In the gram-negative bacterium *Escherichia coli* K-12 the introduction of disulfide bonds into folding proteins is mediated by the Dsb family of enzymes, primarily through the actions of the highly oxidizing protein EcDsbA. Homologues of the Dsb catalysts are found in most bacteria. Interestingly, pathogens have developed distinct Dsb machineries that play a pivotal role in the biogenesis of virulence factors, hence contributing to their pathogenicity. *Salmonella enterica* serovar (sv.) *Typhimurium* encodes an extended number of sulphydryl oxidases, namely SeDsbA, SeDsbL, and SeSrgA. Here we report a comprehensive analysis of the sv. *Typhimurium thiol oxidative system through the structural and functional characterization of the three *Salmonella* DsbA paralogues. The three proteins share low sequence identity, which results in several unique three-dimensional characteristics, principally in areas involved in substrate binding and disulfide catalysis. Furthermore, the *Salmonella* DsbA-like proteins also have different redox properties. Whereas functional characterization revealed some degree of redundancy, the properties of SeDsbA, SeDsbL, and SeSrgA and their expression pattern in sv. *Typhimurium* indicate a diverse role for these enzymes in virulence.

Thioredoxin (TRX)$^2$-like oxidoreductases play a major role in controlling the redox environment of the cell. These enzymes catalyze thiol-disulfide oxidoreductase reactions that are important in enzyme catalysis and in the maintenance of the correct thiol redox state in proteins. Dsb (disulfide bond) proteins are a specific of TRX-like proteins that are essential for the oxidative folding of secreted proteins in many Gram-negative bacteria (1–3). In *Escherichia coli* K-12 five different Dsb proteins catalyze the correct introduction of disulfide bonds in substrate proteins (4). DsbA and DsbB (5, 6) form the oxidase pathway that introduces disulfide bonds into target proteins, whereas DsbC, DsbG and DsbD (7) comprise the isomerase pathway which proofreads and corrects incorrect disulfide bonds in proteins with multiple cysteines. Like all TRX-related proteins, Dsb enzymes contain a conserved CXXC catalytic motif and a *X*-cisProline loop in their active site. The identity of the XX dipeptide located between the cysteines as well as the residue preceding the cisProline residue determines the properties and function of these enzymes; variation in these residues accounts for the wide range of redox activities described above (8–11).

Until recently it was considered that the paradigm redox system present in *E. coli* K-12 was conserved in all bacteria. However, recent studies on Dsb proteins in other bacteria show that these systems can vary considerably between different genera and even strains of the same species (12, 13). This is observed for many Gram-negative pathogenic bacteria, some of which encode multiple Dsb proteins (14, 15). Significantly, there is growing evidence that a range of virulence-related functions depend on the strict redox control carried out by Dsb enzymes. Thus, these catalysts are required for the correct folding (and therefore function) of many secreted or surface exposed virulence determinants. Examples include, the flagellar P-ring motor protein FlgI, the secretin component of Type III secretion systems (T3SS) and a range of toxins and secreted enzymes (13, 16).

The Gram-negative bacterium *Salmonella enterica* serovar (sv.) *Typhimurium* is an important causative agent of acute human gastroenteritis and can cause life-threatening bacteremia in immuno-compromised individuals (17). It is an intracellular pathogen that resides in macrophages. In addition to the oxidase and isomerase systems described above for *E. coli* K-12, sv. *Typhimurium* also has a DsbL/DsbI pair, similar to the uropathogenic *E. coli* (UPEC) CFT073 DsbL/DsbI system (14, 18), and a virulence plasmid-encoded DsbA-like protein, called SrgA (19). The sv. *Typhimurium* DsbA paralogues, termed SeDsbA, SeDsbL, and SeSrgA, share low sequence identity with each other (between 18 and 34%). They each contain different residues in the CXXC redox active site as well as in the cisProline. These differences suggest that the three DsbA paralogues have different functions and most likely act on dif-
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Diferent target protein(s). In support of this notion, characterization of SeDsBA and SeSrgA showed that these two disulfide oxidoreductases have specificity for different substrates (20). SeSrgA is required for the biogenesis of the major structural subunit of the plasmid-encoded fimbriae, PefA (19). Together with SeDsBA it also catalyzes the folding of the outer membrane secretin SpiA, a component of the T3SS encoded in Salmonella Pathogenicity Island 2 (SPI2) (21). SeDsBA is required for the folding of Fgl (20) and most likely many other periplasmic secreted proteins. The change in the periplasmic redox state upon disruption of the dsbA gene also affects the expression of the SPI1 T3SS (22). Although no substrate is known for SeDsBL, this protein shares 93% sequence identity with its E. coli CFT073 orthologue, and could most likely fold the periplasmic enzyme arylsulfate sulfotransferase (AssT) (14). Moreover, as in UPEC CFT073, the sv. Typhimurium assT gene is encoded immediately upstream of dsbL and dsbI. Despite these differences in substrate specificity, there is indication that some overlap also exists between the activities of SeDsBA, SeDsBL, and SeSrgA (21).

The specificity of disulfide catalysis in Gram-negative pathogens with an extended collection of Dsb proteins remains mostly uncharacterized. To further investigate oxidative folding processes in these organisms we have focused on the three sv. Typhimurium DsbA paralogues. Here, we perform a structural, biochemical and functional characterization of SeDsBA, SeDsBL, and SeSrgA, which identifies important structural differences in areas surrounding the catalytic sites. These differences result in diverse redox properties and are likely to underline the distinct substrate specificities and redox functions.

