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DsbL and DsbI contribute to periplasmic disulfide bond formation in *Salmonella enterica* serovar Typhimurium

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Disulfide bond formation in periplasmic proteins is catalysed by the DsbA/DsbB system in most Gram-negative bacteria. *Salmonella enterica* serovar Typhimurium also encodes a paralogous pair of proteins to DsbA and DsbB, DsbL and DsbI, respectively, downstream of a periplasmic arylsulfate sulfotransferase (ASST). We show that DsbL and DsbI function as a redox pair contributing to periplasmic disulfide bond formation and, as such, affect transcription of the *Salmonella* pathogenicity island 1 (SPI1) type three secretion system genes and activation of the RcsCDB system, as well as ASST activity. In contrast to DsbA/DsbB, however, the DsbL/DsbI system cannot catalyse the disulfide bond formation required for flagellar assembly. Phylogenic analysis suggests that the *assT dsbL dsbI* genes are ancestral in the *Enterobacteriaceae*, but have been lost in many lineages. Deletion of *assT* confers no virulence defect during acute *Salmonella* infection of mice.

INTRODUCTION

Disulfide bond formation in periplasmic proteins is catalysed primarily by the DsbA/DsbB system (Kadokura *et al.*, 2003). DsbA, a 21 kDa periplasmic protein with a catalytic CXXC motif, exchanges its disulfide bond with pairs of cysteines in substrate proteins (Kadokura *et al.*, 2003). DsbA is reoxidized by DsbB, an inner-membrane protein with two periplasmic loops, each containing a pair of cysteine residues (Collet & Bardwell, 2002). DsbB oxidizes DsbA by a disulfide exchange with the cysteine pair in one periplasmic loop, and then transfers the electrons, via disulfide exchange in the second periplasmic loop, to quinones in the cytoplasmic membrane (Collet & Bardwell, 2002; Inaba & Ito, 2008).

Mutants lacking DsbA or DsbB exhibit pleiotropic phenotypes due to defects in the folding of numerous cell envelope proteins. For example, in *Escherichia coli*, alkaline phosphatase is inactive and unstable without disulfide bonds (Bardwell *et al.*, 1991). These mutants also show reduced motility because of improper assembly of the flagellum (Dailey & Berg, 1993). In pathogens, mutations in *dsbA* can result in decreased virulence because factors such as pili, toxins and type III secretion systems (T3SSs) require disulfide bonds to function properly (Ellermeier & Slauch, 2004; Ha *et al.*, 2003; Jackson & Plano, 1999; Miki *et al.*, 2004; Peek & Taylor, 1992; Watarai *et al.*, 1995).

Some Gram-negative bacteria produce DsbA and/or DsbB paralogues that either supplement or replace DsbA and DsbB activity (Grimshaw *et al.*, 2008; Kwon & Choi, 2005; Raczko *et al.*, 2005). For example, the *Salmonella enterica* virulence plasmid encodes a DsbA paralogue, SrgA, whose activity is dependent on functional DsbB (Bouwman *et al.*, 2003). The genome sequence suggests that *S. enterica* serovar Typhimurium also encodes an additional oxidative system. DsbL, a DsbA paralogue, and DsbI, a DsbB paralogue, are encoded downstream of a periplasmic arylsulfate sulfotransferase (ASST), which catalyses the transfer of a sulfate group among phenolic compounds (Kim *et al.*, 1992). Highly similar gene clusters are found throughout the genus *Salmonella* and in a subset of *Enterobacteriaceae*. ASST homologues exist in other Gram-negative proteobacteria, Gram-negative bacteroidetes, and the Gram-positive firmicutes (Grimshaw *et al.*, 2008). DsbL and DsbI homologues, not associated with ASST enzymes, are also apparent in a variety of Gram-negative organisms (Kimball *et al.*, 2003; Raczko *et al.*, 2005).

Our previous work showed that *dsbA* in *Salmonella* is transcriptionally controlled by the *Salmonella* pathogenicity island 1 (SPI1) T3SS regulatory circuit (Ellermeier & Slauch, 2004). Loss of DsbA results in both dysfunction of the secretion apparatus and transcriptional downregulation

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Abbreviations: ASST, arylsulfate sulfotransferase; i.p., intraperitoneal(ly); SPI1, *Salmonella* pathogenicity island 1; T3SS, type III secretion system; UPEC, uropathogenic *E. coli*.

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of the SPI1 system (Lin *et al.*, 2008). SPI1 transcriptionally responds to disulfide bond status through the flagellar apparatus and the RcsCDB system (Lin *et al.*, 2008). Here we present evidence that DsbL and DsbI also contribute to disulfide bond formation in the *Salmonella* periplasm. We further prove that the *Salmonella assT* gene encodes an ASST, dependent on a functional disulfide bond oxidoreductase system.

METHODS

Media and reagents. Luria–Bertani (LB), M63 glucose minimal, or low-phosphate (0.1 mM) glucose MOPS minimal media were used for growth of bacteria (Neidhardt *et al.*, 1974; Silhavy *et al.*, 1984). DTT was added to LB cultures to prevent free cystine from contributing to disulfide bond formation. Motility agar contained 0.3 % (w/v) agar in M63 glucose minimal medium. Bacterial strains were grown at 37 °C, except for those containing the temperature-sensitive plasmids pCP20 and pKD46 (Cherepanov & Wackernagel, 1995; Datsenko & Wanner, 2000), which were grown at 30 °C. Antibiotics were used at the following concentrations: 100 μg ampicillin ml^{-1}, 10 μg chloramphenicol ml^{-1}, 50 μg kanamycin ml^{-1}, 50 μg apramycin ml^{-1}. Enzymes were from Invitrogen and used according to the manufacturer's recommendations. Primers were from IDT.

