DsbA2 (27-kDa Com1-Like Protein) of *Legionella pneumophila* Catalyses Extracytoplasmic Disulfide-Bond Formation in Proteins Including the Dot/Icm Type IV Secretion System

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Summary

In Gram negative bacteria, thiol oxidoreductases catalyze the formation of disulfide bonds (DSB) in extracytoplasmic proteins. In this study, we sought to identify DSB-forming proteins required for assembly of macromolecular structures in *Legionella pneumophila*. Here we describe two DSB forming proteins, one annotated as *dsbA1* and the other annotated as a 27-kDa outer membrane protein similar to Com1 of *Coxiella burnetii*, which we designate as *dsbA2*. Both proteins are predicted to be periplasmic, and while *dsbA1* mutants were readily isolated and without phenotype, *dsbA2* mutants were not obtained. To advance studies of DsbA2, a *cis*-proline residue at position 198 was replaced with threonine that enables formation of stable disulfide-bond complexes with substrate proteins. Expression of DsbA2 P198T-mutant protein from an inducible promoter produced dominant-negative effects on DsbA2 function that resulted in loss of infectivity for amoeba and HeLa cells and loss of Dot/Icm T4SS-mediated contact hemolysis of erythrocytes. Analysis of captured DsbA2 P198T-substrate complexes from *L. pneumophila* by mass spectrometry identified periplasmic and outer membrane proteins that included components of the Dot/Icm T4SS. More broadly, our studies establish a DSB oxidoreductase function for the Com1 lineage of DsbA2-like proteins which appear to be conserved among those bacteria also expressing T4SS.

Introduction

Legionella pneumophila, the etiological agent of Legionnaires' disease (McDade *et al*., 1977), resides in aquatic environments as an obligate intracellular pathogen of amoebic hosts (Fliermans *et al*., 1981; Rowbotham, 1980; Fields, 1996). Within these hosts, *L. pneumophila* displays a dimorphic life cycle, alternating between intracellular vegetative replicating forms (RFs) and extracellular planktonic non-replicating cysts that are metabolically dormant, resilient, and highly infectious (Garduno *et al*., 2002). The ability to differentiate appears to be a fundamental survival characteristic of this species which can also be activated in RFs upon: (i) suspension in water (Garduno *et al*., 2002; and Hiltz *et al*., 2004); (ii) ingestion by inappropriate hosts such as ciliates (Berk *et al*., 2008, Faulkner *et al*., 2008); or (iii) within the gut of nematodes (Brassinga *et al*., 2010). Ultrastructural studies of cysts reveal extensive remodeling of the typical Gram negative envelope to a

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complex thickened cell wall overlaying laminations of intracytoplasmic membranes (Faulkner *et al*., 2002, Garduno *et al*., 2002). Comparative proteomic analyses of these forms identified 30 proteins that were more abundant in the cyst than in stationary phase *in vitro*-grown bacteria, including MagA, which is a marker of differentiation that is located in a genomic island (Brassinga *et al*., 2003; Hiltz *et al*., 2004). Consistent with remodeling of the cell envelope were changes in the pattern of outer membrane proteins and enrichment of the HtpB invasin on the cyst surface (Garduno *et al*., 1998a; Garduno *et al*., 1998c; Garduno *et al*., 2002). The molecular mechanisms associated with the extensive remodeling of the cell envelope during morphogenesis are poorly understood.

Other macromolecular complexes associated with virulence traits of *L. pneumophila* include components of the Dot/Icm type IV secretion system (T4SS) (Berger and Isberg, 1993; Brand *et al*., 1994), whose secreted effectors are enriched for post-exponentially (Franco *et al*., 2009) as are components of the flagellar system (Byrne and Swanson, 1998; Albert-Weissenberger *et al*., 2010). In addition, the most abundant protein in the outer membrane of *L. pneumophila* (OmpS) is cross-linked by inter-chain disulfide bonds, forming high molecular weight complexes that are covalently anchored to the underlying peptidoglycan (Butler and Hoffman, 1990). The OmpS disulfide-bonds are susceptible to the action of strong reducing agents (sensitive to alkylation by iodoacetamide) and can reform following removal of the reducing agent with no deleterious effect on viability (Butler *et al*., 1985; Butler and Hoffman, 1990). Proteins associated with assembly of OmpS monomers into multimeric complexes or with maintenance of disulfide bonds among the subunits are not known.

In *E. coli* and other Gram negative bacteria, the extracytoplasmic formation of disulfide bonds in proteins is catalyzed by disulfide bond oxidoreductases such as DsbA (Bardwell *et al*., 1991; Heras *et al*., 2009). DsbA-catalyzed disulfide bond formation is required for activation of enzymes such as alkaline phosphatase (Bardwell *et al*., 1991), for the assembly of secreted virulence factors such as pertussis and cholera toxins (Peek and Taylor, 1992; Stenson and Weiss, 2002) and for the assembly of fimbrial adhesins and components of flagella motors (Peek and Taylor, 1992; Zhang and Donnenburg, 1996; Dailey and Berg, 1993). DSB oxidoreductases of the DsbA type introduce disulfide bonds in periplasmic proteins while DSB isomerases, represented by DsbC enzymes, reduce inappropriately formed disulfides to facilitate proper oxidative folding of proteins (Heras *et al*., 2009). While members of the *dsbA* family are not essential for viability, their function is required for virulence in many bacteria (Heras *et al*., 2009; Kadokura and Beckwtih, 2010). In *E. coli*, DsbA is re-oxidized by membrane spanning DsbB that is part of the electron transport chain, while DsbC is reduced by membrane spanning DsbD which in turn is reduced by cytoplasmic thioredoxin reductase (Heras *et. al*., 2009; Kadokura and Beckwith, 2010). Analysis of whole genomic sequences of *L. pneumophila* strains reveals DSB orthologs including: DsbA, DsbB, DsbD, and a gene involved with cytochrome *c* biogenesis termed DsbE, but no orthologues of DsbC or DsbG. DsbE and DsbD were discovered in virulence and differential essentiality screens (Conover *et al*., 2003; Viswanathan *et al*., 2002), but only DsbE has been extensively studied (Viswanathan *et al*., 2002). The role of redox-active disulfide bond oxidoreductases and isomerases in the assembly and activation of extracytoplasmic proteins associated with differentiation and virulence have not been examined in *L. pneumophila*.

In this report we explore the role of periplasmic DsbA-like oxidoreductases in assembly of outer membrane proteins and extracytoplasmic virulence factors. Our studies will show that deletion of *dsbA* (*dsbA1*) previously annotated in *L. pneumophila* (absent in the genome of close relative *C. burnetii*), retains both motility and infectivity for amoeba and cell-based models, and otherwise exhibits no quantifiable phenotypes. A search for other *dsbA*-like

genes identified a gene encoding a 27 kilodalton outer membrane protein of unknown function related to <u>Co</u>xiella <u>o</u>uter <u>m</u>embrane protein 1 (Com1) of *C. burnetii* that contained a CXXC motif typical of members of the thioredoxin superfamily. Based on demonstrated oxidoreductase activity, cellular location and protein structural criteria, we named the protein DsbA2. Unlike *dsbA1*, *dsbA2* appeared to be essential for viability.

To advance functional studies of DsbA2 and based on related studies of DsbA in *E. coli* (Kadokura *et al*., 2004; Kadokura *et al*., 2005), we expressed a DsbA2 P198T *cis*-proline mutant protein in *L. pneumophila* which exhibited a dominant negative effect on DsbA2 function. By exploiting this dominant negative phenotype, we provide evidence to support a role for DsbA2 in the proper assembly and function of the Dot/Icm T4SS as well as for motility. Our studies not only assign a biological function to the 27-kDa Com1 family of proteins as disulfide bond oxidoreductases, but suggest from phylogenetic analysis that the DsbA2 linage is conserved among those bacteria, with a few exceptions (*Bordetella* spp. and *Helicobacter pylori*), expressing T4SS.

Results

Genetic analysis of DsbA alleles

Analysis of the sequenced genomes of *L. pneumophila* strains revealed an orthologue of the *dsbA* gene of *E. coli* (*lpg0123* in Philadelphia-1 strain) (see Fig. 1A). A deletion mutant was constructed by vector-free allelic replacement mutagenesis (Chalker *et al*., 2001) with a *Gent^r* cassette in *L. pneumophila* strain 130b (AA100Δ*dsbA1*). This mutant was indistinguishable from wild type strain AA100 by growth *in vitro*, and in an *Acanthamoeba castellanii* infection model. Since loss of motility is a common phenotype of *dsbA* mutants (Dailey and Berg, 1993), we examined stationary phase bacteria for motility by wet mount as described by Swanson (Byrne and Swanson, 1998). In contrast to *dsbA* mutants of *E. coli*, the AA100Δ*dsbA1* mutant exhibited no defect in motility. Our studies, while not exhaustive, found no phenotypes attributable to the *dsbA1* mutant and further studies of this mutant were not pursued.

Since it is not uncommon for bacteria to contain multiple *dsbA* alleles (Tinsley *et. al*., 2004; Jarrot *et. al*., 2010), we re-examined the genomes of *L. pneumophila* strains for related genes. A PSI BLAST search for proteins containing the conserved CXXC thioredoxin fold common to members of the DsbA family identified a gene encoding a 27- kDa outer membrane protein (*lpg1841* in Philadelphia-1) that is closely related to the Com1 (<u>Co</u>xiella <u>o</u>uter <u>m</u>embrane) protein of *C. burnetii* (Hendrix *et al*., 1993) as a potential DsbA candidate. As seen in Fig 1A, the Com1-like protein exhibited little similarity in amino acid sequence with LpDsbA1 and EcDsbA proteins, but showed alignments of key residues in the CXXC thioredoxin motif and in a *cis* conformation proline region that are common to and required for DsbA function (Kadokura *et al*., 2004; Kadokura and Beckwith, 2010). These functional alignments were also observed with Phyre software by threading Com1onto the EcDsbA crystal structure (data not shown). Interestingly, the Com1 protein of *L. pneumophila* had been previously identified in a 2D gel proteomic screen of proteins enriched for in differentiated cyst forms (compared to stationary phase forms) of *L. pneumophila* (Garduno *et al*., 2002). Phylogenetic analysis (see Fig. 1B) indicated that Lpg1841 was more closely related to a family of Com1/DsbA-like proteins found in *Agrobacterium tumifaciens*, *Francisella tularensis*, *C. burnetii*, *Rickettsia sp*, and other bacteria than to DsbA of *E. coli*. Based on structural alignments of key functional elements with the DsbA of *E. coli*, we annotated Lpg1841 as DsbA2. It is noteworthy that, with the exception of *Bordetella spp*. and *Helicobacter pylori*, the DsbA2 lineage appears to be conserved in, but limited to, those bacteria expressing T4SS.

DsbA2 functional activity

To test for thiol oxidoreductase activity, recombinant His$_6$-tagged DsbA2 was purified from *E. coli* by Ni-interaction chromatography. In an insulin-based precipitation assay in the presence of dithiothreitol, recombinant DsbA2 accelerated the reduction disulfide bonds recorded spectrophotometrically as an increase in precipitation of insoluble insulin subunits with time (Holmgren, 1979) (see Fig. 1C). Thioredoxin (positive control) also catalyzed the reduction of disulfide bonds in insulin. While DsbA2 exhibits disulfide bond oxidoreductase activity, this assay does not distinguish between oxidase and reductase function.

dsbA2 appears to be essential

In *L. pneumophila*, *dsbA2* is flanked by *uvrD* (upstream) and *glyS* (downstream). Promoter elements identified in the intergenic region suggest that *dsbA2* is likely transcribed independently of *uvrD*. Using a vector-free allelic-replacement mutagenic strategy as described for producing deletions of *dsbA1*, we set out to delete *dsbA2*. However, we were unable to isolate *dsbA2* deletion mutants in either AA100 or Lp02 genetic backgrounds. A second strategy involved cloning of flaking regions of *dsbA2* (replaced with kanamycin cassette) into suicide vector pRDX (Morash *et al*., 2009) and following electroporation into Lp02 or AA100 strains, the bacteria were plated for selection on Km-containing BCYE medium. Selected Km^r colonies were scored for resistance to metronidazole and susceptibility to chloramphenicol that indicate loss of vector and allelic replacement. No colonies displayed the correct phenotypes indicating that allelic replacement had not occurred. We were, however, able to introduce a gentamicin cassette downstream of the coding sequence of *dsbA2*, diminishing the possibility of polar effects on the expression of downstream genes (see Fig. 1D). Moreover, attempts at trans-complementation of a genomic deletion with a plasmid-borne copy of *dsbA2* were also unsuccessful, consistent with our previous experience with both essential genes (LeBlanc *et al*. 2008, Hoffman *et al*., 1992b) and genes that become conditionally lethal in certain mutant backgrounds (LeBlanc *et al*., 2006). Additionally, since some Dot/Icm mutants display conditional lethality depending on functional status of Dot/Icm system (Conover *et al*., 2003), we also attempted to construct a *dsbA2* knockout mutation in a *dotA* mutant, without success. Taken together, these studies suggest that DsbA2 provides an essential function in *L. pneumophila*; whereas, DsbA1 is nonessential and without phenotype. It is also noteworthy that an orthologue of DsbA1 is absent from the genome of close relative *C. burnetii*.