EXPERIMENTAL PROCEDURES

Production of Native and Variant Proteins—Native SeDsBA, SeDsBL, and SeSrgA were expressed and purified as previously described (24). Briefly, the coding DNA sequences for the three DsbA homologues were cloned into a pET21a based LIC vector. The recombinant proteins containing a N-terminal hexahistidine tag were expressed using autoinduction (25) and then purified by nickel-chelate chromatography. Upon removal of the tagged signal by cleavage with tobacco etch virus (TEV) protease, the proteins were further purified by gel filtration and ion exchange chromatography. Proteins were oxidized before crystallization by addition of 1.7 mM copper(II) 1,10-phenanthroline.

Selenomethionine (SeMet)-labeled SeSrgA was expressed from E. coli BL21(DE3) pLysS in minimal medium containing 50 µg ml⁻¹ SeMet (L/D mixture) using methods similar to those described previously (26). Briefly, cultures were grown to exponential phase (A420 = 0.6) and induced with a final concentration of 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG). Cells were harvested 4 h post-induction, and SeMet SeSrgA was purified following the same procedures described for native SeSrgA (24).

Crystallization and Diffraction Data Collection—SeDsBA, SeDsBL, and SeSrgA crystals were obtained from: 200 mM sodium acetate, 20% (w/v) polyethylene glycol 3,350, 100 mM 1,3-bis(tris(hydroxymethyl)methylamino)propane, pH 8.5; 2.6–3.4 M sodium malonate pH 7.0–7.4; and 1.45 M sodium malonate, 0.5% (w/v) Jeffamine ED-2001, 100 mM HEPES pH 7, respectively. Diffraction data for all three native proteins and for SeMet SeSrgA were collected at the protein crystallography beamline (MX1 beamline 3BM1) at the Australian Synchrotron (Melbourne, Australia). In all cases the crystal-to-detector distance was 150 mm; 1° oscillation images were collected for a total of 180°, in the case of native crystals, and 720° for SeMet SeSrgA. Diffraction data were integrated and scaled with HKL2000 (27).

Structure Determination—The structures of SeDsBA and SeDsBL were solved by molecular replacement using E. coli K-12 DsbA (PDB code 1FVK, 85% sequence identity) and E. coli CFT073 DsbL (PDB code 3C7M, 93% sequence identity) respectively as search models and the program Phaser (28). The crystal structure of the virulence plasmid encoded SeSrgA was determined using seleno-methionine single wavelength anomalous dispersion (SAD). Phase calculation, density modification and preliminary model building were carried out using PHENIX AutoSol and AutoBuild suite of programs (29). Phenix AutoSol found 48 heavy atom sites, 40 of those are Se, 35 expected, and 5 that are the result of alternate configuration of the SeMet residues. Eight sites are atoms other than selenium. SeSrgA crystals contained 5 molecules in the asymmetric unit; the electron density maps for 4 of the subunits was clear and unmistakable, but the density for the insertion domain of subunit E was poor. The three Salmonella structures were completed by iterative cycles of manual building and refinement using the programs Coot (30) and phenix.refine (29). In the case of SeSrgA, monomer E was refined using ten TLS groups which were determined with the TLSMD server (31). Table 1 provides the statistics for the x-ray data collection and final refined model.

The quality of the SeDsBA, SeDsBL, and SeSrgA models was assessed by MolProbity (32). Additional information about the surface residues with weak electron density modeled with reduced occupancies for the side chain atoms and residues with alternate conformations is provided as supplemental data. Superposition of molecules was carried out using the LSQ options from the program Coot (30). Molecular figures were generated using PyMOL (33) and figures of the electrostatic potential were generated using APBS (34). The coordinates and structure factors for SeDsBA, SeDsBL, and SeSrgA structures have been submitted to the PDB with accession codes 3L9S, 3L9U, and 3L9V, respectively.

Determination of the Equilibrium Constants with Glutathione—The redox equilibrium of SeDsBA, SeDsBL, and SeSrgA with glutathione was determined as described previously (11). Briefly, proteins at 1.5 μM concentration were incubated for 16–20 h at 25 °C in 100 mM sodium phosphate, 0.1 mM EDTA (pH 7.0) containing 1 mM GSSG and increasing concentrations of GSH. The redox state of SeDsBA, SeDsBL, and SeSrgA was followed by fluorescence emission at 330, 340, and 332 nm, respectively (excitation at 280 nm). The equilibrium constant Kₐ and redox potential were determined from standard thermodynamic equations (10, 35).

Determination of pKₐ Values—The pKₐ of the nucleophilic cysteine in SeDsBA, SeDsBL and SeSrgA was determined by monitoring the specific absorbance of the thiolate anion at 240
Measurements were carried out at room temperature in a buffer system consisting of 10 mM K$_2$HPO$_4$, 10 mM boric acid, 10 mM sodium succinate, 1 mM EDTA, and 200 mM KCl, pH 7.5. Oxidized and reduced protein samples (final concentration of 200 μM) were prepared by incubating the proteins with 1.7 mM Cu(II)Phen$_3$ or 10 mM DTT, respectively. Upon removal of the oxidizing and reducing agents using a PD10 column (GE Healthcare, Piscataway, NJ), the absorbance of the samples at 240 and 280 nm was recorded as the pH of the protein solution was lowered to 2.2 by the stepwise addition of aliquots of 0.2 M HCl. The pH dependence of the thiolate-specific absorbance signal ($S = (A_{240}/A_{280})_{reduced}/(A_{240}/A_{280})_{oxidized}$) was fitted according to the Henderson-Hasselbalch equation.