Bacterial strains and plasmid construction. Bacterial strains, listed in Table 1, were isogenic derivatives of *S. enterica* serovar Typhimurium strain 14028, *E. coli* K-12 strain DH5α or K-12 strain MC4100. Deletions of genes and concomitant insertion of an antibiotic resistance cassette were performed using λ Red-mediated recombination (Datsenko & Wanner, 2000; Yu *et al.*, 2000), as described previously (Ellermeier *et al.*, 2002), and were verified by PCR analysis. The end points of each deletion are indicated. In each case, the resulting constructs were P22-transduced into a wild-type background (Maloy *et al.*, 1996). In some cases, antibiotic resistance cassettes were removed using plasmid pCP20 encoding FLP recombinase (Datsenko & Wanner, 2000). The *wca–lacZ*, *dsbL–lacZ* and *assT–lacZ* transcriptional fusions were created using FLP/FRT-mediated site-specific recombination, as described previously (Ellermeier *et al.*, 2002). Note that these fusion constructs create null mutations in these genes. The *phoA*–6 × His–*lacZ* fusion was constructed by first inserting the sequences encoding 6 × His at the 3′ end of the *phoA* ORF followed immediately by a Kn cassette via λ Red-mediated recombination. The *lacZ* fusion construct was subsequently introduced as described above. The *hilA–lacZ* fusion, integrated at the lambda attachment site, had been constructed previously (Lin *et al.*, 2008). The following genes were amplified using primers carrying *Eco*RI or *Bam*HI sites and then cloned into vector pWKS30 (Wang & Kushner, 1991) with base-pairs indicated relative to the start site of translation of the first gene in the construct: *assT* (−14→+1808); *assT dsbLI* (−14→+3194); *dsbL* (−14→+687); *dsbI* (−14→+693); *dsbLI* (−14→+1379); *dsbA* (−30→+645); *dsbB* (−21→+547). All bacterial strains were rebuilt at least once and experiments were repeated with the independent constructs.

Enzymic assays. *β*-Galactosidase assays were performed using a microtitre plate assay, as described previously (Slauch & Silhavy, 1991). Strains were cultured overnight in LB medium, then subcultured in LB containing 1 % (w/v) NaCl and 0.5 mM DTT and grown statically overnight. *β*-Galactosidase activities were calculated as (μmol *o*-nitrophenol formed min^{-1}) × 10^6/(OD_{600} × ml cell suspension). The values are presented as mean ± SD, where *n*=4. Phosphatase assays were performed in an essentially identical manner except that cells were grown in MOPS medium with limited phosphate to induce *phoA* expression. Cells were resuspended in 1 M Tris buffer and *p*-nitrophenylphosphate was used as substrate.

ASST activity was quantified by monitoring the production of *p*-nitrophenol from the hydrolysis of *p*-nitrophenylsulfate (PNS), using α-naphthol as an acceptor (Kwon *et al.*, 2001). Cultures were grown overnight at 37 °C in LB broth with 2 mM DTT and 100 μg ampicillin ml^{-1}. A 100 μl aliquot of each culture was inoculated into 900 μl M63 glucose minimal medium with 0.1 mM PNS, 0.2 mM α-naphthol, 2 mM DTT and 100 μg ampicillin ml^{-1}, and incubated with shaking at 37 °C for 1 h. To stop the reaction, 1 ml 1.0 M NaOH was added to each culture. The A_{420} was measured. ASST activities were calculated as (μmol *p*-nitrophenol formed min^{-1}) × 10^6/(OD_{600} × ml cell suspension). The values are presented as mean ± SD, where *n*=4.

To assay *β*-galactosidase activity in bacteria grown in macrophages, RAW264.7 macrophage cells (~10^5 per well) grown in Dulbecco's minimal essential medium (DMEM) were infected with *Salmonella* opsonized in 50 % mouse serum (Equitech-bio) at an m.o.i. of 10. After 30 min, cells were rinsed with PBS three times and incubated for 20 min in DMEM containing 12.5 μg gentamicin ml^{-1}. Macrophages were then washed with PBS and incubated in DMEM containing 12.5 μg gentamicin ml^{-1} for 16 h. Cells were washed with PBS three times and macrophages were scraped in PBS to remove them from the dish. Macrophage cells were disrupted with cold distilled H_2O and bacteria were recovered by centrifugation and resuspended in PBS. The number of viable bacterial cells recovered was determined by diluting and plating for c.f.u. The remaining samples were used to measure *β*-galactosidase activity with the chemiluminescent substrate Lumigal 530 according to the manufacturer's instructions (Lumigen). The *β*-galactosidase activity was calculated as luminescent units per c.f.u. of bacteria in each sample. The *β*-galactosidase activity of bacteria grown *in vitro* in LB medium (16 h of growth) was measured using the same method. The values are presented as mean ± SD, where *n*=3.

PhoA immumoblot. Strains containing the phoA–6 × His–*lacZ* fusions were grown overnight in MOPS medium with limiting phosphate to induce *phoA* expression. Each sample was assayed for *β*-galactosidase activity and phosphatase activity (see above). In addition, 20 μl of each culture was combined with loading buffer and proteins were separated on a 4–20 % gradient polyacrylamide gel containing SDS. After electrophoresis, proteins were electrotransferred to nitrocellulose. Mouse monoclonal anti-6-His IgG antibody (Zymed) was used as primary antibody. The secondary antibody was horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (Abcam). The Western blotting and the detection of HRP-conjugated antibodies with a chemiluminescent system were done according to the instructions of the manufacturer (Amersham).

Mouse virulence assays. Mutant and wild-type *Salmonella* were grown for 16 h in LB medium. Equal volumes of each culture were mixed and then diluted in sterile 0.2 M sodium phosphate buffer (pH 8.0) to 5 × 10^8 c.f.u. ml^{-1} (oral) or in sterile 0.15 M NaCl to 5 × 10^3 c.f.u. ml^{-1} [for intraperitoneal (i.p.) administration]. Six to eight-week-old female BALB/c mice (Harlan) were inoculated either orally or i.p. with 0.2 ml of a bacterial suspension. The actual c.f.u. and relative percentage of each strain were determined by direct plating of the inoculum. After 4–5 days, the mice were sacrificed by CO_2 asphyxiation and the entire intestine (oral) and/or spleen (oral and i.p.) were harvested. These organs were homogenized, and serial dilutions were plated on the appropriate medium to recover bacteria. Replica plating on appropriate antibiotic medium was used to determine the percentage of each strain. Competitive index was calculated as (percentage strain A recovered/percentage strain B