DsbA2 is not an outer membrane protein

DsbA2 is annotated in databases as a 27-kDa protein of unknown function, based on a prior report that Com1 of *C. burnetii* is an immunodominant outer membrane protein (Hendrix, *et al*., 1993). Since DsbA-like proteins typically reside in the periplasm of Gram negative bacteria (Tinsley *et al*., 2004) we next investigated its cellular location. *In silico* analysis of the primary amino acid sequence of DsbA2 identified a sec-dependent leader sequence typical of DsbA proteins (PSORTb v3.0) (Yu *et al*., 2010) and predicted with high confidence a periplasmic location (score of 9.84 and similar to that predicted for DsbA1). To confirm a periplasmic location, whole cell extracts were subjected to cellular fractionation protocols routinely used to localize proteins in *L. pneumophila* (Butler *et al*., 1985, Lema *et al*., 1988, Matthews and Roy, 2000; Vincent *et al*., 2006). To identify DsbA2 in these fractions, monospecific mouse polyclonal antibodies raised against purified His$_6$-tagged DsbA2 protein (no cross-reactivity with DsbA1) was used. As a control, antibodies against periplasmic protein LepB, cytosolic protein IHF, and outer membrane protein OmpS were used (Butler *et al*., 1985; Vincent *et al*., 2006; Morash *et al*., 2009). As depicted in Fig. 2A, DsbA2 was distributed between the soluble fraction (Lane S) (cytoplasmic and periplasmic proteins) and in the Triton-soluble fraction (Lane E: inner membrane), but was absent in the Triton-insoluble fraction (Lane N: outer membrane). As a control for the cytoplasmic

fraction, IHF was exclusive to the soluble fraction whereas LepB, a periplasmic protein was associated with the Triton-soluble fraction. The major outer membrane protein OmpS was present in the Triton-soluble as well as the Triton-insoluble fractions as noted previously (Vincent *et al*., 2006). If DsbA2 were a lipoprotein, it would have been located in the Triton-soluble fraction as was LepB. Our studies therefore support a periplasmic location for DsbA2.

To confirm that DsbA2 is not an outer membrane protein, immunogold electron microscopy of ultra-thin sections of *L. pneumophila* were analyzed. As seen in Fig. 2B panel 1, sections probed with DsbA2-specific antibodies indicated that gold particles (10 nm gold conjugated to goat anti-mouse or rabbit immunoglobulin) were predominantly periplasmic, residing there 63% of the time with a 95% confidence interval based on the method of Mallette (Mallette, 1969) and previously validated as described (Garduno *et al*. 1998c). As seen in Fig. 2B, panel 2, immunogold labeling with OmpS-specific antibodies shows gold particles associated with the outer membrane. As a further test for whether DsbA2 localizes to the bacterial surface, whole cell surface labeling with DsbA2-specific antibody (panel 3) or OmpS-specific antibody (panel 4) indicated that DsbA2 is not located on the surface of *L. pneumophila*. These results, together with the cellular localization studies, establish that DsbA2 is located in the periplasm and not in the outer membrane.

Redox status of periplasmic DsbA2

We next assessed the redox status of periplasmic DsbA2 by AMS alkylation and immunoblot with whole cells of *L. pneumophila* collected in early stationary phase. DsbA proteins are typically maintained as the disulfide by DsbB which oxidizes reduced DsbA and is itself oxidized by the electron transport chain (Kadokura and Beckwith, 2010). As seen in Fig. S1, lane 1, AMS fully alkylated DsbA2 in cells pretreated with 10 mM DTT to reduce all disulfide bonds. Lane 2 depicts untreated DsbA2 that runs at a lower molecular weight (non-alkylated). AMS treatment of whole cells (Lane 3) revealed that DsbA2 redox status is mixed (60% thiol and 40% disulfide), similar to the *E. coli* DsbA where the oxidized form is slightly favored (Kadokura et al., 2004). While many factors likely influence the relative redox status of periplasmic proteins, our findings are consistent with an oxidase function.

DsbA2 dominant negative mutants inhibit intracellular replication

Advancing functional studies of essential genes is often dependent on genetic systems that can control promoter function so that phenotypic changes can be correlated with levels of gene expression (Msadek, 2009). Alternatively, dominant negative phenotypes can sometimes be produced through mutations that alter function of the gene product in ways that interfere with the function of the native protein. We noted from *in silico* structural analysis of DsbA2 that the thioredoxin fold and a key *cis*-proline residue near the CXXC domain were highly conserved. We reasoned that it might be possible to create similar functional mutations in DsbA2 based on analogous mutant constructs in DsbA of *E. coli* (Kadokura *et al*., 2004 and Kadokura *et al*., 2005) and in thioredoxin (Motohashi *et al*., 2001). In this regard, site directed mutagenesis was used to change the second cysteine residue in the active CXXC site to serine (C89S), which was used to capture substrate proteins by thioredoxin (Motohashi *et al*., 2001) and by DsbG in *E. coli* (Depuydt *et al*., 2009). This mutation renders the protein incapable of catalyzing oxidative protein folding. A second mutation in DsbA proteins changes a conserved *cis*-proline residue at position 198 in DsbA2 to threonine (P198T) (see Fig. S2). P198T is predicted to stabilize disulfide bonds between enzyme and protein substrates (Kadokura *et al*., 2004), but not with its dedicated oxidant DsbB.

To assess whether expression of C89S or P198T mutant proteins in *L. pneumophila* strain AA100 were deleterious for growth, the genes encoding C89S and P198T mutant proteins were cloned into the pMMB206 vector that enables control of gene expression by IPTG (Morales et al., 1991). *In vitro* growth characteristics in BYE broth over a range of IPTG concentrations were monitored. As seen in Fig. S3A, expression of these mutant proteins resulted in no deleterious effects on bacterial growth. To confirm expression of C89S and P198T mutant proteins during *in vitro* growth, whole cell fractions grown with and without IPTG (0, 0.5, and 5 mM) were analyzed by SDS-PAGE and immunoblot with DsbA2-specific antibody. As seen in Fig. S3B, DsbA2 protein levels increased over time with increasing IPTG concentration as compared with un-induced controls. It is noteworthy, that at 10 h post induction, a doublet can be observed in the P198T panels with the upper band attributed to the recombinant his-tagged protein (Fig. S3B). C89S mutant protein does not contain a His_6-tag. These studies established that over expression of these proteins in *L. pneumophila* had no measurable effects on *in vitro* growth.

To test whether expression of C89S or P198T mutant proteins might affect periplasmic functions related to virulence, AA100 strains expressing these constructs were pre-induced with 0, 0.5 and 5 mM IPTG and used to infect amoeba. IPTG levels were maintained throughout the infection. As seen in Fig. 3A (un-induced), intracellular growth rates for the C89S and P198T mutant strains and controls (pMMB:*dsbA2* and the empty vector), when adjusted for CFU differences at zero time, were not significant. Upon induction with 0.5 mM IPTG (Fig. 3B), the growth rates for the over-expressing wild type (pMMB::*dsbA2*) and C89S expressing strains were unchanged when compared to the uninduced rates depicted in Fig. 3A. However, the strain expressing the P198T mutant protein exhibited a measurable growth defect out to 48 h post infection. At 5 mM IPTG, the growth defect for the P198T mutant was much more pronounced; whereas, growth rates for strains over-expressing the C89S mutant protein and wild type DsbA2 protein were unchanged when compared with un-induced growth rates (see Fig. 3C). To confirm that mutant proteins were expressed, especially with the C89S mutant, cellular extracts from infected amoeba were analyzed by non-reducing SDS-PAGE and immunoblot. As depicted in Fig. 3D, DsbA2 protein levels were detected in all extracts except for the P198T mutant (5 mM IPTG) lane 4, due to the absence of bacteria in the amoebae. Comparison of protein levels for DsbA2 in lanes 1 and 2 with the vector control (genomic expression of DsbA2) in lane 3 are consistent with IPTG induced over expression. Immuno-reactive bands noted at molecular masses higher than that of DsbA2 for strains over-expressing DsbA2 (lane 1) and particularly for the C89S mutant protein (lane 2; major complexes denoted by asterisks) likely represent captured protein substrates. These experiments demonstrate that over-expression of the P198T mutant protein, but not C89S mutant protein, resulted in a dominant negative effect on intracellular multiplication, supporting a role for DsbA2 function in *Legionella* pathogenesis.

DsbA2 P198T expression affects invasion of HeLa cells

Since amoebae are naturally phagocytic, it was not possible to distinguish whether the dominant negative effect on intracellular growth was due to defects in primary attachment or in invasion. To address these possibilities a HeLa cell invasion model was used (Garduno *et al*., 1998b; McCusker *et al*., 1991; Newton *et al*., 2008). As seen in Fig. 4A and 4B, *L. pneumophila* strain AA100 pMMB:empty vector under inducing conditions readily attached to and invaded HeLa cells, while the P198T-expressing mutant exhibited a 60% reduction in attachment efficiency and nearly 90% decrease in internalization. These findings are consistent with previous reports that multiple surface proteins mediate attachment, while only Hsp60 (HtpB) whose surface location and function is dependent on a functional Dot/Icm T4SS mediates invasion of HeLa cells (Garduno *et al*., 1998a).

DsbA2 P198T dominant negative effect on Dot/Icm function

Since a functional Dot/Icm T4SS is essential for invasion and intracellular multiplication of *L. pneumophila* in host cells (Brand *et al*., 1994; Berger and Isberg, 1993; Franco *et al*., 2009), we considered it likely that the dominant negative effect of the P198T mutant protein was due to interference with either assembly or function of the Dot/Icm secretion apparatus. To directly test this possibility, we employed a well established contact hemolytic assay that was previously validated with specific Dot/Icm mutants (Kirby *et al*., 1998; Charpentier *et al*., 2009). In this assay, AA100 pMMB:empty vector mediated lysis of erythrocytes (see Fig. 4C). In contrast, bacteria expressing the dominant negative P198T mutant protein did not exhibit contact-dependent hemolysis, which was equivalent to that of a Δ*dotA* mutant control (Fig. 4C). Longer incubations (>24 hrs) also showed no erythrocyte lysis by bacteria expressing P198T (data not shown). Whether P198T mutant protein exhibits a dominant negative effect on other secretion systems was not investigated, though Zn-metalloprotease activity, a type II secretion system indicator (Rossier et al., 2008; Keen and Hoffman, 1989) was unaffected relative to controls (data not presented). We suggest that the dominant negative avirulence phenotype of the P198T mutant protein most likely results from disruption of assembly or function of the Dot/Icm T4SS.

DsbA2 function is required for flagella assembly

In earlier studies we observed that a *dsbA1* mutant retained motility. To test whether DsbA2 function was required for motility, we examined wet mounts of stationary phase bacteria (AA100 P198T) grown under inducing and non-inducing conditions. Bacterial motility (wet mounts viewed microscopically) was evident for the P198T mutant grown under non-inducing conditions. The same strain grown to the same OD under IPTG inducing conditions was nonmotile. To confirm the presence or absence of flagella as well as other proteins, induced and uninduced bacteria were subjected to osmotic shock in Tris-EDTA buffer (Kadokura, *et al*., 2004) and following centrifugation to remove whole bacteria, the shockates were precipitated with TCA and examined by SDS-PAGE. As seen in Fig. 5, a prominent 55 kDa protein was notably absent from bacteria expressing P198T mutant protein. The 55 kDa protein was verified as flagellin (*fliC or flaA*) by MALDI-ToF MS with high confidence, as most major ions were mapped to this protein. Whether P198T mutant protein affects oxidative folding of flagellin monomers or proteins associated with the motor as noted with *dsbA* mutants of *E. coli* was not pursued. While not the focus of this particular experiment, P198T mutant protein was detected in the shockate fraction by western blot (see Fig. 5, WB). Taken together, these studies indicate that DsbA2 function, and not that of DsbA1, is required for both motility and Dot/Icm function.