**Insulin Reduction Assay**—We determined the disulfide oxi-doreductase activity of SeDsbA, SeDsbL, and SeSrgA by measuring their ability to catalyze insulin reduction in the presence of DTT (37). Reaction mixtures were prepared containing various concentrations of catalyst (5–10 μM) in 0.1 M phosphate buffer, pH 7.0, 2 mM EDTA, and 0.35 mM DTT. The reactions were started by adding insulin to a final concentration of 131 μM and the reduction of insulin was monitored by measuring the optical density of the samples at 650 nm for 80 min at 30-s intervals. The non-catalyzed reduction of insulin by DTT was monitored in a control reaction without catalyst.

**Bacterial Strains, Plasmids, and Culture Conditions**—*sv.* Typhimurium SL1344 strains (wild type and deletion mutants) and *E. coli* strains (BL21(DE3) and DH5α) were routinely cultured at 37 °C on solid or in liquid Luria Bertani (LB) medium supplemented with the appropriate antibiotics; kanamycin (km, 50 μg ml$^{-1}$), ampicillin (amp, 100 μg ml$^{-1}$ or chloramphenicol (cam, 30 μg ml$^{-1}$). Culture media were supplemented with 1 mM IPTG to induce expression of DsbA, DsbL-DsbI, SrgA, and AssT from plasmids pDsbA, pDsbLI, pSrgA and pAssT, respectively. Plasmids pDsbA, pDsbLI and pSrgA were generated by PCR amplification of dsbA (primers dsbA-FW and dsbA-RV), dsbLI (primers dsbLI-FW and dsbLI-RV) and srgA (primers srgA-FW and srgA-RV) genes from the chromosome of SL1344 and subsequent cloning into EcoRI-Xhol-digested pWSK29 (38). Plasmid pAssT was generated by PCR amplification of the assT gene (primers assT-1569 and assT-1570) and subsequent cloning into Xhol-HindIII-digested pSU2718 (39). In both vectors expression of cloned genes was controlled by the inducible lac promoter. All primers used in this section are shown in supplemental Table S1.
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**TABLE 1**

| Data collection | SeSrgA | SeDsbA | SeDsbL |
|-----------------|--------|--------|--------|
| Wavelength (Å)  | 0.956661 | 0.956667 | 0.956667 |
| Resolution range (Å) | 20–2.15 | 50–1.58 | 50–1.57 |
| Space group      | C2     | P 21   | P 21 2 |
| Unit cell dimensions (Å) | a = 185.4, b = 80.4, c = 104.9 | a = 37.3, b = 61.7, c = 42.8 | a = 85.6, b = 46.6, c = 60.2 |
| Observed reflections | 1050164 | 184714 | 246716 |
| Unique reflections | 67898 | 25131 | 34491 |
| \( R_{merge} \) | 0.08 (0.48) | 0.08 (0.19) | 0.06 (0.46) |
| Completeness (%) | 98.1 (97.2) | 95.3 (87.4) | 100 (99.9) |
| \( \langle I/|I| \rangle \) | 27.6 (5.6) | 19.2 (10.3) | 29.6 (4.1) |
| Redundancy | 15.5 (15.6) | 7.4 (6.8) | 7.2 (7.0) |

**SAD Phasing**

| Resolution (Å) | 2.15 |
| Number of selenium sites found | 48 |
| Mean figure of merit | 0.43 |

**Refinement**

| Resolution (Å) | 36.5–2.15 | 21.1–1.58 | 28.4–1.57 |
| Completeness for range (%) | 98.0 | 95.2 | 99.8 |
| \( R_{merge} \) (%) | 21.3 (25.4) | 14.5 (10.9) | 15.2 (13.9) |
| \( R_{free} \) (%) | 26.2 (34.4) | 18.9 (17.4) | 18.4 (21.7) |
| Number of non-H protein atoms | 7361 | 1517 | 1609 |
| Number of waters | 628 | 259 | 299 |
| Wilson B | 38.11 | 10.77 | 15.83 |
| Average B factor (Å²)–all atoms | 48.98 | 17.89 | 20.51 |
| Average B factor (Å²)–water | 48.83 | 31.70 | 33.73 |
| Average B factor (Å²)–non solvent | 50.72 | 15.53 | 18.05 |

