Characterization of SrgA, a *Salmonella enterica* Serovar Typhimurium Virulence Plasmid-Encoded Parologue of the Disulfide Oxidoreductase DsbA, Essential for Biogenesis of Plasmid-Encoded Fimbriae

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Disulfide oxidoreductases are viewed as foldases that help to maintain proteins on productive folding pathways by enhancing the rate of protein folding through the catalytic incorporation of disulfide bonds. SrgA, encoded on the virulence plasmid pStSR100 of *Salmonella enterica* serovar Typhimurium and located downstream of the plasmid-borne fimbrial operon, is a disulfide oxidoreductase. Sequence analysis indicates that SrgA is similar to DsbA from, for example, *Escherichia coli*, but not as highly conserved as most of the chromosomally encoded disulfide oxidoreductases from members of the family *Enterobacteriaceae*. SrgA is localized to the periplasm, and its disulfide oxidoreductase activity is dependent upon the presence of functional DsbB, the protein that is also responsible for reoxidation of the major disulfide oxidoreductase, DsbA. A quantitative analysis of the disulfide oxidoreductase activity of SrgA showed that SrgA was less efficient than DsbA at introducing disulfide bonds into the substrate alkaline phosphatase, suggesting that SrgA is more substrate specific than DsbA. It was also demonstrated that the disulfide oxidoreductase activity of SrgA is necessary for the production of plasmid-encoded fimbriae. The major structural subunit of the plasmid-encoded fimbriae, PefA, contains a disulfide bond that must be oxidized in order for PefA stability to be maintained and for plasmid-encoded fimbriae to be assembled. SrgA efficiently oxidizes the disulfide bond of PefA, while the *S. enterica* serovar Typhimurium chromosomally encoded disulfide oxidoreductase DsbA does not. *pefA* and *srgA* were also specifically expressed at pH 5.1 but not at pH 7.0, suggesting that the regulatory mechanisms involved in *pef* gene expression are also involved in *srgA* expression. SrgA therefore appears to be a substrate-specific disulfide oxidoreductase, thus explaining the requirement for an additional catalyst of disulfide bond formation in addition to DsbA of *S. enterica* serovar Typhimurium.

The ability of the bacterial cell to synthesize and secrete specific proteins is dependent upon several aspects of protein secretory pathways. While the passage of proteins through the secretory apparatus in the inner membrane has been relatively well studied (reviewed in references 18, 42, and 60), the events occurring once proteins reach the periplasm of gram-negative cells are less well understood. Even though the periplasm may not be their final destination, proteins begin the process of conformational folding once they reach the periplasm (74). This folding is accompanied by proline isomerization and disulfide bond formation, both necessary to attaining and maintaining native structure (23). These processes are mediated by peptidyl-prolyl isomerases (45) and disulfide oxidoreductases (2, 50, 56) that are present in the periplasm.

Many proteinaceous structures, such as fimbriae (39, 78), flagella (14), and several bacterial toxins (46, 53, 65, 77), either contain disulfide bonds or require disulfide bonds in some component of their assembly pathway. The disulfide bond formation system that is most extensively characterized in *Escherichia coli* can be considered to be composed of two pathways, an oxidating pathway that includes proteins DsbA and DsbB, and an isomerization pathway utilizing DsbC, DsbG, DipZ

(DsbD), and TrxA (13). A number of in vitro studies have examined the intrinsic properties of the major protein involved in disulfide bond formation, DsbA (15, 24, 28–31, 35, 40, 43, 75), and many others have identified specific cellular defects related to the lack of disulfide bond formation. For example, DsbA is required for introducing a disulfide bond that stabilizes the structural subunit of the type IV bundle-forming pili in enteropathogenic *E. coli* (17, 78), and disulfide bond-forming mutants of *Vibrio cholerae* are avirulent due to an inability to secrete cholera toxin and form functional pili that aid in colonization (54, 67).

It was recently found that pertussis toxin, a multisubunit complex with several intramolecular disulfide bonds, is dependent upon DsbA for toxin assembly and upon DsbC for toxin secretion (65). Mutations in *dsbA* often have pleiotropic effects, as these mutations influence many proteins external to the inner membrane that contain disulfide bonds. However, only those disulfide bonds that are essential for native protein folding and stability or are involved more directly in the function of a particular protein have a phenotype when they are not oxidized.