Table 1. Strains and plasmids

Strain	Genotype*	Deletion end points†	Source or reference‡
14028	Wild-type serovar Typhimurium		ATCC§
JS107	*zjg8101*::Kn		Mann & Slauch (1997)
JS326	Δ*dsbA100*::Cm	4 204 198–4 204 820	Ellermeier & Slauch (2004)
JS741	Δ*srgA1*::Kn	6670–7315 (plasmid pSLT)	Lin *et al.* (2008)
JS748	Δ*dsbA100*::Cm Δ*srgA1*::Kn		Lin *et al.* (2008)
JS749	*att*λ::pDX1::*hilA′–lac*$^+$		Lin *et al.* (2008)
JS754	Δ*dsbA100*::Cm *att*λ::pDX1::*hilA′–lac*$^+$		Lin *et al.* (2008)
JS759	Δ*srgA1*::Kn *att*λ::pDX1::*hilA′–lac*$^+$		Lin *et al.* (2008)
JS761	Δ*dsbA100*::Cm Δ*srgA1*::Kn *att*λ::pDX1::*hilA′–lac*$^+$		Lin *et al.* (2008)
JS849	Δ*dsbB102*::Kn	1 908 514–1 909 037	
JS850	Δ*dsbL103*	3 356 318–3 356 996	
JS851	Δ*dsbI104*	3 357 005–3 357 681	
JS852	Δ*assT*::Kn	3 354 505–3 356 308	
JS853	Δ(*assT dsbLI*)*2919*	3 354 505–3 357 681	
JS854	Δ*assT*::Cm	3 354 509–3 354 550	
JS855	Δ*stm4098*::Cm	4 306 884–4 308 668	
JS856	Φ(*wca–lac*$^+$)		
JS857	Φ(*dsbL–lac*$^+$)*103*		
JS858	Φ(*assT–lac*$^+$)		
JS859	Δ*dsbL103 att*λ::pDX1::*hilA′–lac*$^+$		
JS860	Δ*dsbA100*::Cm Δ*dsbL103 att*λ::pDX1::*hilA′–lac*$^+$		
JS861	Δ*dsbB102*::Kn *att*λ::pDX1::*hilA′–lac*$^+$		
JS862	Δ*dsbI104 att*λ::pDX1::*hilA′–lac*$^+$		
JS863	Δ*dsbB102* Δ*dsbI104 att*λ::pDX1::*hilA′–lac*$^+$		
JS864	Δ*dsbA100* Φ(*wca*–lac$^+$)		
JS865	Δ*dsbA100* Δ*srgA1* Φ(*wca*–lac$^+$)		
JS866	Δ*dsbL103* Φ(*wca*–lac$^+$)		
JS867	Δ*dsbA100*::Cm Δ*dsbL103* Φ(*wca*–lac$^+$)		
JS868	Δ*dsbA100*::Cm Δ*srgA1* Δ*dsbL103* Φ(*wca*–lac$^+$)		
JS869	Δ*dsbB102* Φ(*wca*–lac$^+$)		
JS870	Δ*dsbI104* Φ(*wca*–lac$^+$)		
JS871	Δ*dsbB102* Δ*dsbI104* Φ(*wca*–lac$^+$)		
JS872	Δ*dsbA100*::Cm Δ*dsbB102* Φ(*wca*–lac$^+$)		
JS873	Δ*dsbA100*::Cm Δ*dsbB102*::Kn		
JS874	Δ*dsbA100*::Cm Δ(*assT dsbLI*)*2919*		
JS875	Δ*dsbA100*::Cm Δ*srgA1*::Kn Δ(*assT dsbLI*)*2919*		
JS876	Δ*srgA1*::Kn Δ(*assT dsbLI*)*2919*		
JS877	Δ*dsbB102*::Kn Δ(*assT dsbLI*)*2919*		
JS878	Δ*assT*::Kn Δ*stm4098*::Cm		
JS879	Δ*assT*::Kn Δ*stm4098*::Cm *zjg8101*::Kn		
DH5αλpir	*E. coli* K-12 *endA1 hsdR17 supE44 thi-1 recA1 gyrA relA1* Δ(*lac–argF*)*U169 deoR* ϕ80 Δ(*lac*)M15 λ*pir*$^+$		Laboratory stock
DX1616	DH5αλpir Δ*dsbA*::Kn	4 041 443–4 042 077	
DX1617	DH5αλpir Δ*dsbB*::Cm	1 231 727–1 232 200	
MC4100	F^- *araD139* Δ(*argF–lac*)*U169 rpsL150 relA flhD5301 ptsF25 deoCI*		Silhavy *et al.* (1984)
BK683	MC4100 Φ(*phoA–6×his–lac*$^+$)		
BK685	MC4100 Φ(*phoA–6×his–lac*$^+$) Δ*dsbA*		

Plasmid	Relevant characteristics	Cloned end points†	Reference‡
pKD46	*bla* P_{BAD} *gam bet exo* pSC101 oriTS		Datsenko & Wanner (2000)
pCP20	*bla cat cI*857 λP_R *flp* pSC101 oriTS		Cherepanov & Wackernagel (1995)
pAH125	*lacZ* t_{L3} λ*attP* oriR6K Kan t_{mgB}		Haldimann & Wanner (2001)
pDX1	*lacZ* t_{L3} λ*attP* oriR6K *aacIV* t_{mgB}		

Table 1. cont.

Plasmid	Relevant characteristics	Cloned end points†	Reference‡
pWKS30	pSC101 ori, Ap^r		Wang & Kushner (1991)
pDsbA	pWKS30 : : *dsbA*	4 204 168–4 204 843	
pDsbB	pWKS30 : : *dsbB*	1 908 492–1 909 060	
pDsbL	pWKS30 : : *dsbL*	3 356 302–3 357 003	
pDsbI	pWKS30 : : *dsbI*	3 356 988–3 357 695	
pDsbLI	pWKS30 : : *dsbLI*	3 356 302–3 357 695	
pAssT	pWKS30 : : *assT*	3 354 487–3 356 309	
pAssT DsbLI	pWKS30 : : (*assT dsbLI*)	3 354 487–3 357 695	

*All *Salmonella* strains are isogenic derivatives of serovar Typhimurium strain 14028, unless otherwise noted.
†Numbers indicate the base pairs (inclusive) that are deleted or cloned as defined in the *S. enterica* serovar Typhimurium LT2 or *E. coli* K-12 MG1655 genome sequences (National Center for Biotechnology Information database).
‡This study unless specified otherwise.
§American Type Culture Collection.

recovered)/(percentage strain A inoculated/percentage strain B inoculated). Student's *t* test was used for statistical analysis. Animal work was reviewed and approved by the University of Illinois Institutional Animal Care and Use Committee (IACUC) and performed under protocol 07070.