**Ramachandran plot**

| Bonds (Å) | 0.008 | 0.005 | 0.006 |
| Angles (°) | 1.060 | 0.903 | 0.944 |

**Construction of sv. Typhimurium SL1344 Mutants**—To delete the dsbA, dsbLI, and srgA genes, respectively, a 3-step PCR procedure was employed to generate an amplification product that contained the kanamycin cassette from pKD4 (40) flanked on both sides by ~500 bp of DNA sequence homologous to the target gene to be modified. The following sets of primers were used for dsbA (dsbAP1F2, dsbAP2R2; dsbAP5F2 and dsbAP6R2), dsbLI (stm3193_P1F2; stm3193_P2R; stm3194_P5F and stm3194_P6F2) and srgA (srgAP1F3; srgAP2R; srgAP5F and srgAP6R2) (supplemental Table S1). These primers were used in combination with the kanamycin cassette amplification primers KanP3 and KanP4 (supplemental Table S1). The final 3-step PCR product for each target gene was gel-purified and cloned into pT7Blue (Merck KGaA, Darmstadt, Germany) according to the manufacturer’s instructions to generate a donor plasmid. SL1344 single, double, and triple gene deletion mutants were constructed using the “gene gorging method” described previously (41). Briefly, SL1344 cells were electrophoresed with a donor plasmid (pT7Blue::dsbA::kan, pT7Blue::dsbLI::kan, or pT7Blue::srgA::kan) and a putative deletion plasmid (pACBSR; (41)). Cells containing both plasmids were cultured for 9 h in LB broth supplemented with 25 μg ml⁻¹ chloramphenicol and 0.2% (w/v) L-arabinose to induce the A Red and I-SceI genes on pACBSR. Induction of these genes leads to linearization of the donor plasmid and a double recombination event with the regions flanking dsbA/dsbLI/srgA, resulting in disruption of the respective gene. Clones sensitive to ampicillin and chloramphenicol (but resistant to kanamycin) were selected and deletion of dsbA/dsbLI/srgA was confirmed by sequencing. For the construction of double and triple gene deletion mutants the kanamycin cassette was removed using plasmid pCP20 as described (40). All deletion mutants were confirmed by PCR and subsequent DNA sequencing.

**Polyclonal Sera Production and Immunoblotting**—Polyclonal anti-DsbA, anti-DsbL, and anti-SrgA sera were raised in rabbits by the Institute of Medical and Veterinary Sciences (South Australia) following a standard protocol. For immunoblotting, whole cell lysates were subjected to SDS-PAGE using NuPAGE® Novex® 10% Bis-Tris precast gels with NuPAGE® MES SDS running buffer and subsequently transferred to polyvinylidene difluoride microporous membrane filters using the iBlot™ dry blotting system. Western blotting was performed using rabbit polyclonal anti-DsbA, anti-DsbL, or anti-SrgA sera followed by incubation with goat anti-rabbit IgG-alkaline phosphatase (AP) conjugate secondary antibody and development with BCIP/NBT solution (Sigma, Castle Hill, Australia). Western blotting was performed using rabbit polyclonal anti-DsbA, anti-DsbL, or anti-SrgA sera followed by incubation with goat anti-rabbit IgG-alkaline phosphatase (AP) conjugate secondary antibody and development with BCIP/NBT solution (Sigma, Castle Hill, Australia).

**Motility Assays**—The motility of the SL1344 triple mutant (SL1344dsbA,dsbLI,rsrgA) and the four complemented strains (pDsbA, pDsbLI, pSrgA, and pWSK29, respectively) was assessed by inoculation of 2 μl of overnight liquid culture onto the surface of semi-solid (0.25% w/v) agar. The diameter of bacterial outward growth was recorded after 8 h of incubation at 37 °C. Plasmid-complemented deletion mutants were...
assessed in the presence and absence of 1 mM IPTG. All experiments were performed in triplicate.

**AssT Activity Assays**—AssT enzyme activity was monitored at colony level using an agar plate assay, as described previously (42). Briefly, bacterial strains were streaked on LB agar plates containing 0.1 mM 4-methylumbelliferyl sulfate (MUS, Sigma, Castle Hill, Australia) and cultured at 37 °C overnight. The sulfate of the 4-MUS in the medium is cleaved by AssT forming 4-methylumbelliferone, a fluorescent product that can be detected under UV light (320 nm). Strains producing functional AssT enzyme have colonies that fluoresce brightly under UV light. sv. Typhimurium strain SL1344dsbA, dsbLsrgA and the complemented strains (with pDsbA, pDsbLI, pSrgA, and pWSK29, respectively) were transformed with pAssT plasmid containing the assT gene. Expression of assT was induced with 1 mM IPTG.

**Immunofluorescence Labeling and Microscopy of Pef Expression**—Bacterial strains were cultured in liquid LB-MES (pH 5.1) broth statically at 37 °C for 2 days (optimal conditions for Pef expression; (43)). Cell suspensions (adjusted at an A600 of 0.6) were washed twice with PBS and loaded onto microscope slides (ProSciTech, Thuringowa, Australia). Slides were saturated for 15 min with 0.5% w/v bovine serum albumin before incubation with a 1:1,000 dilution of the primary polyclonal anti-PefA serum (43), followed by a 1:10,000 dilution of the secondary goat anti-rabbit IgG-FITC conjugate (Sigma, Castle Hill, Australia). Cells were fixed with 4% w/v paraformaldehyde (PFA). Slides were mounted with ProLong-Gold (Invitrogen, Mulgrave, Australia) and examined under a Zeiss-Axioplan2 fluorescence microscope. Image acquisition and analysis was performed using AxioVisionAC software.