DsbA2 P198T mutant protein competes with DsbA2 protein in vitro

Our studies suggest that the dominant negative effect of the P198T mutant protein results from interference with wild type DsbA2 activity. To directly test this hypothesis we employed a modified insulin reduction assay. We first determined whether the P198T mutant protein retained oxidoreductase activity and as seen in Fig. 6A, it did not catalyze reduction of insulin when compared to functional DsbA2 and a DTT control. Even extending the assay for several hours did not enable P198T catalyzed insulin precipitation to increase above that of DTT controls. Since the P198T mutant protein is predicted to form stable disulfide bond complexes with substrate proteins, we considered the possibility that such complexes might ablate insulin precipitation. To test this possibility, P198T mutant protein was added to the insulin assay to evaluate effects on DsbA2 activity. As seen in Fig. 6B, increasing concentrations of P198T mutant protein partially inhibited wild type enzyme activity by appearing to limit available substrate to DsbA2, consistent with the notion that the mutant protein formed disulfide bonded complexes with insulin. An alternative possibility that might explain these results is that P198T mutant protein nonspecifically

contributed to the solubility of reduced insulin proteins. To rule this out, comparable concentrations of bovine serum albumin (5xBSA) were added to the DsbA2 insulin reduction assay. BSA concentrations had no effect on the reaction (See Fig. 6A). These results suggest that while P198T mutant protein lacks enzymatic activity, it appears to interfere with DsbA2 activity, possibly by forming stable complexes with insulin.

DsbA2P198T traps substrate proteins in L. pneumophila

Based on the *in vitro* insulin assay results and work of others (Kadokura, *et al*., 2004), we considered the possibility that P198T mutant protein might form stable complexes with DsbA2 substrate proteins in *L. pneumophila* and enable their identification. To test this possibility, protein complexes obtained from AA100 strains grown in the presence or absence of IPTG were analyzed by non-reducing SDS PAGE and immunoblot for P198T-substrate complexes. As seen in Fig. 7A, protein complexes of higher molecular weight than His-tagged P198T were not observed in extracts from un-induced bacteria (lane 2). In contrast, immuno-reactive proteins of higher molecular weight were detected under inducing conditions (lane 3) and are indicative of captured substrates. These complexes disappeared under reducing conditions as shown in Fig. 7A, lane 4, consistent with reduction of cross-linking disulfide-bonds. To ensure that the proteins noted were indeed cross-linked to P198T mutant protein, His$_6$-tagged P198T protein-complexes were affinity purified by nickel (Ni^{++}) interaction chromatography and the pattern of high molecular weight complexes resolved by non-reducing SDS-PAGE and immunoblot were similar to those depicted in Fig. 7A (see Fig. 7B). As a control for nonspecific binding of *L. pneumophila* proteins to the Ni^{++} column, equivalent extracts from the vector control strain AA100 were analyzed and as depicted in Fig. 7C (lanes 5–8), no proteins were captured. We chose not to pursue further experiments with the C89S mutant protein at this time because proteins captured by the free thiol likely differ from those substrates captured by oxidative disulfide bond formation by DsbA2.

To identify substrate proteins, Ni^{++} affinity-purified P198T complexes were run on SDS PAGE diagonal gels (Hoffman *et al*., 1992a; Kadokura *et al*., 2004) with the first dimension non-reducing and the second dimension under reducing conditions that dissociate disulfide-bond cross-linked complexes. As seen in Fig. 7D, dissociated proteins running off the diagonal are indicative of captured proteins. The extended band noted by arrow at ~30 kDa is P198T protein. Proteins running off the diagonal line were identified by MS-MS and yielded almost exclusively periplasmic and membrane-associated proteins, including several virulence factors, all of which contained cysteine residues with the one exception being HtpA (Table 2). Some important captured substrates included: Dot/Icm T4SS core structural proteins DotC, DotG, and DotK and IcmX; the periplasmic and surface localized invasin HtpB (Hsp60) and its co-chaperone HtpA (Hsp10) which lacks cysteine; and subunits of OmpS of the MOMP complex. While some of the captured proteins are indicated as cytoplasmic based on PSORTb predictions, this does not necessarily exclude a periplasmic location. It is important to note that other Dot/Icm proteins that do not contain cysteine residues (DotD, DotF, IcmW and IcmY) or cytoplasmic proteins that do contain cysteine residues (isocitrate dehydrogenase) were not captured in these studies. Finally, the Dot/Icm proteins captured through interaction with P198T mutant protein are considered essential for assembly and function of the T4SS (Vincent *et al*., 2006). Further study will be required to determine whether any of the captured substrates are essential for viability. These studies support a functional role for DsbA2 in the oxidative folding of proteins destined for assembly into the Dot/Icm T4SS.

Discussion

Here we report that a 27-kDa outer membrane protein of unknown function is a member of the DsbA family of oxidoreductases. Based on phylogenetic and functional criteria, as well as confirmation of a periplasmic location, we have named this gene *dsbA2*. While *L. pneumophila* contains two genes encoding DsbA-like proteins, *dsbA1* mutants were essentially wild type for infectivity of amoeba and exhibited no discernable phenotypes *in vitro*. To overcome the apparent essentiality of DsbA2, we constructed a P198T mutant that in *E. coli* DsbA (P151T) formed stable complexes with substrate proteins (Kadokura *et al.*, 2004). In *L. pneumophila*, expression of the DsbA2 P198T mutant protein produced a dominant negative effect on several virulence phenotypes including: (i) loss of intracellular multiplication in amoeba; (ii) loss of attachment and invasiveness of HeLa cells; (iii) loss of contact (Dot/Icm dependent) lysis of erythrocytes; and (iv) loss of flagella-driven motility. Disruption of the Dot/Icm complex was inferred from the above results and confirmed by capture of DotC, DotG, DotK and IcmX substrates by P198T mutant protein. The abundance and diversity of captured proteins (see Table 2) suggests that *L. pneumophila* is highly dependent on DsbA2 for oxidative folding of periplasmic proteins, including those destined for assembly into macromolecular structures. These studies not only revealed important biological functions for DsbA2 in *L. pneumophila*, but also the likelihood that DsbA2 provides similar function in a very broad range on microbes, including many also expressing T4SS.

In retrospect, the original annotation of Com1 as an outer membrane protein was clearly misleading, but not uncommon. In reviewing the history, we note that i*n silico* analysis of the primary amino acid sequence of DsbA2 identified a sec-dependent leader sequence typical of DsbA proteins and predicted with high confidence a periplasmic location (score of 9.84 and similar to that predicted for DsbA1). The periplasmic location was confirmed in our studies by cell fractionation and immunogold electron microscopy. In contrast, the cellular location of Com1 could not be predicted by PSORTb analysis, despite sharing 48% identity and 69% similarity with DsbA2. There was ambiguity in the original communication on the cellular location as recombinant Com1 expressed in *E. coli* appeared in the Sarkosyl-soluble (inner membrane) fraction, while surface iodination and protinease K treatments of whole *C. burnetii* cells indicated an outer membrane association (Hendrix *et al.*, 1993). Com1 is an immunodominant antigen of *C. burnetii* that is useful in the diagnostics of Q fever (Hendrix, *et al.*, 1993), while DsbA2 is not an immunodominant antigen based on screening of convalescent sera from patients with confirmed Legionnaires' disease (personal communication, Claressa Lucas, Centers for Disease Control and Prevention).

In addition to the misleading annotation, DsbA2 also differs sufficiently in amino acid sequence from the canonical DsbA that it is now listed by the NCBI as a separate family. Simple BLAST searches with DsbA2 do not capture DsbA, although domain searches with the thioredoxin fold of DsbA2 would have placed it in the thioredoxin super family, but not necessarily in the DsbA family. Most genera expressing DsbA2, like *L. pneumophila*, also expressed a DsbA (DsbA1) orthologue (the exception being *C. burnetii*), but without exception all lacked an orthologue of DsbC that provides protein disulfide isomerase activity. Ironically, comparative BLAST searches with *E. coli* DsbA and DsbA2 (any member of this group) using a cut-off of e^{-24}, will reveal the DsbA2 group to be far more conserved through evolution than the DsbA group. Much of the amino acid conservation noted for DsbA2 members is due to a 50 – 60 amino acid extension N-terminal to the CXXC motif compared with the shorter $30-34$ amino acids for DsbA members. In this respect, DsbA2 may be more similar to DsbC (107 amino acids to CXXC motif), but unlike DsbC, DsbA2 lacks a well defined dimerization domain. Other similarities with DsbC include a

common amino acid residue adjacent to the conserved *cis*-proline (T̲PA) instead of V̲PA found in DsbA (Ren *et al*., 2009). The similarly to DsbC isomerases might explain the ability of the free thiol of C89S mutant protein to form complexes with substrates as demonstrated in Fig. 5D lane 2. However, the redox status of DsbA2 in the periplasm was more typical of DsbA than DsbC. If DsbA2 was DsbC-like, most of the protein would have existed in the reduced state. Our studies do not exclude the possibility that DsbA2 may be bifunctional or exist in different redox states as a monomer or potential dimer, all of which might vary with growth stage. We are currently investigating the nature of the substrates captured by DsbA2 C89S mutant protein that might support disulfide isomerase activity.

To our knowledge, all of the *dsb* genes studied to date are nonessential for viability, though it is not uncommon for Gram-negative bacteria to express several DsbA-like enzymes. For example, *Neisseria meningitidis* contains three DsbA-like homologues (Tinsley, *et al*., 2004), *Salmonella enterica* expresses SeDsbA, SeDsbL, and SeSgrA that have been crystallized and shown to be DsbA-like (Jarrott *et al*., 2010), and in uropathogenic *E. coli* a second DsbA/B like system, DsbI/L has been identified (Grimshaw, *et al*., 2008). In the case of *N. meningitidis*, single and double deletion mutants retained full virulence indicating considerable redundancy in function (Tinsley *et al*., 2004). It is noteworthy that the triple mutant, while viable, exhibited a temperature sensitive growth phenotype (Tinsley, *et al*., 2004). Several studies have shown that DsbA is required for assembling various extra-cytoplasmic virulence factors including components of T2 and T3 secretion systems and toxins (Heras *et al*., 2009). Our inability to isolate mutants of *dsbA2* suggests that DsbA2 must provide an as yet undefined essential function(s) in *L. pneumophila* and possibly other bacteria expressing a *dsbA2* orthologue.

In addition to *L. pneumophila* and *C. burnetii*, other human and plant pathogens expressing T4SS include: *B. suis*, *Bartonella henslae*, *Ehrlichia sp*., *Anaplasma sp*., *R. prowazekii* and *Agrobacterium tumefactions* (Llosa *et al*., 2009). All of these bacteria express an orthologue of DsbA2, suggesting a role in assembling components of the respective T4SS. Bacterial genera not containing T4SS, but expressing DsbA2 include: *Caulobacter, Bradyrhizobium, Azospirillum, Paracoccus, Methylobacterium*, and *Magnetospirillum*. Two notable exceptions in bacteria that express T4SS systems include *B. pertussis* (Ptl T4SS) which expresses DsbA1 and DsbC orthologues and *H. pylori* in which an orthologue of DsbA is absent. In the case of *H. pylori*, the acidic gastric mucosa, like anaerobic environments, do not favor stability of disulfide bonds. Further phylogenetic analyses of bacteria containing T4SS will likely confirm an evolutionary relationship with DsbA2. The phenotype of the P198T mutant suggests that the conserved *cis*-proline mutation first exploited in *E. coli* might be applied to other Gram-negative bacteria as a strategy to capture protein substrates associated with virulence or other cellular functions.

In addition to P198T capture of Dot/Icm components, a wide range of substrate proteins were captured (Table 2), including enhanced entry proteins (Enh) that are required for entry into amoeba (Liu *et al*., 2008). Perhaps the reduced attachment and invasion seen with P198T mutant protein might be due to impairment of Enh function (Fig. 6). Heat shock proteins HtpA and HtpB (GroES and GroEL), previously reported as periplasmic proteins, were captured in this study (Hoffman *et al*., 1990; Garduno *et al*., 1998a and 1998c) and it is noteworthy that the localization of HtpB onto the cell surface is Dot/Icm dependent (Garduno *et al*., 1998c). HtpB contains four cysteine residues (*E. coli* GroEL contains two) whose oxidation might be important for function and like DsbA2 is enriched in the hyper virulent cyst form. Ironically, HtpA contains no cysteine residues and raises the possibility of co-capture (with HtpB) of molecular complexes. It is noteworthy that other Dot/Icm proteins that contain no cysteine residues such as DotD, IcmW and IcmY were not captured, nor were cytoplasmic proteins rich in cysteine residues like isocitrate dehydrogenase.