Homologues of DsbA have been found in *Haemophilus influenzae* (69), *V. cholerae* (36, 54, 77), *Legionella* spp. (H. A. Shuman, 1994, submission to GenBank), *Azotobacter vinelandii* (51), *Shigella flexneri* (72), *Erwinia* spp. (61), and *Pseudomonas aeruginosa* (M. Leipelt, B. Schneidinger, and K.-E. Jaeger, 1997, submission to GenBank), to name a few, emphasizing

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that similar mechanisms are available to a wide range of bacteria to incorporate disulfide bonds into their secreted proteins. Considering that the substrates that DsbA acts on can be quite different from organism to organism and that many proteins contain cysteines that do not become disulfide bonded, it is interesting to contemplate whether or not these various forms of disulfide oxidoreductases have any substrate specificity. Also, if they do have substrate specificity, what aspects are important for recognizing particular substrates? It became of interest to examine this question when it was discovered that *Salmonella* spp. possessed a second disulfide oxidoreductase that was quite different from the chromosomally encoded *Salmonella enterica* serovar Typhimurium DsbA homologue (71).

Friedrich et al. (25) previously isolated and sequenced a 13.9-kb segment of the 90-kb plasmid from *S. enterica* serovar Typhimurium that contains a 7-kb region bearing the genes for a novel type of fimbriae named plasmid-encoded fimbriae. In this region, five open reading frames are related in sequence to genes in previously characterized fimbrial biosynthetic operons, such as the *pap* operon (37). Baumlér et al. (6, 7) have since demonstrated that the plasmid-encoded fimbriae mediate adherence to the mouse small intestine and seem to be involved in the initiation of fluid accumulation. The presence of the 90-kb plasmid also confers on the bacteria an increased ability to spread to the mesenteric lymph nodes, spleen, and liver after the initial invasion of the intestinal epithelium (32), and therefore, the overall virulence of *S. enterica* serovar Typhimurium is determined by both chromosomal and plasmid-borne genes. Recently, a study on the regulation of plasmid-encoded fimbriae has shown that fimbria production is subject to phase variation via DNA methylation, and plasmid-encoded fimbriae were expressed only under conditions of low pH and O₂ in rich medium (52).

The sequence identified by Friedrich et al. (25) also contained an open reading frame downstream of the region containing the regulatory gene *pefI* (52) that showed no homology to previously identified fimbrial genes. This open reading frame was identified as a periplasmic protein, as three independent *TnphoA* fusions generated within this open reading frame gave rise to active alkaline phosphatase (25). At the time, this protein was not thought to be required for expression of plasmid-encoded fimbriae, as plasmid-encoded fimbriae were assembled with either the wild-type or the *TnphoA*-interrupted version of this gene in an *E. coli* background strain (25).

In this paper we demonstrate that this open reading frame, called *srgA* (1), is actually required for plasmid-encoded fimbria expression in *S. enterica* serovar Typhimurium and that it encodes a functional disulfide oxidoreductase. SrgA appears to be a substrate-specific oxidoreductase involved in oxidation of a component of the plasmid-encoded fimbrial system.

MATERIALS AND METHODS

Bacterial strains and plasmids. All bacterial strains and plasmids are listed in Table 1. NZ, LB (27), and M63 minimal, low-phosphate (63) media were used with the appropriate antibiotics as required.

Complementation of *dsbA* and *srgA* function for the electron microscopy study was done with *E. coli* MC1061 (11). The *dsbA::Km* allele was introduced by P1-mediated transduction from strain MC4100 *dsbA::Km* (4) with selection for kanamycin resistance.