**RESULTS**

**Crystal Structures of sv. Typhimurium SrgA, DsbA, and DsbL**—To obtain a more complete picture of the oxidative folding machinery in *Salmonella* we pursued the structural characterization of all *Salmonella* DsbA paralogues. SeSrgA crystals belong to the space group *C*2, diffracted to 2.2 Å and contained 5 molecules in the asymmetric unit. After subsequent steps of building and refinement, the structure of SeSrgA corresponding to residues 3–184 was refined to a final Rfactor of 21.3 (Rfree = 26.2) (Fig. 1, Table 1). Pairwise comparison of the 5 symmetrically independent monomers gave r.m.s.d. values for all Cα atoms of less than 0.5 Å. Because no significant differences were observed between molecules in the asymmetric unit all subsequent structural
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comparisons were performed with monomer A. Similar to other DsbA-like proteins (44), the structure of SrgA incorporates a TRX domain (residues 15–56) forming the classic $\beta$$\alpha$ $\beta$ motif and residues 133–188 form the $\beta$$\beta$ motif preceded by the connecting helix) and an inserted $\alpha$-helical domain (residues 57–132) (Fig. 1A). SeDsbA and SeDsbL crystals belonged to $P2_1$ and $P_2_1_2_1$ space groups respectively and contained one molecule in the asymmetric unit. The structures of SeDsbA (corresponding to residues 3–187) and SeDsbL (corresponding to residues 1–199) were refined at 1.6 Å resolution to $R_{free}$ values of 18.9 and 18.4%, respectively (Table 1). SeDsbA and SeDsbL largely resemble their E. coli counterparts with overall r.m.s.d. values of 0.9 Å (185 Ca aligned) for the structural superposition of SeDsbA and EcDsbA and 0.4 Å (195 Ca aligned) for the structural superposition of SeDsbL and CFT073 DsbL.

Structural Comparison of sv. Typhimurium SrgA, DsbA, and DsbL—SeSrgA, SeDsbA, and SeDsbL all adopt the same architecture comprising the two domain structure described for the prototypic EcDsbA (Fig. 1) (44). Structural superposition of SeSrgA and its paralogues SeDsbA and SeDsbL resulted in overall r.m.s.d. values of 1.5 Å (180 Ca aligned) and 2.4 Å (157 Ca aligned), respectively, indicating a closer structural relationship with SeDsbA than with SeDsbL (Fig. 2C). Indeed, only small differences can be observed between the overall folds of SeSrgA and SeDsbA, specifically for loops connecting $\beta_1$-$\beta_2$, $\alpha_3$-$\alpha_4$, and $\alpha_4$-$\alpha_5$ (Fig. 1). Another probably more relevant discrepancy between these two structures resides in the region immediately preceding $147$Thr-$\text{cis-Pro}$ (between $\alpha_6$ and $\beta_4$). This region delineates one edge of a hydrophobic peptide-binding groove in EcDsbA (44) that binds to DsbB (45) and is proposed to interact with unfolded protein substrates. In SeDsbA and EcDsbA, this loop points away from the hydrophobic groove residues that precede the $\text{cis-Pro}$ loop, whereas in SeSrgA this section of the protein is oriented toward the $\beta_1$ strand at the N terminus of the TRX-fold, positioning the side chain of $145$Arg at the correct distance to form a salt bridge with $7$Glu (3.7 Å) (Fig. 1B) and thereby truncating the putative peptide binding groove. Incidentally, electron density maps revealed extra density corresponding to a PEG molecule bound in the peptide-binding groove of SeSrgA, which may indicate the binding mode of unfolded peptides to this redox catalyst (supplemental Fig. S1).

The overall structure of SeDsbL diverges considerably from SeDsbA and SeSrgA (Fig. 1). The helical domain of SeDsbL has extended loops between $\alpha_2$-$\alpha_3$ and $\alpha_3$-$\alpha_4$ (Fig. 2C), with the latter including an acidic protrusion at the C terminus of $\alpha_3$, which is located adjacent to the $32$Cys-Pro-Phe-Cys$^{35}$ catalytic site. Unlike SeSrgA and SeDsbA, the SeDsbL connecting helix $\alpha_6$ bends toward the $\beta_4$$\beta_5$a7 motif of the TRX fold, a position that is fastened by a hydrogen bond between residue $164$Gln at the C terminus of $\alpha_6$ and the main chain amide nitrogen of $179$Thr (B5) (Fig. 1B). Additionally, the $\beta_4$$\beta_5$a7 motif, which forms one edge of a hydrophobic peptide-binding groove, is 6 residues shorter in SeDsbL; it has a shorter loop connecting $\beta_4$ and a7 and a shorter a7 helix (by two turns) (Figs. 1, A and C, 3rd panel and 2A).

sv. Typhimurium DsbA Paralogues Have Different Redox Active Sites—SeDsbA, SeDsbL, and SeSrgA have different dipeptides in the catalytic Cys-$X$-$X$-Cys sites, which are PH, PF, and PP for SeDsbA, SeDsbL, and SeSrgA, respectively. Despite this, they all adopt the usual right-hand hook conformation (46) that is found in other TRX-like redox proteins (Fig. 2B). These enzymes also differ in the identity of the residue before the cis-Proline; SeSrgA has a Thr ($147$Thr-$\text{cis-Pro}$ loop), which is within hydrogen bonding distance of the nucleophilic N-terminal $27$Cys (3.2 Å), while SeDsbA and SeDsbL have a valine in that position. These different active sites are likely to give rise to different redox properties among the sv. Typhimurium DsbA enzymes.
SeDsbA (Fig. 4). Conversely, SeSrgA is about 10 times more reducing than SeDsbA, which has a redox potential similar to EcDsbA (49). The pH dependence of the thiolate-specific absorbance signal (S = (A280/440)/(A280/(A440/440))) was fitted according to the Henderson-Hasselbalch equation (the oxidized proteins were used as a reference). Right panel, catalyzed reduction of insulin. Reduction of insulin (131 µM) was measured in 0.1 M phosphate buffer, pH 7.0, 2 mM EDTA. The assay was performed in the absence (x) or presence of 10 µM SeSrgA (.), 10 µM SeDsbA (.), or 10 µM SeDsbL T150V (○). 5 µM EcDsbC (●) and 10 µM EcDsbA (∆) were used as positive controls. At time 0, DTT was added to each reaction to a final concentration of 0.35 mM, and the catalyzed reduction of insulin was measured as an increase in absorbance at 650 nm.