However, as noted previously (Kadokura *et al.*, 2004), despite the addition of excess iodoacetamide to alkylate free thiols to limit capture of cytoplasmic proteins, some cytoplasmic proteins were captured by this procedure. In retrospect, one might limit cytoplasmic contamination by isolating captured complexes by osmotic shock, though these strategies could potentially limit capture of membrane associated complexes. While subunits of OmpS were also captured in these studies, it is unlikely that DsbA2 plays a major role in catalyzing disulfide bond cross-linking of the subunits as one might expect the over expression of the P198T mutant to have resulted in restricted *in vitro* growth or changes in the OmpS profile. However, further studies will be required to fully explore the role of DsbA2 in assembly of the OmpS oligomer.

The lack of motility and assembled flagella in the P198T over-expressing mutant described here mirrors what is known in other Gram-negative bacteria with DsbA mutations: Δ*dsbA E. coli* produce no T4 pili (BFP) (Vogt *et al.*, 2010), and *Salmonella* lacking the DsbA deriviative SrgA produce no fimbriae (Bouwman *et al.*, 2003). The absence of flagellin in the periplasmic shockate with P198T mutant protein suggests that DsbA2 either catalyzes a disulfide bond required for flagella biosynthesis or indirectly, as through a defect in FlgI assembly (flagella motor defect) triggers degradation of subunits and or activation of regulatory systems like CpxR that might repress the flagella system (Ravio, 2005).

In summary, our studies assign biological functions to a family of proteins previously annotated as 27-kDa outer membrane proteins similar to Com1 as disulfide bond oxidoreductases. The DsbA2 lineage is phylogenetically distinct from members of the DsbA family. Remarkably, all members of this group also lack orthologues of DsbC and DsbG, but retain the DsbD reductase, suggesting the possibility that DsbA2 might be bifunctional, providing disulfide-bond oxidizing as well as editing functions (protein disulfide isomerase) required for repairing nonconsecutive disulfide bonds. Finally, the DsbA2 lineage is conserved among those bacterial species that also express a T4SS, suggesting a likely role for DsbA2 in assembly or function of these macromolecular structures.

Experimental procedures

Bacterial strains and growth conditions

Legionella pneumophila serogroup 1 strains 130b (also known as AA100, ATCC BAA-74) and *dotA* mutant (kind gift of Dr. Abu-Kwaik), and the Philadelphia-1 strain Lp02 and Lp03 (Berger and Isberg, 1993) were grown aerobically at 37°C on buffered charcoal yeast extract agar (BCYE) (Pasculle *et al.*, 1980) or in buffered yeast extract (BYE) broth and supplemented with α-ketoglutaric acid (1 mg/ml), ferric pyrophosphate (250 µg/ml), L-cysteine (40 µg/ml), thymidine (100 µg/ml), and antibiotics where required. Starter cultures were prepared as previously described (LeBlanc *et al.*, 2006) and used to inoculate pre-warmed BYE to an optical density at 620 nm (OD_{620}) of 0.2. For growth curve determinations, samples were taken every two hours (triplicate) and optical density was determined at 620 nm. *E. coli* strain DH5α (Clontech Laboratories, Mountain View, CA) and BL21(DE3) CodonPlus™RIL (Stratagene) were grown at 37°C on Luria-Bertani (LB) agar or in LB broth supplemented with the appropriate antibiotics. Antibiotics (Sigma-Aldrich Ltd.) were added to media at the following concentrations, when appropriate: streptomycin (100 µg/ml), kanamycin (40 µg/ml), gentamicin (10 µg/ml), chloramphenicol (20 µg/ml), and ampicillin (100 µg/ml). All strains were stored at −85°C in nutrient broth containing 10% dimethyl sulfoxide.

Phylogenetic analysis of DsbA1 and DsbA2

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 *Legionella pneumophila* Philadelphia-1. Phylogenetic trees were generated by multiple sequence alignment of unedited DsbA2 sequences using ClustalW. Protein prediction software Phyre (www.sbg.bio.ic.ac.uk/~phyre) was used align DsbA2 onto the crystal structure of DsbA of *E. coli*.

Construction of Lp02ΔdsbA1 and Lp02ΔdsbA2

Allelic replacement was performed as described previously (Chalker *et al.*, 2001;Hiltz *et al.*, 2004). Briefly, 700 bp flanking regions of the *dsbA* gene were amplified with primers pairs DSBA1-1F and DSBA1-2R and DSBA1-3F and DSBA1-4R (see Table 1). The internal primers DSBA1-2R and DSBA1-3F were complementary to 5' and 3' regions of a gentamicin resistance cassette amplified from pPH1J1 (Hirsh and Beringer, 1984), so that the PCR products of the *dsbA1* flanking regions would anneal to the gentamicin resistance cassette in a three way PCR. A final PCR reaction using outside primers DSBA1-1F and DSBA1-4R generated the linear DNA fragment that was electroporated into Lp02 as described previously (Hiltz *et al.*, 2004) and plated on BCYE with thymidine and gentamicin. Colonies were screened by PCR for deletion of *dsbA1* using both flanking primers and primers designed to internal sequences of *dsbA1*. Allelic replacement strategies to produce deletions of *dsbA2* were essentially as described for *dsbA1*: primers DSBA2-1F, DSBA2-2R, DSBA2-3F, and DSBA2-4R were used to generate *dsbA2* flanking regions and to incorporate a kanamycin resistance cassette between them. The final construct was electroporated into Lp02 and Lp03 (a *dotA* mutant strain kindly provided by Michelle Swanson). A second *dsbA2* knockout strategy utilized native NsiI restriction sites in the middle of the *dsbA2* gene to insert a kanamycin resistance cassette. The commercial vector pBlueScript SK+ (Stratagene) was restricted with BamHI and EcoRI, and a PCR product generated with DsbA21-F and DsbA24-R (containing the *dsbA2* gene and flanking regions) was similarly restricted with BamHI and EcoRI and ligated to produce pBS:*dsbA2*. This vector was restricted with NsiI, blunted, and a blunted kanamycin cassette was inserted, effectively disrupting the *dsbA2* coding sequence. PCR was performed to amplify the kanamycin disrupted *dsbA2* and flanking regions and the linear DNA was electroporated into Lp02 and Lp03 as above. Both linear DNA constructs were also used for natural transformation of Lp02 and Lp03 and in the AA100 strains (130b) as described previously (Sexton and Vogel, 2004).

A suicide vector (pRDXA) developed previously (Morash, *et al.*, 2009) was used to disrupt the *dsbA2* coding sequence using the two constructs described above. This vector utilizes metronidazole as a counter-selectable marker to confirm loss of the plasmid and in conjunction with a positive-selection marker, in this case kanamycin, can be used to increase the likelihood of obtaining low frequency recombination events. Approximately 10 μg of plasmid DNA was electroporated into electrocompetent *L. pneumophila* Lp02, plated on BCYE medium supplemented with streptomycin, kanamycin, and thymidine, and incubated for 3 to 4 days. The resulting transformants were replica plated and screened for loss of metronidazole sensitivity on BCYE supplemented with 20 μg/ml of metronidazole (loss of the plasmid vector) and on chloramphenicol (12 μg/ml) as a second marker for the plasmid.

Construction of AA100ΔdsbA1

Natural competence was induced in *L. pneumophila* strain AA100 (130b) as described previously (Sexton and Vogel, 2004). Briefly, dime-sized patches were made on BCYE of AA100 from freshly grown plates. These patches were allowed to grow for 16 h at 25°C,

and then 5 µg of genomic DNA from Lp02Δ*dsbA1* was carefully dropped onto the surface of the patch. The patches were left to grow for 48–72 hours, and then resuspended in water and plated on BCYE containing gentamicin. Colonies were screened by PCR for deletion of *dsbA1*.

Construction of C89S and P198T-His$_6$ mutants

To create mutant alleles of DsbA2, cysteine at position 89 was changed to serine using a PCR based strategy with primer pairs C89S-F and C89S-R and similarly for DsbA2P198T mutants, primer pairs P198T-F and P198-R were used. The PCR amplicons were restricted with EcoR1/BamHI and cloned into pMMB206 to enable expression under a controllable promoter (*lacI^q* and IPTG inducible promoter) (Morales *et al*., 1991). For the P198T construct, 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.

DsbA2 antibody production

dsbA2 was amplified with primers DsbA2expNde1 and DsbA2expBamHI, digested with appropriate enzymes, and cloned into the pET15b expression system. An N-terminal His$_6$-DsbA2 was over-expressed and purified by Ni $^{++}$ chromatography. Purified protein was used to generate polyclonal antibodies in mice as previously described (Helsel *et al*., 1987) and all western blotting was performed with DsbA2-specific antibody diluted 1:10,000 in PBS with 0.1% Tween 20. Immunoblots of whole cell lysates of Lp02 were used to confirm specificity of the antibody. DsbA2 antibody was monospecific for a 30 kDa protein with no cross-reactivity with DsbA1.

Cell fractionation

Subcellular localization of DsbA2 protein was conducted using *L. pneumophila* harvested from 50-ml cultures that were grown to stationary phase in BYE broth at 37°C. Bacterial cells were disrupted by sonication and lysates fractionated into soluble and membrane-associated proteins by ultracentrifugation at 100,000 × g (Butler *et al*., 1985; Vincent *et al*., 2006). Total membrane fractions were treated with 1% Triton X100 and the suspension subjected to ultracentrifugation to separate the soluble fraction (inner membrane and periplasmic proteins) from the outer membrane pellet. Fractions were analyzed by SDS-PAGE and immunoblot using the following monospecific antisera: DsbA2, and OmpS (Butler *et al*., 1985), LepB (Vincent, *et al*., 2003) and IHF (Morash *et al*., 2009).

Electron microscopy and immunogold labeling

Bacteria were prepared for electron microscopy by embedding in TAAB resin and sectioned or directly to grids (whole cells) as previously described (Garduno *et al*., 2002). Immunogold labeling was carried out at room temperature as previously described (Fernandez *et al*., 1996). Antibodies used in these studies included rabbit OmpS-specific antibody (1/400) and mouse DsbA2-specific antibody (1/400 to 1/50 dilutions) and the secondary antibody (anti-mouse IgG or anti-rabbit IgG) conjugated to 10-nm colloidal gold spheres [Sigma Immunochemicals]) was routinely diluted 1:100. After the labeling procedure, the specimens were fixed by floating the grids on drops of 2.5% (vol/vol) glutaraldehyde in PBS for 10 min, repeatedly washed on drops of deionized water, and then stained with 2% (wt/vol) aqueous uranyl acetate. Statistical analysis on gold particle localization was performed using the method of Mallette (Mallette, 1969) as described previously (Garduno *et al*., 1998c).

Insulin assay

Reductase activity was assessed by an insulin precipitation assay (Bardwell *et al*., 1991) with minor modifications. 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 DTT, and 2 mM EDTA and incubated in 96 well plate format at room temperature, taking OD_{650} readings at time intervals noted. DsbA2, P198T mutant protein and a bovine serum albumin control were added to the assay in equimolar concentrations.

Competition assay

Purified DsbA2 and P198T mutant protein were added to a modified insulin precipitation reaction by first establishing a rate with DsbA2 and then adding equivalent and higher concentrations of the P198T mutant protein. Reactions were performed as described in the insulin assay, except the concentration of DTT was increased to 0.75 mM and the insulin decreased to 86 μM.

Periplasmic redox status of DsbA2 by AMS/gel-shift

The 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 (Kadokura *et al*., 2004). Briefly, *L. pneumophila* was grown to stationary phase (~24 h) when DsbA2 is most abundant and whole cells were divided into aliquots, one of which was first treated with 10 mM DTT, then TCA precipitated, and alkylated with 100 mM AMS; one TCA precipitated, then alkylated with AMS; and one served as an untreated control. Each of the samples was analyzed by SDS-PAGE and immunoblot. AMS shifts the mass by 490 Da leading to a band shift.

Acanthamoeba castellanii infection

A. castellanii (ATCC 30010) was maintained in ATCC medium 354 at 25°C. For infection, a 48-hr culture of *A. castellanii* was washed and resuspended in Tris-buffered salt solution containing 2 mM KCl, 1mM $CaCl_2$, 0.5 mM $MgCl_2$, and 1 mM Tris, with the pH adjusted to 6.8 to 7.2 (Berk *et al*., 1998). Approximately 10^5 *A. castellanii* per ml were infected with 10^3 CFU/ml of *L. pneumophila* from an overnight culture grown in the presence or absence of IPTG. All experiments were performed in triplicate at 30°C, and at each time point wells were scraped to remove adherent amoeba, and 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.