Phylogenetic analysis. Sequences were aligned using CLUSTAL W (Thompson *et al.*, 1994). Phylogeny was inferred by maximum-likelihood under the KY85 model in PAUP* v4b10 (Wilgenbusch & Swofford, 2003). The robustness of each alignment was determined through non-parametric bootstrap analyses consisting of 1000 replicates (Felsenstein, 1985).

RESULTS

Loss of DsbL or DsbI affects transcription of *hilA* and *wca* genes

We previously showed that loss of DsbA, the primary disulfide bond oxidoreductase in the periplasm, or the cognate DsbB, decreases transcription of the SPI1 genes in serovar Typhimurium (Ellermeier & Slauch, 2004; Lin *et al.*, 2008). During our studies, we recognized a pair of DsbA and DsbB paralogues encoded together in the *Salmonella* genome. DsbL (STM3193) is 27 % identical in amino acid sequence to DsbA, and has a putative CPFC active site motif. DsbI (STM3194) is 30 % identical in sequence to DsbB, and has two pairs of cysteine residues like DsbB. To test the ability of DsbLI to form disulfide bonds, we took advantage of the well-established fact that both the activity and the stability of the *E. coli* periplasmic alkaline phosphatase PhoA are dependent on disulfide bond formation in the protein (Bardwell *et al.*, 1991). To monitor PhoA activity, protein levels, and expression at the same time, we tagged the C terminus of the PhoA protein with 6 × His and inserted a promoterless *lacZ* gene immediately downstream of the *phoA* ORF. We then constructed isogenic strains that were $dsbA^+$, $dsbA^-$ or $dsbA^-$ containing a low-copy-number plasmid in which the *dsbL* and *dsbI* genes were cloned downstream of a *lac* promoter (pDsbLI). These strains were grown in phosphate-limiting minimal medium, the resulting cells were assayed for alkaline phosphatase activity and β-galactosidase activity, and Western blot analysis was performed to monitor PhoA protein levels. As shown in Fig. 1, the $dsbA^+$ cells had robust alkaline phosphatase and β-galactosidase activities and the PhoA protein was evident in the cell extracts. The *dsbA* mutant showed increased β-galactosidase activity, suggesting that transcription of the *phoA* gene was increased in this background. However,

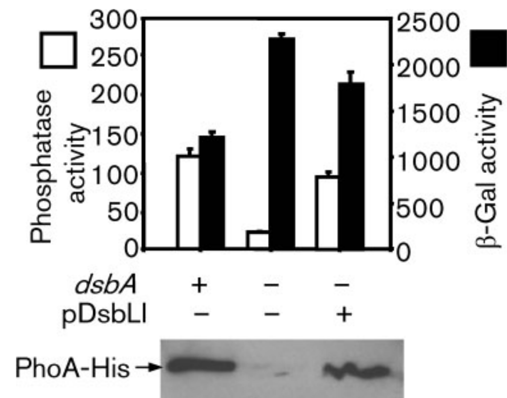

Fig. 1. DsbLI have oxidoreductase activity. All strains contained a *phoA*–6×His construct followed immediately by a promoterless *lacZ* gene. The *dsbA* gene was deleted in the designated strains and the pDsbLI plasmid was introduced as indicated. Bacteria were grown overnight in low-phosphate minimal glucose MOPS medium. Cells were assayed for phosphatase activity (white bars) and β-galactosidase activity (β-Gal; black bars), and subjected to Western blot analysis using anti-6×His antibody (lower panel). Both phosphatase and β-galactosidase activities are defined as (μmol *o*-nitrophenol formed min^{-1})×10^6/(OD_{600}×ml cell suspension). Data are presented as mean ± SD, where n=4. The strains used were BK683, BK685 and BK685 pDsbLI.

phosphatase activity was low and the PhoA protein was barely detectable by Western blot analysis. This is consistent with earlier results showing that the PhoA protein is unstable in the absence of disulfide bonds (Bardwell *et al.*, 1991). Introduction of the pDsbLI plasmid into the *dsbA* mutant restored both phosphatase activity and PhoA protein almost to wild-type levels. These data suggest that DsbLI are able to replace DsbA and introduce disulfide bonds into the PhoA protein.

In order to determine the function of these two proteins in *Salmonella*, we first tested the effect of *dsbL* or *dsbI* deletions on expression of a *hilA–lacZ* fusion. The *hilA* gene, encoding the master regulator of the SPI1 T3SS (Bajaj *et al.*, 1995), is transcriptionally controlled by a complex regulatory circuit (Ellermeier *et al.*, 2005). Consistent with our previous data (Lin *et al.*, 2008), deletion of *dsbA* or *dsbB* significantly decreased the expression of *hilA* (Fig. 2a). SrgA is a periplasmic disulfide bond oxidoreductase encoded on the *Salmonella* virulence plasmid. Deletion of *srgA* alone had no effect on the expression of *hilA*, while a Δ*dsbA* Δ*srgA* double mutation led to a greater decrease in expression than the Δ*dsbA* single mutation, as we have previously shown (Lin *et al.*, 2008). Similar to *srgA*, deletion of *dsbL* or *dsbI* alone did not have much effect on *hilA* expression, but Δ*dsbA* Δ*dsbL* or Δ*dsbB* Δ*dsbI* double mutations reduced *hilA* expression more than either the Δ*dsbA* or the Δ*dsbB* single mutation.