**Surface Properties of sv. Typhimurium DsbA Paralogues**—In accordance with their low sequence identity (SeSrgA shares 36 and 18% sequence identity with SeDsbA and SeDsbL, respectively, Fig. 2A), the three *Salmonella* DsbA proteins have different surface properties, principally in regions surrounding the catalytic sites that are predicted to be important for activity (Fig. 3). SeDsbA shares the surface features of its closest structural homologues EcDsbA, *Vibrio cholerae* DsbA (TcpG) (47) and *Neisseria meningitidis* NmDsbA3 (15, 48), which include a hydrophobic patch and a peptide-binding groove incorporating a hydrophobic pocket (Fig. 3). By contrast, the surface of the hydrophobic patch is to some extent positive in SeSrgA and more so in SeDsbL (Fig. 3). Furthermore, both SeSrgA and SeDsbL have truncated peptide binding grooves compared with SeDsbA (Fig. 1, B and C). Differences in the region preceding the β6β5α7 motif of SeSrgA (distinct orientation of the turn preceding the 147Thr-cis-Pro loop fixed by the electrostatic interaction between 145Arg and 17Glu (Fig. 1B)) and SeDsbL (bent α6 helix (Fig. 1B) and 6-residue deletion at C terminus) alter the shape and size of the hydrophobic peptide-binding groove (1785, 891, and 1022 Å3 for SeDsbA, SeSrgA, and SeDsbL, respectively, Fig. 1C). Moreover, SeDsbL lacks a hydrophobic pocket in the peptide-binding groove (Fig. 3). Finally, both SeSrgA and SeDsbL lack the acidic cavity present at the opposite side of the active site of SeDsbA (Fig. 3).

*SeDsbA, SeDsbL, and SeSrgA Have Very Different Redox Properties*—The oxidizing power of SeDsbA, SeDsbL, and SeSrgA was determined by measuring the equilibrium constants (K<sub>ox</sub>) for the thiol–disulfide exchange reaction with glutathione (Fig. 4A). The measured constants are 12.8 × 10<sup>-5</sup> m<sup>s</sup>, 12.7 × 10<sup>-6</sup> m<sup>s</sup>, and 11.3 × 10<sup>-6</sup> m<sup>s</sup> for SeDsbA, SeDsbL, and SeSrgA, respectively. These K<sub>ox</sub> values convert into intrinsic redox potentials of −126 mV, −97 mV, and −154 mV, respectively (Table 2). SeDsbL is about 10 times more oxidizing than SeDsbA, which has a redox potential similar to EcDsbA (49). Conversely, SeSrgA is about 10 times more reducing than SeDsbA (Fig. 4A, Table 2).

**TABLE 2**

| Enzyme | K<sub>eq</sub> (M) | Redox potential (mV) | pK<sub>a</sub> |
|--------|-----------------|---------------------|--------------|
| SeDsbA | 12.8 ± 0.3 × 10<sup>-5</sup> | −126 | 3.3 ± 0.06 |
| SeDsbL | 12.7 ± 0.6 × 10<sup>-6</sup> | −97 | 3.8 ± 0.14 |
| SeSrgA | 11.3 ± 0.3 × 10<sup>-4</sup> | −154 | 4.7 ± 0.07 |
| EcDsbA | 8.1 ± 0.2 × 10<sup>-5</sup> | −119 | 3.3 ± 0.09 |

* Ref. 49.

The more N-terminal of the two cysteines in the CXXC motif of TRX fold proteins is the nucleophilic cysteine, it is solvent-exposed and reactive. Its reactivity is in part because of a lower p<sub>K<sub>a</sub></sub> (~4–5) compared with most cysteines (~8.5) (36). We measured the reactive cysteine p<sub>K<sub>a</sub></sub> of all three *Salmonella* DsbA-like proteins. Like the EcDsbA (36), the two stronger oxidants SeDsbA and SeDsbL showed very low p<sub>K<sub>a</sub></sub> values, 3.6 and 3.8, respectively (Table 2). However, the more reducing SeSrgA has a p<sub>K<sub>a</sub></sub> of 4.7, which is considerably high for a DsbA-like protein and denotes a less reactive nucleophilic cysteine.

**sv. Typhimurium DsbA Paralogues Have Different Disulfide Oxidoreductase Activity in Vitro**—Most TRX-like oxidoreductases, including DsbC and to some extent DsbA, catalyze DTT-induced insulin reduction. SeDsbA, SeDsbL, and SeSrgA showed very different activities in this standard disulfide reductase assay. Whereas SeDsbA had similar activity to EcDsbA, the less oxidizing SeSrgA reduced insulin rather rapidly and showed an activity more similar to that of the disulfide isomerase DsbC (Fig. 4). By contrast, SeDsbL was completely inactive in this assay. Furthermore, unlike *E. coli* CFT073 DsbL (14), SeDsbL did not prevent unspecific aggregation of reduced insulin.