HeLa cell infection

HeLa cells were grown in minimal essential medium (MEM) as described previously (Garduno *et al*., 1998b). HeLa cells were resuspended in MEM without antibiotics to a concentration of 10^6 cells/ml and left to adhere for 1 to 2 hrs. *L. pneumophila* was standardized to an OD_{600} of 1.0. One hundred microliters of the bacterial suspension was added to triplicate wells to a final inoculum 10^8 bacteria/ 10^6 HeLa cells. Plates were centrifuged at 500 rcf for 5 minutes at room temperature in a clinical centrifuge to maximize contact of bacteria with the HeLa cell monolayer and then incubated for 3 hrs to facilitate infection. For attachment assays, monolayers were washed six times with PBS, lysed with water and vigorous pipetting, and decimal dilutions were plated in triplicate on BCYE agar for bacterial enumeration. For invasion assays, monolayers were washed six times with PBS, treated for 1 hr with MEM containing 50 μg/ml gentamicin, washed six times with PBS, lysed and enumerated as detailed above.

Hemolysis assay

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 50:1 in a final volume of ~1 ml as generally described (Kirby et al., 1998). *Legionella* strains and RBCs were mixed, pelleted for 3 min at 10,000 g, and after 2 hours 100 µl aliquots of supernatants were transferred to microtitre plates, and absorbance measured at OD_{415} for hemoglobin release.

SDS-PAGE and diagonal gel electrophoresis of cellular extracts

Preparation of cellular extracts and diagonal gel electrophoresis were as described by Kadokura (Kadokura *et al.*, 2004). Briefly, 4L of AA100 [strain with P198T] was induced at an OD_{600} of 1.0 with 1 mM IPTG for 15 hours. The cells were centrifuged and washed once with $1/10^{th}$ volume $ddiH_20$, then re-suspended in $1/10^{th}$ volume cold $ddiH_2O$. Dry TCA was added to ~8%, the cells were lysed on ice for 20 minutes, centrifuged for 10 minutes, and the pellet washed twice with acetone and dried. The pellet was alkylated in 100 mM TrisHCl (pH 8.0) containing 0.5% SDS and 100 mM iodoacetamide. The alkylated lysate was then diluted four times with 50 mM TrisHCl (pH 8.0) containing 300 mM NaCl and centrifuged at $10,000 \times g$ for 20 min at 4°C to pellet insoluble material. The cleared lysate was loaded onto 4 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 8 column volumes of Buffer A containing 20 mM imidazole and protein fractions eluted with 5 column volumes of Buffer A + 250 mM imidazole. Fractions containing DsbA2 P151T – 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 minutes under non-reducing conditions. The lane was excised and incubated in warm 100 mM DTT for 15 minutes, then placed in Laemmli loading buffer without bromophenol blue + 100 mM iodoacetamide for 5 minutes. 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.*, 1992a). The gel slice was locked in place by addition of stacking gel, and electrophoresed at 200 V for 45 minutes, followed by silver staining.

Protein identification by mass spectrometry

Gel slices containing proteins of interest were transferred to siliconized tubes and prepared for mass spectrometry by standard procedures in the Biomolecular Core facility of the University of Virginia School of Medicine. Tryptic digests were analyzed by LC-MS on a Thermo Electron LTQFT mass spectrometer. The digest was analyzed using the double play capability of the instrument acquiring full scan mass spectra to determine peptide molecular weights and product ion spectra to determine amino acid sequence in sequential scans. The data were analyzed by database searching using the Sequest search algorithm against *L. pneumophila*. MALDI ToF MS was also used to identify proteins in some experiments and also performed by the Biomolecular Core facility.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

References

Albert-Weissenberger C, Sahr T, Sismeiro O, Hacker J, Heuner K, Buchrieser C. Control of flagellar gene regulation in *Legionella pneumophila* and its relation to growth phase. J Bacteriol. 2010; 192:446–455. [PubMed: 19915024]

Bardwell JC, McGovern K, Beckwith J. Identification of a protein required for disulfide bond formation in vivo. Cell. 1991; 67:581–589. [PubMed: 1934062]

Berger KH, Isberg RR. Two distinct defects in intracellular growth complemented by a single genetic locus in *Legionella pneumophila*. Mol Microbiol. 1993; 7:7–19. [PubMed: 8382332]

Berk SG, Faulkner G, Garduno E, Joy MC, Ortiz-Jimenez MA, Garduno RA. Packaging of live *Legionella pneumophila* into pellets expelled by *Tetrahymena* spp. does not require bacterial replication and depends on a Dot/Icm-mediated survival mechanism. Appl Environ Microbiol. 2008; 74:2187–2199. [PubMed: 18245233]

Berk SG, Ting RS, Turner GW, Ashburn RJ. Production of respirable vesicles containing live *Legionella pneumophila* cells by two *Acanthamoeba* spp. Appl Environ Microbiol. 1998; 64:279–286. [PubMed: 9435080]

Bouwman CW, Kohli M, Killoran A, Touchie GA, Kadner RJ, Martin NL. Characterization of SrgA, a *Salmonella enterica* serovar typhimurium virulence plasmid-encoded paralogue of the disulfide oxidoreductase DsbA, essential for biogenesis of plasmid-encoded fimbriae. J Bacteriol. 2003; 185:991–1000. [PubMed: 12533475]

Brand BC, Sadosky AB, Shuman HA. The *Legionella pneumophila icm* locus: A set of genes required for intracellular multiplication in human macrophages. Mol Microbiol. 1994; 14:797–808. [PubMed: 7891565]

Brassinga AK, Hiltz MF, Sisson GR, Morash MG, Hill N, Garduno E, Edelstein PH, Garduno RA, Hoffman PS. A 65-kilobase pathogenicity island is unique to Philadelphia-1 strains of *Legionella pneumophila*. J Bacteriol. 2003; 185:4630–4637. [PubMed: 12867476]

Brassinga AKC, Kinchen JM, Cupp ME, Day SR, Hoffman PS, Sifri CD. *Caenorhabditis* is a metazoan host for *Legionella*. Cell Microbiol. 2010; 12:343–361. [PubMed: 19863556]

Butler CA, Hoffman PS. Characterization of a major 31-kilodalton peptidoglycan-bound protein of *Legionella pneumophila*. J Bacteriol. 1990; 172:2401–2407. [PubMed: 2332403]

Butler CA, Street ED, Hatch TP, Hoffman PS. Disulfide-bonded outer membrane proteins in the genus *Legionella*. Infect Immun. 1985; 48:14–18. [PubMed: 3980079]

Byrne B, Swanson MS. Expression of *Legionella pneumophila* virulence traits in response to growth conditions. Infect Immun. 1998; 66:3029–3034. [PubMed: 9632562]

Chalker AF, Minehart HW, Hughes NJ, Koretke KK, Lonetto MA, Brinkman KK, Warren PV, Lupas A, Stanhope MJ, Brown JR, Hoffman PS. Systematic identification of selective essential genes in *Helicobacter pylori* by genome prioritization and allelic replacement mutagenesis. J Bacteriol. 2001; 183:1259–1268. [PubMed: 11157938]

Charpentier X, Gabay JE, Reyes M, Zhu JW, Weiss A, Shuman HA. Chemical genetics reveals bacterial and host cell functions critical for type IV effector translocation by *Legionella pneumophila*. PLoS Pathog. 2009; 5(7):e1000501. [PubMed: 19578436]

Christie PJ, Atmakuri K, Krishnamoorthy V, Jakubowsk iS, Cascales E. Biogenesis, architecture, and function of bacterial type IV secretion systems. Annu Rev Microbiol. 2005; 59:451–485. [PubMed: 16153176]

Conover GM, Derre I, Vogel JP, Isberg RR. The *Legionella pneumophila* LidA protein: A translocated substrate of the Dot/Icm system associated with maintenance of bacterial integrity. Mol Microbiol. 2003; 48:305–321. [PubMed: 12675793]

Dailey FE, Berg HC. Mutants in disulfide bond formation that disrupt flagellar assembly in *Escherichia coli*. Proc Natl Acad Sci U S A. 1993; 90:1043–1047. [PubMed: 8503954]

Davis AJ, Diaz DA, Mecsas J. A dominant-negative needle mutant blocks type III secretion of early but not late substrates in *Yersinia*. Mol Microbiol. 2010; 76:236–259. [PubMed: 20199604]

Depuydt M, Leonard SE, Vertommen D, Denoncin K, Morsomme P, Wahni K, Messens J, Carrol KS, Collet J-F. A periplasdmic reducing system protects single cysteine residues from oxidation. Science. 2009; 326:1109–1111. [PubMed: 19965429]

Faulkner G, Garduno RA. Ultrastructural analysis of differentiation in *Legionella pneumophila*. J Bacteriol. 2002; 184:7025–7041. [PubMed: 12446652]

Faulkner G, Berk SG, Garduno E, Ortiz-Jimenez MA, Garduno RA. Passage through *Tetrahymena tropicalis* triggers a rapid morphological differentiation in *Legionella pneumophila*. J Bacteriol. 2008; 190:7728–7738. [PubMed: 18805971]

Fernandez R, Logan S, Lee S, Hoffman P. Elevated levels of *Legionella pneumophila* stress protein Hsp60 early in infection of human monocytes and L929 cells correlate with virulence. Infect Immun. 1996; 64:1968–1976. [PubMed: 8675295]

Fields BS. The molecular ecology of *Legionellae*. Trends in Microbiology. 1996; 4:286–290. [PubMed: 8829338]

Fliermans CB, Cherry WB, Orrison LH, Smith SJ, Tison DL, Pope DH. Ecological distribution of *Legionella pneumophila*. Appl Environ Microbiol. 1981; 41:9–16. [PubMed: 7013702]

Franco IS, Shuman HA, Charpentier X. The perplexing functions and surprising origins of *Legionella pneumophila* type IV secretion effectors. Cell Microbiol. 2009; 11:1435–1443. [PubMed: 19563462]

Garduno RA, Garduno E, Hoffman PS. Surface-associated Hsp60 chaperonin of *Legionella pneumophila* mediates invasion in a HeLa cell model. Infect Immun. 1998a; 66:4602–4610. [PubMed: 9746556]

Garduno RA, Quinn FD, Hoffman PS. HeLa cells as a model to study the invasiveness and biology of *Legionella pneumophila*. Can J Microbiol. 1998b; 44:430–440. [PubMed: 9699298]

Garduno RA, Garduno E, Hiltz M, Hoffman PS. Intracellular growth of *Legionella pneumophila* gives rise to a differentiated form dissimilar to stationary-phase forms. Infect Immun. 2002; 70:6273–6283. [PubMed: 12379706]

Garduno RA, Faulkner G, Trevors MA, Vats N, Hoffman PS. Immunolocalization of Hsp60 in *Legionella pneumophila*. J Bacteriol. 1998c; 180:505–513. [PubMed: 9457851]

Grimshaw JPA, Stirnimann CU, Brozzo MS, Malojcic G, Grutter MG, Capitani G, Glockshuber R. DsbL and DsbI form a specific dithiol oxidase system for periplasmic arylsulfate sulfotransferase in uropathogenic *Escherichia coli*. J Mol Biol. 2008; 380:667–680. [PubMed: 18565543]

Hendrix LR, Mallavia LP, Samuel JE. Cloning and sequencing of *Coxiella burnetii* outer membrane protein gene *com1*. Infect Immun. 1993; 61:470–477. [PubMed: 8423075]

Heras B, Shouldice SR, Totsika M, Scanlon MJ, Schembri MA, Martin JL. DSB proteins and bacterial pathogenicity. Nat Rev Microbiol. 2009; 7:215–225. [PubMed: 19198617]

Helsel LO, Bibb WF, Butler CA, Hoffman PS, McKinney R. Recognition of a genus-wide antigen of *Legionella* by a monoclonal antibody. Current Microbiology. 1987; 16:201–208.

Hiltz MF, Sisson GR, Brassinga AKC, Garduno E, Garduno RA, Hoffman PS. Expression of *magA* in *Legionella pneumophila* philadelphia-1 is developmentally regulated and a marker of formation of mature intracellular forms. J Bacteriol. 2004; 186:3038–3045. [PubMed: 15126465]

Hirsch PR, Beringer JE. A physical map of pPH1JI and pJB4JI. Plasmid. 1984; 12:139–141. [PubMed: 6095352]

Hoffman PS, Seyer JH, Butler CA. Molecular characterization of the 28- and 31-kilodalton subunits of the *Legionella pneumophila* major outer membrane protein. J Bacteriol. 1992a; 174:908–913. [PubMed: 1310095]

Hoffman PS, Houston L, Butler CA. *Legionella pneumophila htpAB* heat shock operon: Nucleotide sequence and expression of the 60-kilodalton antigen in *L. pneumophila*-infected HeLa cells. Infect Immun. 1990; 58:3380–3387. [PubMed: 2205580]

Hoffman PS, Ripley M, Weeratna R. Cloning and nucleotide sequence of a gene (*ompS*) encoding the major outer membrane protein of *Legionella pneumophila*. J Bacteriol. 1992b; 174:914–920. [PubMed: 1732223]

Holmgren A. Thioredoxin catalyzes the reduction of insulin disulfides by dithiothreitol and dihydrolipoamide. J Biol Chem. 1979; 254:9627–9632. [PubMed: 385588]

Jarrott R, Shouldice SR, Guncar G, Totsika M, Schembri MA, Heras B. Expression and crystallization of SeDsbA, SeDsbL and SeSrgA from *Salmonella enterica* serovar Typhimurium. Acta Crystallogr Sect F Struct Biol Cryst Commun. 2010; 66:601–604.

