et al*., 2012.

4.6 *In vivo* characterization of DsbA2 forms for virulence in *L. pneumophila*

In addition, we characterized the monomer form of oxidoreductase DsbA2, DsbA2ΔN, and its contributing role to the bifunctional activity of DsbA2 for virulence in the periplasm of *L. pneumophila*. Consistent with DsbA-like activity, a DsbA2 *cis*-proline (P198T) mutant protein formed stable disulfide cross-linked complexes with substrate proteins, but not with its dedicated oxidant DsbB (Kadokura *et al*., 2004; Jameson-Lee *et al*, 2011), and further exhibited a dominant negative effect that was due to interference with either assembly or function of the Dot/Icm T4SS apparatus (Jameson-Lee *et al*., 2011). DsbA2 was reported in (Jameson-Lee *et al*., 2011) to capture some important substrates including, Dot/Icm T4SS core structural proteins DotC, DotG, DotK and IcmX, the periplasmic and surface-localized invasin HtpB (Hsp60), and its co-chaperone HtpA (Hsp10), and subunits of Omps of the MOMP complex. It is also important to note that other Dot/Icm proteins that do not contain cysteine residues such as DotD, DotF, IcmW and IcmY, or cytoplasmic proteins that do contain cysteine residues were not captured by DsbA2 P198T mutant protein. The Dot/Icm proteins captured through interaction with P198T muntant were considered essential for assembly and function of the T4SS. Also, *L. pneumophila* DsbA1 has been reported to be dispensable for growth, motility, and infectivity in cell-based assays and this gene is naturally absent from the genome of *C. burnetti*, which also express *dsbB* and *dsbD* genes, while DsbA2 is essential for virulence (Jameson-Lee *et al*., 2011; Kpadeh *et al*., 2013).

In order to characterize the importance of the monomer form of DsbA2 on pathogenesis, we utilized DsbA2ΔN as a tool to assess this effect by constructing a P198T mutation in DsbA2ΔN from (Kpadeh *et al*., 2013) to overcome the apparent essentiality of DsbA2. We examined DsbA2ΔN P198T mutant *in vitro* and by protein expression on SDS-PAGE and immunoblotting with anti-DsbA2 serum, and no defect in growth was seen. However, we show

here that expression of the DsbA2ΔN P198T mutant protein in *L. pneumophila* strain AA100 produced a dominant-negative effect on DsbA2 function resulting to several virulence phenotypes including: (i) defect of intracellular multiplication in amoeba, (ii) a significant defect in attaching and invading HeLa cells similarly to DsbA2 P198T mutant in (Jameson-Lee *et al.*, 2011), and (iii) a greater defect in contact dependent hemolysis of human erythrocytes, all of which suggest the mutant has an incomplete and non-functional Dot/Icm T4SS.

Although amoeba has been one of the most recognized model hosts for evaluating *L. pneumophila* infection and growth intracellularly, our amoeba results led us to further question whether the duration of the bacteria in contact with amoeba has any effect on the results generated. To eliminate any factors that may skew the analysis of the infectivity results, we infected non-phagocytic HeLa cells with our mutant to investigate the virulence of oxidized DsbA2 *in vivo*. HeLa cell is an established model system to study the invasiveness and biology of *L. pneumophila* (Garduno *et al.*, 1998a & Garduno *et al.*, 1998c). We utilized this model to examine adherence and invasion; virulent strains of *Legionella* are adherent and invasive while nonvirulent strains are adherent but poorly invasive. Invasion is reported to be rapid and does not require *de novo* bacterial protein synthesis (Garduno *et al.*, 1998a), suggesting that the factors necessary for invasion, which require a properly assembled Dot/Icm T4SS apparatus for their translocation, have to be constitutively expressed by virulent strains. Importantly, in addition to the capture of Dot/Icm components by Jameson-Lee *et al.* (2011), a wide range or substrate proteins were captured, including enhanced entry proteins (Ehn) that are required for entry into amoeba (Liu *et al.*, 2008), and HtpB (Hsp60), which facilitates attachment and invasion of non-phagocytic cells (Garduno *et al.*, 1998a&b). Perhaps the reduced attachment and invasion

observed with monomeric DsbA2 P198T mutant protein might be due to impairment of Ehn and/or HtpB function (s), respectively.

Due to similar activity assessed in DsbA2 P198T mutant, these results suggest that DsbA2ΔN P198T interfered with the function of DsbA2, and resulted in a disruption in the Dot/Icm T4SS complex. In addition, these results indicate that Lpn is dependent on the oxidized form of DsbA2 for the proper folding of periplasmic proteins, including those destined for the assembly into macromolecular structures.