Disulfide bond status is one of several signals that can activate the RcsCDB phosphorelay system, composed of the sensor RcsC, the phosphotransfer protein RcsD (YojN), and the response regulator RcsB (Lin *et al.*, 2008; Majdalani & Gottesman, 2005). Activation of the capsule synthesis operon, *wcaA–F* is controlled by RcsCDB (Majdalani & Gottesman, 2005), resulting in a mucoid phenotype. We noticed that the Δ*dsbA* Δ*dsbL* or Δ*dsbB* Δ*dsbI* double mutants are mucoid, just like a Δ*dsbA* Δ*srgA* double mutant. In contrast, serovar Typhimurium *dsbA* or *dsbB* single mutants are not obviously mucoid. Therefore, expression of the *wca* genes can be used to monitor disulfide bond status in the periplasm. As shown in Fig. 2(b), *wca* expression was induced ~37-fold in the *dsbA* mutant, while deletion of both *dsbA* and *srgA* led to more than a 100-fold induction of the *wca* fusion. Consistent with the results shown in Fig. 2(a), deletion of *dsbL* or *dsbI* alone had no effect on *wca* expression. However, a Δ*dsbA* Δ*dsbL* or Δ*dsbB* Δ*dsbI* double mutation activated *wca* expression to a higher level than either the Δ*dsbA* or the Δ*dsbB* single mutation. Furthermore, deletion of *dsbL* in the *dsbA srgA* background led to a further induction of the *wca* fusion. Taken together, these data suggest that DsbL and DsbI contribute to disulfide bond formation in the periplasm.

DsbL and DsbI function as a redox pair for disulfide bond formation

To further understand the role of DsbL and DsbI in *Salmonella*, we investigated the ability of DsbL or DsbI to complement *dsbA* or *dsbB* mutations. First, we tested whether overproduction of DsbL or DsbI was able to suppress the activation of the *wca* genes. We introduced plasmids pDsbA, pDsbB, pDsbL, pDsbI or pDsbLI (expressing both proteins), or the vector control pWKS30 into a *wca–lacZ* fusion strain in wild-type, *dsbA*, *dsbA srgA*, *dsbB* or *dsbA dsbB* deletion backgrounds. These plasmids conferred very minor effects on expression of *wca* genes in an otherwise wild-type background (Fig. 3a, b). As expected, production of DsbA from a plasmid significantly suppressed the induction of *wca* in *dsbA* or *dsbA srgA* deletion backgrounds (Fig. 3a). In contrast, overproduction of DsbL only slightly suppressed *wca* activation in these backgrounds. However, the co-expression from the plasmid of both DsbL and DsbI decreased expression of *wca* similarly to overexpression of DsbA. The DsbB-expressing plasmid was also able to fully overcome the activation of *wca* expression in the Δ*dsbB* mutant (Fig. 3b). Overproduction of DsbI alone also fully suppressed *wca* induction in the Δ*dsbB* mutation. The plasmid pDsbLI completely suppressed the induction in both the Δ*dsbB* and the Δ*dsbA* Δ*dsbB* mutant backgrounds. These results suggest that DsbI is able to reoxidize DsbA in the absence

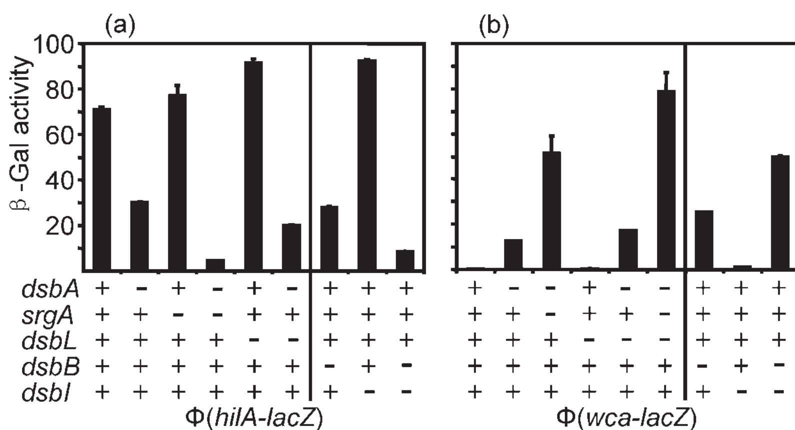

Fig. 2. Effects of oxidoreductase gene deletions on *hilA* and *wca* expression. Strains contained a *hilA–lacZ* fusion (a) or *wca–lacZ* fusion (b) with the indicated genes deleted (denoted by '–'). Cultures were grown statically overnight in LB [1 % (w/v) NaCl and 0.5 mM DTT] and then assayed for β-galactosidase activity, which is defined as (μmol *o*-nitrophenol formed min^{-1})×10^6/(OD_{600}×ml cell suspension). Data are presented as mean ± SD, where $n=4$. The strains used were (a) JS749, JS754, JS759, JS761 and JS859–JS863; (b) JS856 and JS864–JS871.

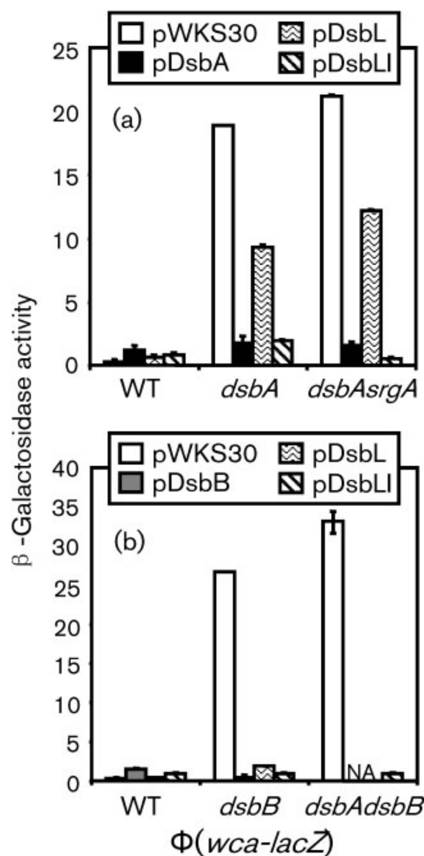

Fig. 3. Complementation of *dsbA* and/or *dsbB* mutations by *dsbL* and *dsbI*. Strains contained the *wca–lac* fusion with the deletions of genes designated below each graph and the indicated plasmids. Cultures were grown statically overnight in LB [1 % (w/v) NaCl and 0.5 mM DTT] and then assayed for β-galactosidase activity, which is defined as (μmol *o*-nitrophenol formed min^{-1})$\times 10^6$/($OD_{600}\times$ml cell suspension). Data are presented as mean ± SD, where $n=4$. The strains used were plasmid-containing derivatives of (a) JS856, JS864 and JS868; (b) JS856, JS869 and JS872. WT, wild-type.

of DsbB, but that DsbL is not efficiently reoxidized by DsbB. However, DsbL does efficiently contribute to disulfide bond formation in the presence of DsbI, suggesting that these proteins function best together as a redox pair in *Salmonella*.