Kadokura H, Beckwith J. Mechanisms of oxidative protein folding in the bacterial cell envelope. Antiox. Redox Signal. 2010; 13:1231–1246.

Kadokura H, Tian H, Zander T, Bardwell JCA, Beckwith J. Snapshots of DsbA in action: Detection of proteins in the process of oxidative folding. Science. 2004; 303:534–537. [PubMed: 14739460]

Kadokura H, Nichols L 2nd, Beckwith J. Mutational alterations of the key *cis* proline residue that cause accumulation of enzymatic reaction intermediates of DsbA, a member of the thioredoxin superfamily. J Bacteriol. 2005; 187:1519–1522. [PubMed: 15687218]

Keen MG, Hoffman PS. Characterization of a *Legionella pneumophila* extracellular protease exhibiting hemolytic and cytotoxic activities. Infect Immun. 1989; 57:732–738. [PubMed: 2917785]

Kirby JE, Vogel JP, Andrews HL, Isberg RR. Evidence for pore-forming ability by *Legionella pneumophila*. Mol Microbiol. 1998; 27:323–336. [PubMed: 9484888]

LeBlanc JJ, Brassinga AK, Ewann F, Davidson RJ, Hoffman PS. An ortholog of OxyR in *Legionella pneumophila* is expressed postexponentially and negatively regulates the alkyl hydroperoxide reductase (*ahpC2D*) operon. J Bacteriol. 2008; 190:3444–3455. [PubMed: 18359810]

LeBlanc JJ, Davidson RJ, Hoffman PS. Compensatory functions of two alkyl hydroperoxide reductases in the oxidative defense system of *Legionella pneumophila*. J Bacteriol. 2006; 188:6235–6244. [PubMed: 16923890]

Lema MW, Brown A, Butler CA, Hoffman PS. Heat-shock response in *Legionella pneumophila*. Can J Microbiol. 1988; 34:1148–1153. [PubMed: 2904297]

Liu M, Conover GM, Isberg RR. *Legionella pneumophila* EnhC is required for efficient replication in tumour necrosis factor alpha-stimulated macrophages. Cell Microbiol. 2008; 10:1906–1923. [PubMed: 18549456]

Llosa M, Roy C, Dehio C. Bacterial type IV secretion systems in human disease. Mol Microbiol. 2009; 73:141–151. [PubMed: 19508287]

Mallette, MF. Chapter XV evaluation of growth by physical and chemical means. Norris, JR.; Ribbons, DW., editors. Academic Press; 1969. p. 521-566.

Matthews M, Roy CR. Identification and subcellular localization of the *Legionella pneumophila* IcmX protein: A factor essential for establishment of a replicative organelle in eukaryotic host cells. Infect Immun. 2000; 68:3971–3982. [PubMed: 10858211]

McCusker KT, Braaten BA, Cho MW, Low DA. *Legionella pneumophila* inhibits protein synthesis in Chinese hamster ovary cells. Infect Immun. 1991; 59:240–246. [PubMed: 1987039]

McDade JE, Shepard CC, Fraser DW, Tsai TR, Redus MA, Dowdle WR. Legionnaires' disease: Isolation of a bacterium and demonstration of its role in other respiratory disease. N Engl J Med. 1977; 297:1197–1203. [PubMed: 335245]

Morales VM, Backman A, Bagdasarian M. A series of wide-host-range low-copy-number vectors that allow direct screening for recombinants. Gene. 1991; 97:39–47. [PubMed: 1847347]

Morash MG, Brassinga AK, Warthan M, Gourabathini P, Garduño RA, Goodman SD, Hoffman PS. Reciprocal expression of integration host factor and HU in the developmental cycle and infectivity of *Legionella pneumophila*. Appl Environ Microbiol. 2009; 75:1826–1837. [PubMed: 19201975]

Motohashi K, Kondoh A, Stumpp MT, Hisabori T. Comprehensive survey of proteins targeted by chloroplast thioredoxin. Proc Natl Acad Sci U S A. 2001; 98:11224–11229. [PubMed: 11553771]

Msadek T. Grasping at shadows: Revealing the elusive nature of essential genes. J Bacteriol. 2009; 191:4701–4704. [PubMed: 19465656]

Newton HJ, Sansom FM, Dao J, Cazalet C, Bruggemann H, Albert-Weissenberger C, et al. Significant role for *ladC* in initiation of *Legionella pneumophila* infection. Infect Immun. 2008; 76:3075–3085. [PubMed: 18426879]

Peek JA, Taylor RK. Characterization of a periplasmic thiol:disulfide interchange protein required for the functional maturation of secreted virulence factors of *Vibrio cholerae*. Proc Natl Acad Sci U S A. 1992; 89:6210–6214. [PubMed: 1631111]

Pasculle AW, Feeley JC, Gibson RJ, Cordes LG, Myerowitz RL, Patton CM, et al. Pittsburgh pneumonia agent: Direct isolation from human lung tissue. J Infect Dis. 1980; 141:727–732. [PubMed: 7391615]

Ravio TL. Envelope stress responses and Gram-negative bacterial pathogenesis. Mol Microbiol. 2005; 56:1119–1128. [PubMed: 15882407]

Ren G, Stephan D, Xu Z, Zheng Y, Tang D, Harrison RS, et al. Properties of the thioredoxin fold superfamily are modulated by a single amino acid residue. J Biol Chem. 2009; 284:10150–10159. [PubMed: 19181668]

Rossier O, Dao J, Cianciotto NP. The type II secretion system of *Legionella pneumophila* elaborates two aminopeptidases, as well as a metalloprotease that contributes to differential infection among protozoan hosts. Appl Environ Microbiol. 2008; 74:753–761. [PubMed: 18083880]

Rowbotham TJ. Preliminary report on the pathogenicity of *Legionella pneumophila* for freshwater and soil amoebae. J Clin Pathol. 1980; 33:1179–1183. [PubMed: 7451664]

Sexton JA, Vogel JP. Regulation of hypercompetence in *Legionella pneumophila*. J Bacteriol. 2004; 186:3814–3825. [PubMed: 15175295]

Stenson TH, Weiss AA. DsbA and DsbC are required for secretion of pertussis toxin by *Bordetella pertussis*. Infect Immun. 2002; 70:2297–2303. [PubMed: 11953363]

Tinsley CR, Voulhoux R, Beretti J, Tommassen J, Nassif X. Three homologues, including two membrane-bound proteins, of the disulfide oxidoreductase DsbA in *Neisseria meningitidis*: Effects on bacterial growth and biogenesis of functional type IV pili. J Biol Chem. 2004; 279:27078–27087. [PubMed: 15105427]

Vincent CD, Friedman JR, Jeong KC, Buford EC, Miller JL, Vogel JP. Identification of the core transmembrane complex of the *Legionella* Dot/Icm type IV secretion system. Mol Microbiol. 2006; 62:1278–1291. [PubMed: 17040490]

Viswanathan VK, Kurtz S, Pedersen LL, Abu Kwaik Y, Krcmarik K, Mody S, Cianciotto NP. The cytochrome c maturation locus of *Legionella pneumophila* promotes iron assimilation and intracellular infection and contains a strain-specific insertion sequence element. Infect Immun. 2002; 70:1842–1852. [PubMed: 11895946]

Vogt SL, Nevesinjac AZ, Humphries RM, Donnenberg MS, Armstrong GD, Raivio TL. The *cpx* envelope stress response both facilitates and inhibits elaboration of the enteropathogenic *Escherichia coli* bundle-forming pilus. Mol Microbiol. 2010; 76:1095–1110. [PubMed: 20444097]

Yu JR, Wagner MR, Laird G, Melli S, Rey R, Lo P, Dao SC, Sahinalp M, Ester LJ, Foster FS, Brinkman L. PSORTb 3.0: Improved protein subcellular localization prediction with refined localization subcategories and predictive capabilities for all prokaryotes. Bioinformatics. 2010; 26:1608–1615. [PubMed: 20472543]