**Expression of DsbA Paralogues in sv. Typhimurium**—To begin to understand the role of each DsbA parologue in *sv. Typhimurium*, we investigated the expression of SeDsbA, SeDsbL, and SeSrgA in *sv. Typhimurium* strain SL1344 using rabbit polyclonal antisera created specifically against each protein. Strain SL1344 expressed SeDsbA and SeSrgA, but not SeDsbL during growth in LB broth (Fig. 5A). To confirm that
the immunoreactive bands were specific to each DsbA paralogue, we constructed single deletion mutants for dsbA, dsbL, and srgA, as well as a dsbA,dsbL,srgA triple mutant in SL1344 (i.e. SL1344dsbA, SL1344dsbL, SL1344srgA, and SL1344dsbA, dsbL,srgA). Loss of SeDsbA and SeSrgA expression was confirmed by Western blot analysis of SL1344dsbA and SL1344srgA, respectively. No immunoreactive bands at ~20 kDa (approximate molecular mass of each DsbA paralogue) were detected in whole-cell samples prepared from above basal levels was observed only upon co-expression with DsbL in the triple mutant, but not with SeDsbA or SeSrgA (Fig. 5B). Lastly, phase-variable expression of Pef on the cell surface of SL1344dsbA,dsbL,srgA was restored by all three DsbA paralogues, as judged by immunofluorescence labeling using primary anti-PefA sera and a secondary FITC-IgG conjugate (Fig. 5C). Taken together, the results suggest redundancy in the catalytic folding of some but not all substrates by SeDsbA, SeDsbL, and SeSrgA.

Specificity of DsbA Paralogues for Native Protein Substrates in sv. Typhimurium—The structural and surface property differences of SeDsbA, SeDsbL, and SeSrgA, particularly in the region around the active site, suggest unique substrate specificities. Specifically, SeDsbA, SeDsbL, and SeSrgA have been independently shown to catalyze the correct folding of the flagella protein FlgI, the periplasmic enzyme AssT and the major fimbrial subunit PefA, respectively (14, 19, 20). However, the specificity of each enzyme for each of these substrates has not been investigated. To test this in the relevant genetic background of a sv. Typhimurium strain, we transiently expressed each DsbA paralogue in the triple mutant SL1344dsbA,dsbL,srgA and assessed each strain for 1) motility, 2) AssT enzyme activity, and 3) phase-variable expression of Pef fimbriae on the cell surface. All three oxidoreductases restored motility in SL1344dsbA,dsbL,srgA to wild-type levels (Table 3). In contrast, catalytic activity of AssT

FIGURE 5. A, Western blot analysis of (i) SeDsbA, (ii) SeSrgA, and (iii) SeDsbL expression in whole cell lysates of sv. Typhimurium SL1344 wild type, dsbA, dsbL, srgA, and dsbA,dsbL,srgA deletion mutants and triple mutant dsbA,dsbL,srgA complemented with pWSK29 (vector control), pDsbA (DsbA expression vector), pDsbLI (DsbLI expression vector), or pSrgA (SrgA expression vector). B, UV illumination images of LB-MUS agar plates containing colonies of the triple mutant strain SL1344dsbA,dsbL,srgA complemented with (i) pAssT + pWSK29 (vector control), (ii) pAssT + pDsbA, (iii) pAssT + pSrgA, and (iv) pAssT + pDsbLI. Production of functional AssT results in cleavage of the sulfate of MUS in the medium forming a fluorescent product (4-methylumbelliferone). C, Immunofluorescence analysis of Pef fimbrial expression in SL1344 strains. Phase contrast (panels A, C, E, G, I) and fluorescence (panels B, D, F, H, J) microscopy images of SL1344 wild type (A and B) and the triple mutant strain SL1344dsbA,dsbL,srgA complemented with pWSK29 (vector control) (C and D), pSrgA (E and F), pDsbA (G and H), or pDsbLI (I and J). Bacterial cells cultured statically in LB-MES pH 5.1 were labeled with rabbit polyclonal anti-PefA sera followed by anti-rabbit IgG-FITC conjugate. No Pef-expressing SL1344dsbA,dsbL,srgA(pWSK29) cells were detected in multiple scanned fields.

Structural and Functional Characterization of Salmonella DsbA Enzymes

VOLUME 285 • NUMBER 24 • JUNE 11, 2010

18430 JOURNAL OF BIOLOGICAL CHEMISTRY
DISCUSSION

The formation of disulfide bonds is a crucial step required for the correct folding of many secreted proteins such as bacterial virulence factors. In prokaryotes disulfide formation machineries show considerable diversity (12), particularly among Gram-negative microbes, which contain an extended arsenal of thiol-disulfide oxidoreductases (14, 15), underscoring the relevance of redox control in these organisms.

In this study we have carried out a comprehensive analysis of the sv. Typhimurium oxidative pathway. Unlike prototypic *E. coli* K-12, which encodes a single thiol oxidase (EcDsbA), sv. Typhimurium possess three DsbA homologues. Structural characterization of SeDsba, SeDsbL, and SeSrgA has shown that they all have the canonical EcDsbA fold consisting of a TRX domain that incorporates a helical insertion. Despite this, the three DsbA paralogues share low sequence identity and it is therefore not surprising that they contain unique structural and enzymatic properties. SeDsba retains all the surface features of the *E. coli* homologue (44), including a hydrophobic groove that binds the key partner protein DsbB (45). SeDsba is postulated to interact with unfolded peptides (44) and possesses a broad hydrophobic region surrounding the active site. SeSrgA and particularly SeDsbL differ from SeDsba in that they have truncated peptide binding grooves and positively charged surfaces surrounding the active site (Figs. 1 and 3). Additionally, SeDsbL incorporates long loops in the helical domain, including the one linking α3 and α4 (Fig. 2C), which is lined with negatively charged residues and generates an acidic protrusion that maps near the reductase catalytic site (Fig. 3).