The DsbLI system is unable to substitute for DsbAB in motility

Loss of DsbA or DsbB prevents proper assembly of the flagellar apparatus, causing a severe motility defect (Dailey & Berg, 1993). In order to determine the ability of DsbL and DsbI to facilitate flagellar assembly, we monitored motility in various mutant backgrounds while overproducing DsbL or DsbI. As expected, deletion of *dsbL* or *dsbI*, like *srgA*, conferred no obvious motility phenotype (Fig. 4a). Loss of DsbA led to a severe motility defect (Fig. 4b), a phenotype exacerbated by also deleting *srgA* (Fig. 4c). Interestingly, while overproduction of DsbA could fully complement the motility defect in the *dsbA* or *dsbA srgA* deletion strains, overproduction of DsbL had no apparent effect (Fig. 4b, c). Moreover, even overproducing DsbL and DsbI together had no significant effect on the motility defect in the *dsbA* or *dsbA srgA* mutant backgrounds.

Loss of DsbB also conferred a significant motility defect (Fig. 4d). Overproduction of DsbI in this background partially suppressed this phenotype (Fig. 4d). Overproduction of DsbL and DsbI together further suppressed the motility defect in the *dsbB* mutant, but not to wild-type levels. These results are consistent with those described above, suggesting that although DsbI can work with DsbA, it functions better with DsbL. However, these data also indicate that the DsbLI system cannot fully overcome the motility deficiency caused by loss of the DsbAB system. To further confirm these results, we also introduced plasmid pDsbLI into a Δ*dsbA* Δ*dsbB* double mutant background. Consistent with our previous observations, the double mutant was mucoid and non-motile. Introduction of the plasmid expressing both DsbL and DsbI apparently relieved the mucoid phenotype, but had little effect on motility (Fig. 4e). Thus, although the DsbLI redox system does contribute to disulfide bond formation, as evidenced by the change in the mucoid phenotype, it cannot substitute for the DsbAB system in flagellar assembly.

DsbL and DsbI contribute to ASST activity

DsbL and DsbI are encoded downstream of a putative ASST encoded by the *assT* gene. It has been reported that in *Enterobacter amnigenus* and uropathogenic *E. coli* (UPEC) disulfide bond formation is required for efficient production of functional ASST in the periplasm (Grimshaw *et al.*, 2008; Kwon & Choi, 2005). Therefore, a physiological role for the DsbLI system is likely to be the activation of ASST in *Salmonella*. In the serovar Typhimurium LT2 genome sequence, *assT* is annotated as two ORFs, STM3191 and STM3192 (McClelland *et al.*, 2001). We cloned and sequenced this region from serovar Typhimurium strain 14028. Sequence analysis showed a single ORF encoding a putative protein homologous over its entire length to known ASST enzymes. Analysis suggested that the LT2 genome has acquired a frame-shift mutation that inactivates *assT* (see below).

E. coli K-12 does not have the *assT dsbLI* or any other apparent redox system besides DsbAB, so we first tested for ASST activity in wild-type, *dsbA* or *dsbB* deletion backgrounds in *E. coli* K-12 strain DH5α. We cloned the putative *assT* gene with or without *dsbLI*. As shown in Fig. 5(a), the introduction of pAssT or pAssT DsbLI into the wild-type strain conferred high-level ASST activity. The deletion of *dsbA* or *dsbB* reduced ASST activity 4.6- and threefold, respectively, in strains making only AssT. In

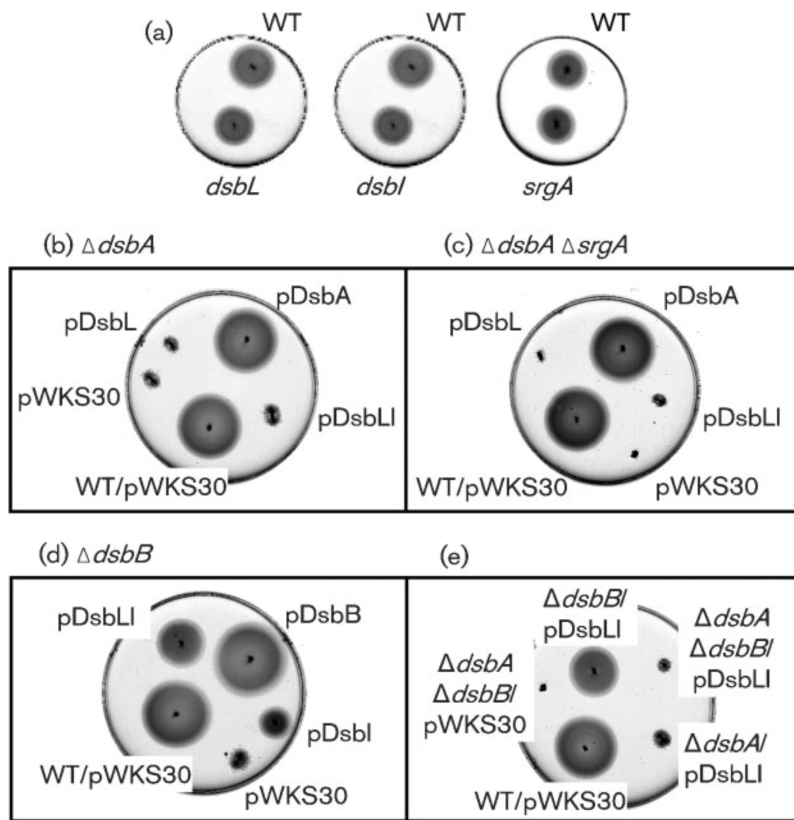

Fig. 4. DsbLI are unable to substitute for DsbAB in motility. Single colonies of the indicated strains were stabbed onto motility agar plates and incubated overnight. The genotype of the background strain is marked for each panel unless otherwise noted in the individual labels. The strains used were: (a) 14028, JS741, JS850 and JS851; plasmid-containing derivatives of (b) JS326, (c) JS748, (d) JS849, (e) JS326, JS849 and JS873, except for WT=14028.

contrast, co-expression of DsbL and DsbI resulted in significant ASST activity even in *dsbA* or *dsbB* mutant backgrounds.