Zhang H, Donnenberg MS. DsbA is required for stability of the type IV pilin of enteropathogenic *Escherichia coli*. Molecular Microbiology. 1996; 21:787–797. [PubMed: 8878041]
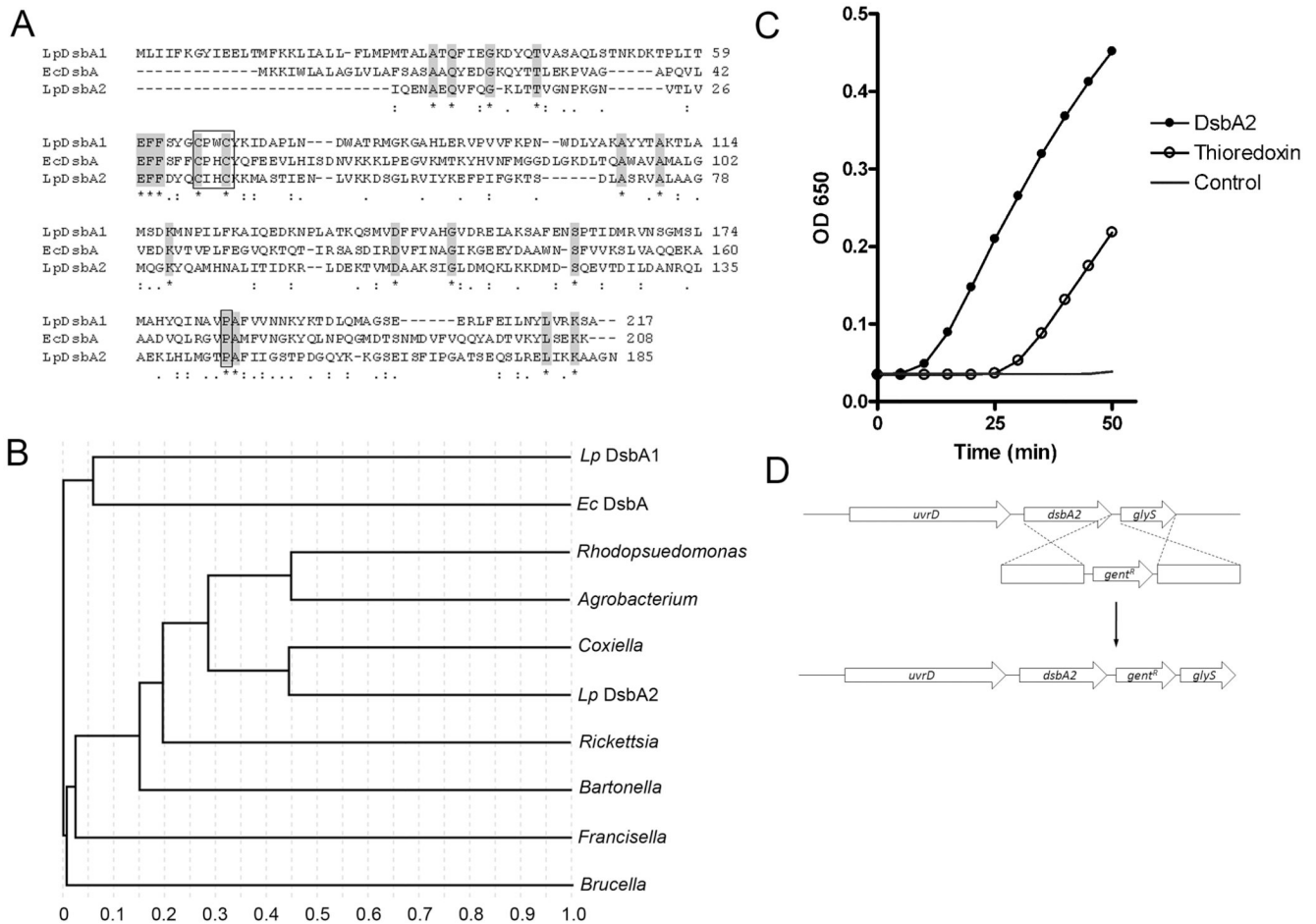
Fig. 1. Phylogenetic analysis and thioredoxin activity of DsbA2 (Lpg1841)
(A) Amino acid sequence alignments: *L. pneumophila* AAU26230.1 (Lp DsbA), *E. coli* CAA56736.1 (Ec DsbA), and *L. pneumophila* YP_095867.1 (Lp DsbA2). Conserved amino acids are highlighted; active site CXXC and conserved *cis*-proline residues are boxed. (B) ClustalW phylogenetic analysis delineates a sub-set of DsbA-like oxidoreductases present in several T4SS containing bacteria. Strains listed in Fig. 1A above and *Rhodopseudomonas palustris* NP_946373.1 (*Rhodopseudomonas*), *Agrobacterium tumafaciens* AAK87124.1 (*Agrobacterium*), *Coxiella burnetti* BAA20499.1 (*Coxiella*), *Rickettsia typhi* YP_067376.1 (*Rickettsia*), *Bartonella henselae* YP_033613.1 (*Bartonella*), *Francisella tularensis* YP_170079.1 (*Francisella*), and *Brucella melitensis* NP_540478.1 (*Brucella*). All proteins contain CxxC active site and conserved *cis*-proline. (C) Precipitation of insulin was followed spectrophotometrically after the addition of 1 µM thioredoxin (open circles), 5 µM DsbA2 (dark circles), or no enzyme, DTT control (no symbol). A representative experiment is shown. (D) Genetic organization of the *dsbA2* locus and allelic replacement strategy to introduce a gentamicin cassette downstream of *dsbA2*. Insertions within *dsbA2* were not obtained and considered lethal.
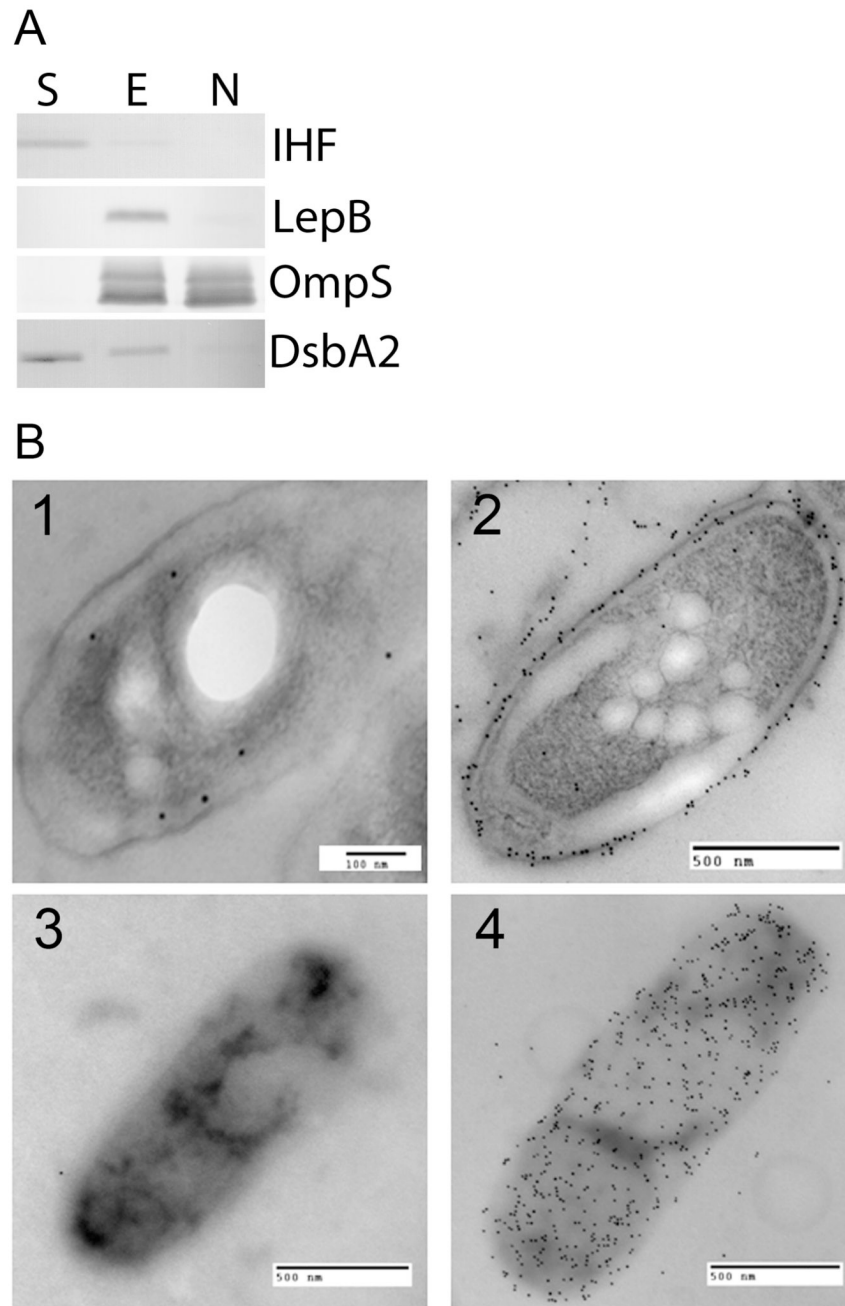
Fig. 2. DsbA2 is not an outer membrane protein
(A) DsbA2 partitions into the soluble phase after Triton X-100 separation. Whole-cell lysates of *L. pneumophila* were cleared by low-speed centrifugation (10,000 × g), and membrane material was pelleted by ultra-centrifugation (100,000 × g). The Triton-extractable inner membrane proteins (Lane E) were re-suspended in 1% Triton X-100, and the Triton-insoluble outer membrane proteins (Lane N) were re-pelleted by ultra-centrifugation. Equivalent protein amounts were loaded in all lanes, and to ensure fraction separation, a known soluble phase protein (IHF), inner membrane protein (LepB), and outer membrane protein (OmpS) were included as controls for the soluble (S), inner membrane (E), and outer membrane protein (N) fractions, respectively. (B) Immunogold electron

microscopy of ultrathin sections and whole cells of *L. pneumophila*. Panels 1 and 3 are developed with DsbA2 primary antibody and 10 nm gold conjugated goat anti-mouse IgG secondary antibodies, while Panels 2 and 4 are developed with OmpS-specific antibody and 10 nm gold conjugated goat anti-rabbit antibodies. Counting procedures (detailed in the Methods) indicate that DsbA2 is located in the periplasm 63% of the time with 95% confidence. The DsbA2-specific antibody does not cross react with DsbA1.
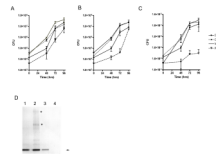
Fig. 3. Expression of P198T mutant protein inhibits intracellular growth in amoeba

Acanthamoeba castellanii were infected with *L. pneumophila* AA100 constructs (A) in the absence of IPTG induction, (B) induction at 0.5 mM IPTG, and (C) at 5 mM IPTG. Colony forming units (cfu) were determined in triplicate at the indicated times in a single experiment: AA100 pMMB:empty (no symbol), DsbA2 (filled circle), DsbA2 C89S (dashed line), or DsbA2 P198T (open triangle). (D) Immunoblot detection of DsbA2 proteins expressed during infection of *A. castellenii*. Lanes 1. pMMB: DsbA2; Lane 2, pMMB:C89S; Lane 3, pMMB:empty; and Lane 4, pMMB:P198T. DsbA2 at 30 kDa is indicated by the arrow. SDS PAGE was run under non-reducing conditions and higher molecular weight complexes than DsbA2 can be seen in bacteria expressing wild type DsbA2 (Lane 1) and C89S (Lane 3) that disappear under reducing conditions (data not shown). Asterisks denote bands unique to the C89S mutant.
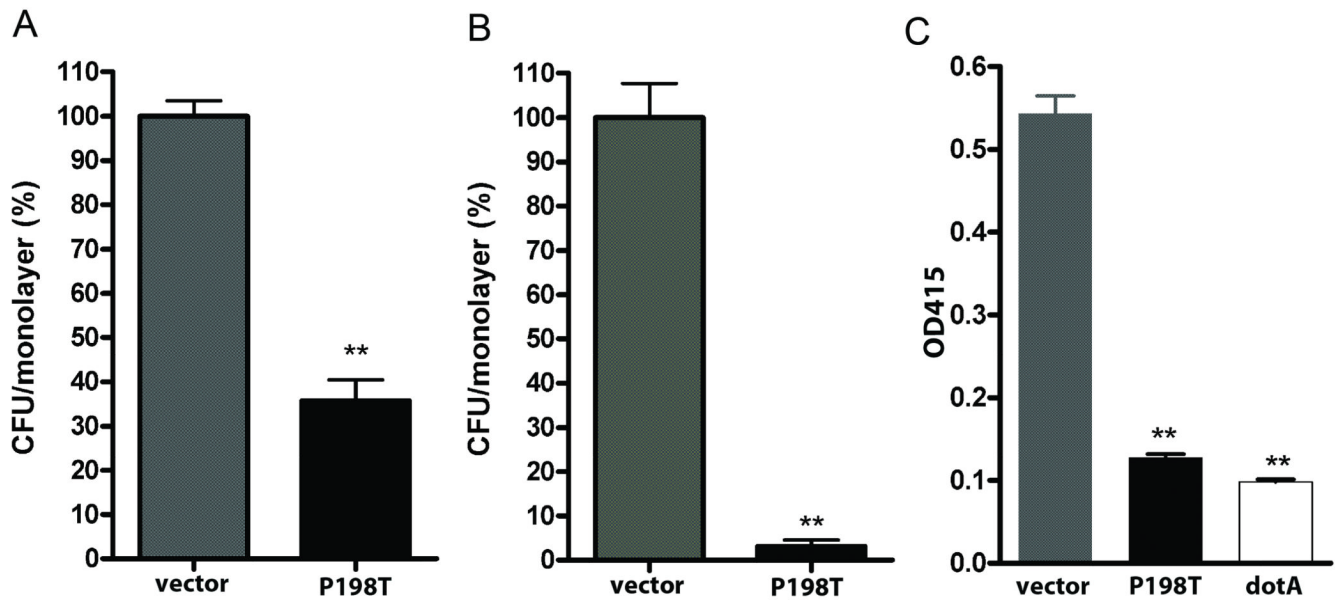
Fig. 4. Expression of P198T mutant protein inhibits infectivity of HeLa cells and contact lysis of erythrocytes