SeSrgA, SeDsba, and SeDsbL have markedly different oxidizing strengths (Fig. 4). SeSrgA, with an $E^{pr}$ of $-154 \text{ mV}$, is one of the most reducing DsbA proteins characterized (Wolbachia *pipientis* α-Dsba1 being the most reducing one, $E^{pr} = -163 \text{ mV}$ (50)). SeDsba is as strong an oxidant as EcDsbA ($E^{pr} = -126 \text{ mV}$ and $-122 \text{ mV}$, respectively (11)), while SeDsbL ($E^{pr} = -97 \text{ mV}$) is together with the three Neisseria Dsba homologues (redundant potentials of $-80 \text{ mV}$) (15) and DsbL from uropathogenic *E. coli* CFT073 ($E^{pr} = -90 \text{ mV}$) (14) one of the most oxidizing proteins so far characterized. Moreover, the reactivity of the nucleophilic cysteine also varies across the three *Salmonella* proteins (Fig. 4). As expected from their intrinsic redox potentials, SeDsba and SeDsbL contain very acidic cysteines ($pK_a$ values, 3.6 and 3.8, respectively) and the more reducing SeSrgA has a less reactive cysteine with a $pK_a$ similar to that of isomerase-like DsbC (8) ($pK_a$ 4.7 and 4.6, respectively).

The different redox properties of SeSrgA seem to be mainly dictated by the dipeptide in the active site. The histidine residue in the Cys-Pro-His-Cys motif of EcDsbA plays a key role in determining the oxidizing properties of this protein and substitution of this residue with a proline dramatically increases the $pK_a$ of the nucleophilic cysteine and reduces the oxidizing strength of EcDsba (11). In the same way, SeSrgA, which naturally has a proline instead of a histidine in the catalytic site (Cys-Pro-Pro-Cys), is more reducing than EcDsba or SeDsba and contains a much less reactive active site cysteine. In the case of SeDsbL, the cluster of positively charged residues surrounding the Cys-Pro-Phe-Cys active site likely stabilize the thiolate form of the protein which results in a decreased $pK_a$ of the nucleophilic active site cysteine and an increase in the redox potential.

The structural differences and divergent redox properties suggest that the three DsbA proteins encoded by sv. Typhimurium may have different substrate specificities and redox function. Indeed, these proteins have distinct *in vitro* oxidoreductase activity; SeSrgA is a strong insulin reductase, with an activity similar to that of the disulfide isomerase EcDsbC. SeDsba is as active as EcDsbA in this assay and SeDsbL did not show any insulin reductase activity (Fig. 4). Despite some similarities between the biochemical properties of SeSrgA and EcDsbC, *in vitro* we found no activity for disulfide bond isomerase in SeSrgA or in any of the *Salmonella* Dsba proteins (data not shown).

To further investigate the functional properties of SeDsba, SeDsbL, and SeSrgA, we examined their expression and target specificity in the context of a sv. Typhimurium background. We were able to detect expression of SeDsba and SeSrgA, but not SeDsbL. The lack of SeDsbL expression is consistent with a previous report that examined SeDsbL expression in uropathogenic *E. coli* (18). All three oxidoreductases were able to restore motility and Pef fimbriae production in the sv. Typhimurium triple mutant SL1344Δdsba,ΔdsbLΔsrgA. In contrast, DsbL exhibited target specificity for AssT.

In conclusion, although SeSrgA, SeDsba, and SeDsbL share the same overall fold, structural differences between these proteins (mainly localized in the redox active site and peptide binding interface) imply that they may target different substrates and have distinct functionality. Biochemical characterization confirmed these postulations, since SeSrgA, SeDsba, and SeDsbL have different redox strengths, the reactivity of their surface exposed cysteine varies and they show dissimilar oxidoreductase activity *in vitro*. Despite some degree of functional redundancy, SeDsba, SeDsbL, and SeSrgA therefore represent thiol oxidases that cover a wide range of structural and biochemical properties to efficiently catalyze the oxidative folding of target proteins. During sv. Typhimurium infection, SeDsba, SeDsbL, and SeSrgA are probably used differently in temporal and spatial regulation and this, together with their different degree of target specificity, emphasizes the importance of this critical folding step in bacterial survival and virulence.

Acknowledgments—We thank Professor Jenny Martin for support and advice. We acknowledge use of the Australian Synchrotron and the support of PX1 3BM1 beamline scientists Julian Adams and Tom Caradoc-Davies. We thank Karl Byriel and Gordon King for support and access to the University of Queensland (UQ) ROCX Diffraction facility. We thank David Low for providing the anti-PefA polyclonal serum.

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**TABLE 3**

Molotivity of sv. Typhimurium SL1344 wild type and isogenic deletion mutants

| SL1344 strain | Average motility radius (mm) ± S.D. |
|---------------|------------------------------------|
| WT            | 21.3 ± 1.5                         |
| dsbA, dsbLI, srgA | 5 ± 2.6                           |
| dsbA, dsbLI, srgA + pDsbA | 26.7 ± 2.3                     |
| dsbA, dsbLI, srgA + pSrgA | 19.7 ± 2.1                      |
| dsbA, dsbLI, srgA + pDSbaLI | 20 ± 1.4                        |
| dsbA, dsbLI, srgA + pWSK29 (vector control) | 2.7 ± 0.6                   |