We then introduced these plasmids into various *Salmonella* strains in which the chromosomal *assT dsbLI* genes were deleted. Fig. 5(b) shows that the plasmid producing AssT conferred robust ASST activity. This activity was reduced 3.7-fold in the *dsbA* mutant and further reduced in a Δ*dsbA* Δ*srgA* double mutant background. Deletion of *srgA* alone had little effect. As expected, a *dsbB* deletion had a similar effect to that of the *dsbA srgA* double mutations. In

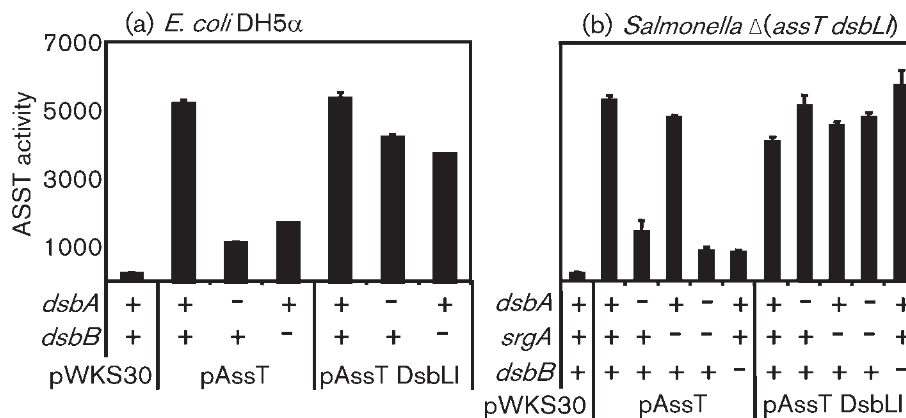

Fig. 5. DsbL and DsbI contribute to ASST activity in *E. coli* (a) and *Salmonella* (b). The strains contained pWKS30 (vector), pAssT or pAssT DsbLI in the mutant backgrounds as indicated below the graphs. Strains were grown overnight at 37 °C in LB broth with 2 mM DTT and 100 μg ampicillin ml^{-1}, and assessed for ASST activity as described in Methods. ASST activity=(μmol *p*-nitrophenol formed min^{-1})×10^6/(OD_{600}×ml cell suspension). The data are presented as mean ± SD, where *n*=4. Strains used were plasmid-containing derivatives of (a) DH5αλpir, DX1616 and DX1617; (b) JS853 and JS874–JS877.

contrast, when we overproduced DsbL and DsbI from the plasmid, the ASST activity was independent of DsbA, SrgA and DsbB, suggesting that DsbL and DsbI efficiently introduce disulfide bounds into the ASST protein in *Salmonella*.

The *assT* and *dsbLI* genes are independently transcribed

Previous studies in UPEC and *Enterobacter amnigenus* imply that *assT* and *dsbLI* are encoded in one operon (Grimshaw *et al.*, 2008; Kwon & Choi, 2005). To determine whether *assT* and *dsbLI* are in one operon in *Salmonella*, we inserted a chloramphenicol resistance cassette into *assT* with transcription of the cassette directed antisense to *assT* transcription. Then we measured the effect of this insertion on the expression of a *lacZ* transcriptional fusion in the downstream gene *dsbL*. The results indicated that there was little effect of the Δ*assT*::Cm mutation on *dsbL* expression (16.58 ± 0.96 β-galactosidase units, $assT^{+}$; 14.01 ± 0.04, *assT*::Cm). This suggests that *assT* and *dsbL* are transcribed independently in *Salmonella*. Further analysis showed that deletion of *dsbA* and *srgA* or *dsbB* had no effect on transcription of *dsbL* or *dsbI* (data not shown). It is not clear if these genes are regulated in response to any particular stimuli.

Although earlier microarray data show that the expression of *assT* in serovar Typhimurium is induced 3.28-fold after infection of macrophages (Eriksson *et al.*, 2003), our data suggested that expression of *assT* was not induced in macrophages (155.6 ± 0.35 units c.f.u.$^{-1}$ in LB; 128.8 ± 5.67 units c.f.u.$^{-1}$ in macrophages). In contrast to *assT*, expression of *dsbL* was slightly induced (467.15 ± 1.06 units c.f.u.$^{-1}$ in LB; 855.8 ± 90.9 units c.f.u.$^{-1}$ in macrophages), consistent with the hypothesis that *assT* and *dsbL* are transcribed independently.

ASST has no apparent function during acute infection

To explicitly test for a role of AssT in virulence, we performed competition assays after either i.p. or oral infection. Deletion of *assT* conferred no virulence defect compared with an isogenic wild-type strain after i.p. infection (Table 2). STM4098 encodes an additional putative ASST in the *Salmonella* chromosome. Deletion of *stm4098* along with deletion of *assT* still conferred no virulence phenotype in bacteria recovered from the spleen after i.p. infection or in the intestines or spleens of mice infected orally (Table 2). However, insertions in *assT* have been identified in a screen for mutations affecting long-term persistence in mice (Lawley *et al.*, 2006). Thus, although loss of AssT activity does not affect acute infection, this enzyme might have some more subtle effect that can only be observed during a longer infection period.

DISCUSSION

The DsbA/DsbB catalytic system in *E. coli* K-12 has been widely studied and serves as the paradigm for disulfide bond oxidoreductases (Kadokura *et al.*, 2003). In *Salmonella*, the virulence plasmid-encoded DsbA paralogue SrgA has also been characterized (Bouwman *et al.*, 2003). Here we show that *Salmonella* encodes an additional pair of DsbA/DsbB paralogues, DsbL and DsbI, in the chromosome associated with an ASST. DsbL/DsbI contribute to overall periplasmic disulfide bond formation, as evidenced by effects on SPI1 expression and activation of the RcsCDB system. However, DsbL/DsbI are unable to compensate for DsbA/DsbB in motility.