Effects of P198T expression on attachment to and invasion of HeLa cell monolayers. (A) Attachment of AA100 expressing P198T (black) to HeLa cells was reduced by 60% compared with the empty vector control (grey). (B) Invasion of HeLa cells (gentamicin treatment) by AA100 P198T (black) was decreased by >90% compared to the empty vector control (grey). (C) Erythrocyte contact lysis assay. Erythrocyte lysis was measured at A_{450} with the AA100 (empty vector) control (grey), AA100 expressing P198T (black) and a *dotA* mutant that is defective in T4SS (white). ** denotes statistical significance of < 0.05 by student t-test.
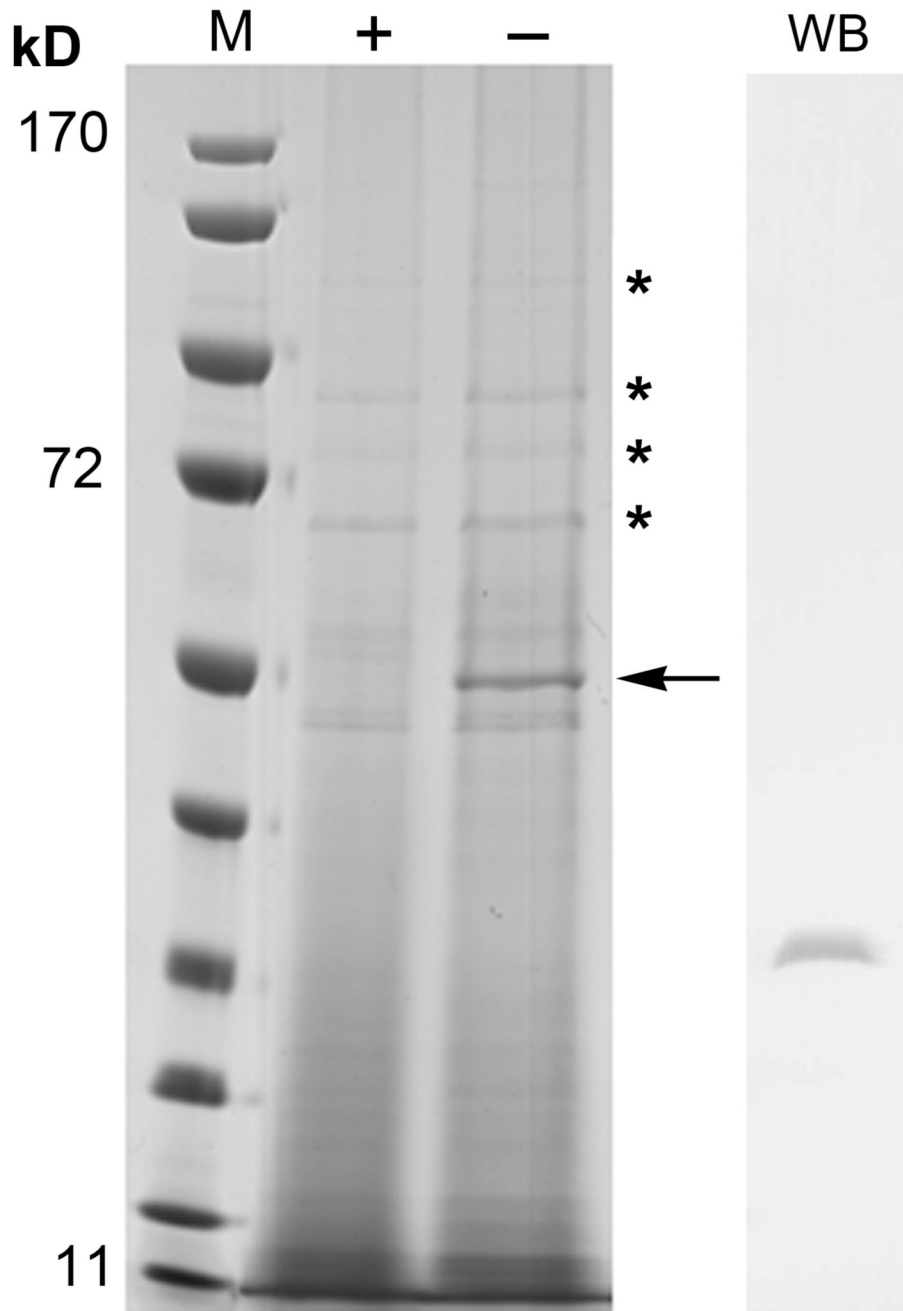
Fig. 5. Expression of P198T mutant protein inhibits flagellar synthesis
Coomassie stained SDS PAGE of the osmotic shockate from the AA100 P198T mutant
strain after induction with 1 mM IPTG (+) or uninduced (−). The 55 kDa band noted by the
arrow in the uninduced lane and absent from the induced lane was identified as flagellin by
MALDI TOF MS. * indicate periplasmic proteins that are identical between induced and
uninduced conditions. Lane M denotes molecular weight markers in kilodaltons (kD). WB is
a western blot with DsbA2-specific antibody indicating that DsbA2 is present in the
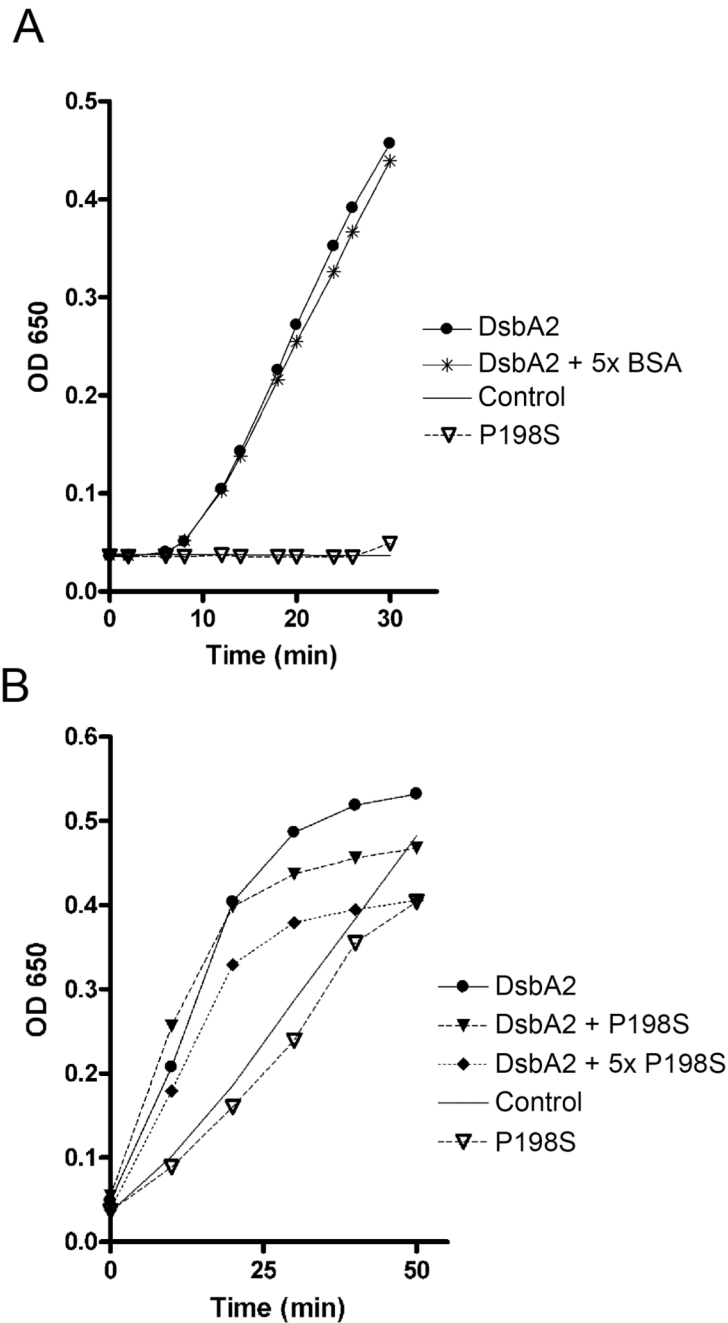shockate.

Fig. 6. DsbA2 P198T inhibits DsbA2 activity in a modified insulin reduction assay
(A) DsbA2 P198T (5 µM, open triangle) has no activity greater than control (solid line) in the insulin reduction assay. DsbA2 activity in this assay (solid circle) is not affected by addition of 25 µM BSA (star). (B) Modified insulin reduction assay (see text): Insulin precipitation by 5 µM DsbA2 (black circle) and competition with 5 µM (dark dashed triangles) or 25 µM P198T (dark dashed diamonds) mutant protein. As noted in panel A, P198T (open triangle) displayed no activity over that of the DTT control (solid line). The dose dependent effect of the P198T mutant protein on DsbA2 activity is consistent with the possibility that the mutant protein, by binding insulin, has a rate limiting effect on the

activity of DsbA2. These experiments were repeated three separate times, representative experiment shown.
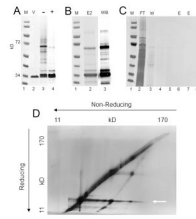
Fig. 7. Substrate-protein capture by P198T-mutant protein in *L. pneumophila*
(A). Immunoblots (DsbA2 antibodies) of whole cell lysates subjected to SDS-PAGE under non-reducing conditions or as indicated: Lane 1 MW markers; Lane 2, AA100 pMMB:P198T un-induced whole cell lysates; Lane 3 AA100 pMMB:P198T induced with 1 mM IPTG; Lane 4, same as Lane 3 except run under reducing conditions (note disappearance of higher molecular weight complexes). (B) His_6 DsbA2-substrate complexes enriched for by Ni^{++} column chromatography and separated by non-reducing SDS PAGE: Coomassie stain (E2, lane 2), and DsbA2 western blot (WB, lane 3). (C) Control NI^{++} column of AA100 lysate, flow through (FT, lane 2), washes (W, lane 3–5), and elutions (E, lanes 6–8) show that there is negligible non-specific binding of *Legionella* proteins to the nickel column. (D) AA100 pMMB:P198T was induced *in vitro* and His_6-tagged DsbA2 P198T substrate complexes were purified by Ni^{++} chromotography. Diagonal gels of captured proteins were run under non-reducing (direction of electrophoresis indicated by arrows), then reducing conditions. Protein spots (silver stained) off the diagonal are DsbA2 interacting proteins whose disulfide bridge had been reduced in the second dimension. The identities of these proteins by MS are listed in Table 2. Arrow points to DsbA2 that has dissociated from interacting proteins in the second dimension.

Table 1

Primers used in this study

DsbA2 F:	GGGAATTCTAAGGGGAATTACGTGAAATTTAC
DsbA2 R:	TAA GGA TCC TTA ATTGCCAGCCGCC
C89S-F:	GGGAATTCTAAGGGGAATTACGTGAAATTTAC
C89S-R:	GATGCCATTTTTTTGGAATGAATGC
P198T-F:	CATTTGATGGGTACAACAGCCTTTATAATTGG
P198T-R:	CCA ATT ATA AAG GCT GTT GTA CCC ATC AAA TG
DsbA2 6his:	TAA GGA TCC TTA GTG ATG ATG ATG ATG ATT GCC AGC CGC C
DsbA1-1F:	AGTACCGCCACCTAAGTCGACCTCAAGCTATAAAGGATATTC
DsbA1-2R:	AAACTCGAGCCCGATGTCGAGCCCTCTG
DsbA1-3F:	TCATGGATCCGCAAGGTATTAGTAC
DsbA1-4R:	ACAGGCTTATGTCAAGTCGACGTTGCCAAAGCAGTCATTGGC
DsbA2-1F:	AACGGATCCCTGAGGTACCCTGACTTG
DsbA2-2R:	GTGGCTTTGAGCTCGGTATTTTCAATGGTAGATGCCA
DsbA2-3F:	CTAAGAGCTCGGTACCCGGAGCAATCACTACGTGAG
DsbA2-4R:	GGCGGATCCGAGTAATACCACCTGAAATTG

Table 2

DsbA2 Interacting Proteins

Protein	Accession	Localized	Size	Reference	Cysteines
IcmE (DotG)	gi52840696	OM	108 kDa	Vogel[a]	16
Major Outer Membrane Protein	gi52843155	OM	32 kDa	Hoffman[b]	4
DotC	gi52842881	OM	34 kDa	Vogel	2
HtpB (GroEL)	gi52840925	P/OM	58 kDa	Hoffman[c]	4
cytochrome c oxidase	gi52843092	IM	45 kDa	predicted	4
Sensory transduction system	gi52840618	IM	24 kDa	predicted	1
aspartate kinase/decarboxylase	gi52842038	C	95 kDa	predicted	12
DnaJ	gi52842241	C/P	41 kDa	predicted	10
Dioxygenase	gi52842491	C	41 kDa	predicted	5
LPS biosynthesis protein	gi52840985	C	54 kDa	predicted	7
penicillin binding protein 1A	gi52841160	IM	89 kDa	predicted	2
Glu/Leu/Phe dehydrogenase	gi52842489	C	38 kDa	predicted	3
co-chaperonin HtpA (GroES)	gi52840924	IM	10 kDa	Hoffman	0
aminopeptidase	gi52843010	OM	47 kDa	predicted	6
cell-division protein FtsA	gi52842816	C	45 kDa	predicted	8
membrane protein YdgA-like	gi52840785	OM	55 kDa	predicted	1
hypothetical lpg1994	gi52842211	OM	45 kDa	predicted	2
S-adenosyl-L-homocysteine hydrolase	gi52842238	C	49 kDa	predicted	8
hypothetical lpg2327	gi52842537	C	34 kDa	predicted	2
malate dehydrogenase	gi52842562	Unk	36 kDa	predicted	6
hypothetical 2519	gi52842727	Unk	21 kDa	predicted	2
IcmX	gi52842895	IM	51 kDa	predicted	3
EnhA	gi52840941	OM	23 kDa	predicted	7
DotK	gi52840692	OM	21 kDa	Vogel[a]	3
D-alanyl-D-alanine carboxypeptidase	gi52841739	IM	48 kDa	predicted	2
SCO1/SenC family (thioredoxin-like) protein	gi52840658	Unk	24 kDa	predicted	2
hypothetical protein lpg0732	gi52840969	OM	23 kDa	predicted	1
hypothetical protein lpg1585 (similar EnhB)	gi52841815	OM	18 kDa	predicted	4

Protein	Accession	Localized	Size	Reference	Cysteines
16kD immunogenic protein	gi52841943	OM	16 kDa	predicted	3
alkylhydroperoxide reductase	gi52842560	C	24 kDa	predicted	3

[a] Vincent *et al.*, 2006

[b] Hoffman et al., 1992b

[c] Hoffman et al., 1990