4.7 Why is DsbA1 maintained in *L. pneumophila*?

In many pathogenic bacteria, DsbA-like proteins are important for assembly of virulence factors and for secretion systems that are dependent on disulfide bond formation (Heras *et al*., 2009). However, previous reports had shown no phenotype for Lpn *dsbA1* mutant (Jameson-Lee *et al*., 2011). This result was not concerning at first since *dsbA1* is absent in *Coxiella*, the most closely related bacteria to *Legionella*. Since DsbA1 showed enzyme activity in the insulin precipitation assay, and was more oxidizing than reducing similar to EcDsbA, we reexamined the biology of the Lpn *dsbA1* mutant. To confirm the role of DsbA1 in the DSB system of *L. pneumophila*, we closely evaluated any difference in *in vitro* broth culture AA100 WT and Δ*dsbA1* constructs. Interestingly, we detected a defect in pigment production in strains lacking *dsbA1*. We also examined the ability for Δ*dsbA1* mutant to grow in regular or cys and iron limited BYE media, and no growth difference was detected compared to AA100 WT, suggesting that the defect in pigment production is not contributed by cys or iron acquisition. Pigment production such as melanin is important in microbial pathogenesis because it has been associated with virulence in many microorganisms, and is linked to protection against environmental insults

(Nosanchuk and Casadevall, 2003). Therefore, the lack of dark-brownish pigment observed following overnight growth in broth might suggest that DsbA1 interacts with protein(s) that are important for modulating the virulence of Lpn. Because DsbA1 appears to have a phenotypic difference from wild type, we wanted to more definitively elucidate the functional activity of DsbA1 for virulence in an infectivity assay. Using L929 cells, we performed plaque assay using different constructs of *Legionella* AA100 strains including *dsbA1* knockout. In these studies, the Δ*dsbA1* mutant demonstrated a hyper-virulent phenotype that disappeared upon complementation with WT DsbA1, indicating a modulating role for *Legionella* virulence. In further studies utilizing contact-dependent lysis of human erythrocytes, we were able to validate this hypervirulent phenotype demonstrated in L929 cells. All together, the defect in pigment production and the hypervirulent phenotype illustrated *in vivo* define a role for DsbA1 in Lpn. Noteworthy, this is the first report indicating an important role of DsbA1 in *L. pneumophila*.

Furthermore, protein capture assays indicated that DsbA1 interacts with fewer substrates in the periplasm of *L. pneumophila* compared to expression in *E. coli*. Knowing that capture studies has been very important for the characterization of oxidase function of DsbA in other pathogens, and DsbA2 in *L. pneumophila* (Jameson-Lee et al, 2011), we can suggest that DsbA1 is not evolutionarily selected against because it contributes to disulfide bond formation in the periplasm. Lastly, we successfully crystalized DsbA1, and comparison analysis of Lpn DsbA1, EcDsbA and EcDsbC showed that Lpn DsbA1 is more similar to EcDsbA structurally than EcDsbC. We concluded from these studies that although DsbA1 may not be as directly involved in protein folding associated with virulence traits. Our studies support a role, perhaps through interference with DsbA2 function, as a modulator of virulence in *L. pneumophila*.

Chapter 5. Conclusion

Our studies contribute new functional knowledge on the biology of the DsbA2 system, a novel class of DSB oxidoreductase common to nearly all bacteria expressing T4SSs and many that do not. We have identified fundamental differences between the *L. pneumophila* DsbA2 system and the DsbA/DsbC system of *E. coli* (Fig. 21). These studies have also revealed that both redox forms of DsbA2 are most important and essential − the oxidase or the PDI reductase activity for Dot/Icm T4SS proper assembly and function. In addition, we have reported here that there is considerable diversity in DSB proteins and their biology, which our work has stimulated the possibility to explore these systems in other bacteria contributing to macromolecular assembly and function of T4SSs. The redundancy of *dsb* genes (*dsbB1, dsbB2, dsbD1* and *dsbD2*) in *L. pneumophila* may provide a cumulative advantage, each contributing to the global quantity of a protein involved in a specific cellular process (Thomas, 1993). It is highly likely that under certain growth conditions, the presence of a single *dsbB gene* or *dsbD gene* is not sufficient to drive disulfide bond formation and that efficient oxidative folding requires the presence of both *dsbB*s or *dsbD*s. Alternatively, possessing two *dsbB* or *dsbD* genes may allow a broader spectrum of regulation than a single one, the expression of two oxidant/redundant operons may be differently modulated according to the environmental conditions, which would *de facto* give rise to more individualized functions for these two genes.

We also find it interesting that DsbA2 related proteins are present in a number of bacterial species instead of the DsbA/DsbC system as a means to manage extracytoplasmic disulfide bonding and protein folding (e.g. *Coxiella burnetti* which only contains DsbA2 and lacks both DsbA1 and DsbC). This genetically diverse group also includes most intracellular parasites that express T4SS, all of which grow very slowly compared to *E. coli*. Perhaps DsbA2 represents an

evolutionary adaptation that is more efficient in orchestrating disulfide bond formation and protein folding that suits the lifestyles of slow-growing bacteria. Finally, we speculate *dsbA2* mutants can be used to probe how Dot/Icm system becomes activated following contact with a host cell, thus making a significant impact in the field of microbiology and microbial pathogenesis. Thus, targeting of disulfide bond formation pathways appears to be an attractive approach for the development of antimicrobials and anti-virulence agents. The future may yield inhibitors of these pathways of both basic and applied interest as some reports in literature have begun to address this concept (Shouldice *et al*, 2010b; Fruh *et al*., 2010). With these ideas in mind, further investigations will surely reveal the roles of DsbA2 for the proper folding of targeted-captured substrates, and essentiality during environmental survival and pathogenesis in humans.

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