Grimshaw *et al.* (2008) have characterized the orthologous system from the UPEC strain CFT073. Although DsbL and DsbI are 88 % and 87 % identical, respectively, between the two bacteria, there are notable phenotypic differences. Consistent with our data, Grimshaw and colleagues concluded that UPEC DsbL/DsbI could not complement *dsbA*/*dsbB* mutants for motility, but could form disulfide bonds in ASST. They suggested that, whereas DsbL is inherently more oxidizing than DsbA, charged amino acids around the active site might limit potential substrates. Indeed, they concluded that DsbL/DsbI are specific for ASST. While it is apparent that neither DsbL orthologue is capable of oxidizing a key flagellar protein(s), motility and ASST activity were the only *in vivo* functional assays

Table 2. Effects of Δ*assT* in mouse virulence assays

Strain*	Route of infection†	Tissue	Median CI‡	Number of mice	*P*§
Δ*assT*	i.p.	Spleen	1.23	4	0.39
Δ*assT* Δ*stm4098*	i.p.	Spleen	3.05	3	0.22
Δ*assT* Δ*stm4098*	Oral	Spleen	1.69	5	0.07
Δ*assT* Δ*stm4098*	Oral	Intestine	3.77	5	0.02

*All inocula contained a 1 : 1 ratio of the designated mutant and isogenic wild-type strain. Strains used: i.p., JS852, JS878 and 14028; oral, JS879 and JS107.

†Doses: 10^3 c.f.u. i.p., 10^8 c.f.u. oral.

‡Competitive index=output(mutant/wild-type)/input(mutant/wild-type).

§Student's *t* test comparing output versus inoculum.

performed in their study and it is not known if DsbLI facilitate disulfide bond formation in additional proteins in the UPEC strain. These authors also noted, as did we, that DsbL and DsbI function best together as a redox pair. However, their data suggest that the UPEC DsbL can partially function with DsbB, while DsbA cannot function with UPEC DsbI. In striking contrast, we noted that *Salmonella* DsbL could function only weakly with DsbB, whereas DsbA provided robust activity in conjunction with *Salmonella* DsbI (Figs 3 and 4).

The *assT* gene was originally identified in various uropathogenic strains of *E. coli* (Lloyd *et al.*, 2007). Mobley and colleagues had shown that *assT* transcription is increased in UPEC cells recovered from the urine of infected mice (Snyder *et al.*, 2004). This result and the fact that the *assT* (and *dsbLI*) genes are missing in several strains of *E. coli*, including K12 strains, led these authors to conclude that *assT* was 'uropathogen-specific', with the implication that this gene is somehow involved in virulence, an assumption that has been propagated in subsequent papers (Grimshaw *et al.*, 2008; Malojcic *et al.*, 2008). However, analysis shows that the *assT dsbLI* genes are present in the same genomic context in a variety of *Enterobacteriaceae*, including: all the *S. enterica* subsp. *enterica* serovars, as well as *S. enterica* subsp. *arizonae* and *Salmonella bongori*; numerous *E. coli* strains, including non-UPEC isolates, but not K12 or O157 isolates; other *Escherichia* species; and *Citrobacter*. Phylogenetic analysis of the *assT* DNA sequence from these various organisms shows a relationship that reflects the accepted phylogeny of the overall genomes (Fig. 6; Vernikos *et al.*, 2007). The simplest interpretation of these data is that the *assT dsbLI* genes were present in the common ancestor of these species, but that these genes were lost during the evolution of certain lineages. In both *S. enterica* serovar Typhimurium strain LT2 (McClelland *et al.*, 2001) and *Salmonella bongori* strain 12149 (Sanger Institute), the *assT* genes apparently contain independent frameshift mutations. As shown here, the *assT* gene encodes a functional enzyme in serovar Typhimurium strain 14028.

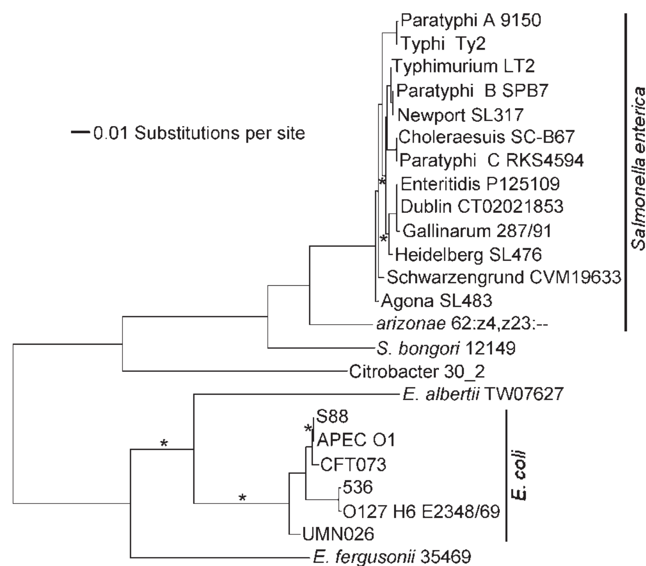

Fig. 6. Maximum-likelihood phylogenetic tree of *assT* DNA sequences. Sequences are from representative genomes in which the *assT dsbLI* genes are in the same overall genomic context. Those branches with <70 % bootstrap support from 1000 replicates are marked with an asterisk.

The presence of *assT* in UPEC strains and its apparent induction during growth in the bladder led to the suggestion that the gene product has a role in UPEC virulence, although no direct assay was performed. Although a number of interesting molecules such as the catecholamine epinephrine (Kobashi *et al.*, 1987), known to affect virulence gene expression in intestinal pathogens (Walters & Sperandio, 2006), and various phenolic antibiotics (Kim *et al.*, 1992) have been shown *in vitro* to be modified by ASST enzymes, we have shown that loss of *assT* confers no phenotype during acute *Salmonella* infection in mice. Mutations in *assT* do confer a defect in long-term infection of mice (Lawley *et al.*, 2006). Thus, any role in virulence is subtle at best and the physiological role of ASST in the *Enterobacteriaceae* is yet to be determined. DsbI has been shown to affect the ability of *Helicobacter pylori* to colonize mouse stomach, but this result is not surprising given that DsbI (not associated with *assT* or *dsbL* genes) appears to be the only DsbB-like protein in this organism (Godlewska *et al.*, 2006).

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