Recombinant DNA techniques. Restriction enzymes and DNA ligase were purchased from New England Biolabs. Isolation of plasmid DNA, digestion with

TABLE 1. Strains and plasmids

Strain or plasmid	Relevant genetic markers ^a	Reference
Strains		
KM1086	MC4100 <i>malT^c λ malF-lacZ102</i>	4
MC1061	<i>araD139 Δ(ara,leu)7697</i> <i>Δlac(IZY)X74 galE15 galK16 rpsL</i> <i>hsdR2 (r_K⁻ m_K⁺) mcrA mcrB1</i> <i>araD139 Δ(argF-lac)U169 rpsL150</i> <i>relA flbB5301 deoC1 ptsF25 rbsR</i>	11
MC4100	KM1086 <i>araΔ714 leu::Tn10</i>	This study
NLM117	<i>dsbA::Km mot⁺</i>	This study
NLM119	KM1086 <i>araΔ714 leu::Tn10</i>	This study
NLM131	NLM117 <i>dsbB::Tet</i>	This study
NLM2153	SL1344 <i>ΔhisDCBHAFIE712 arg-501</i> <i>ser821</i> cured of pStSR100	R. Kelln
NLM2198	NLM2153 <i>dsbA::Km</i>	This study
NLM2217	SL1344 <i>his</i> pStSR100	73
Plasmids		
pRK415-1	Broad-host-range vector	41
pBAD24	pBR322 <i>ori ampR araC</i>	33
pLMN106	<i>dsbA</i> cloned into MCS of pBAD24	This study
pLMN107	<i>srgA</i> with hemagglutinin epitope cloned into MCS of pBAD24	This study
pLMN108	<i>srgA</i> cloned into MCS of pBAD24	This study
p30	14 kb of DNA from virulence plasmid pStSR100 of LT2 strain ST422 encoding the <i>pef</i> operon cloned into pRK415-1	25
p30Tn2.7	p30 with <i>TnphoA</i> inserted in <i>srgA</i> coding region	This study
p16-1	<i>dsbA</i> cloned into pBR322	4
pBAD33	pACYC184 <i>ori Cm^r araC</i>	33
pCB6	<i>srgA</i> cloned into pBAD33	This study

^a MCS, multiple cloning site.

restriction enzymes, gel purification of DNA fragments, ligations, and transformations were performed with standard techniques (58). Plasmids pLMN106 and pLMN108 were made by amplification of *dsbA* and *srgA* with oligonucleotide primers that incorporated an *EcoRI* restriction site and an optimized Shine-Dalgarno region and complementary downstream primers in a thermal cycling reaction. The thermal cycling products were gel purified, ligated into the multi-cloning site of pBAD24, and screened for complementation of activity in a *dsbA::Km* background strain. One *dsbA*-containing construct and one *srgA*-containing construct were chosen and sequenced to ensure that no additions or deletions had occurred during the cloning procedure.

Plasmid pLMN107 was constructed in a similar fashion except that the downstream primer also contained the sequence for the hemagglutinin epitope (20), which was inserted immediately before the stop codon and enabled detection of SrgA by immunoprecipitation and also Western immunoblotting. The epitope protein sequence is YPYDVPDYA. The plasmid pCB6 was constructed by moving the *srgA* coding region from pLMN107 by digestion with *NheI* and *HindIII* and ligating this fragment into *XbaI*- and *HindIII*-cut pBAD33.

Pulse-chase labeling and immunoprecipitations. Exponential-phase cells were pulse labeled for 30 s with 40 mCi of [³⁵S]methionine per ml and chased with a final concentration of 0.1% methionine in large glass tubes. All labeling reactions were terminated by transferring 0.7 ml of bacteria to a 1.5-ml microcentrifuge tube that had been prechilled in an ice-water bath and contained methionine and iodoacetamide to final concentrations of 0.1% and 100 mM, respectively. This method rapidly inhibits further disulfide bond formation while preventing the initiation of translation of nascent chains (10). The bacteria were then pelleted by centrifugation for 2 min at 4°C, resuspended in 50 ml of sodium dodecyl sulfate (SDS) buffer (10 mM Tris [pH 8.0], 1% SDS, 1 mM EDTA), and heated at 80°C for 5 min. The samples were then immunoprecipitated as described by Froshauer et al. (26), with saturating concentrations of antiserum (anti-hemagglutinin or anti-DsbA), and resuspended in 50 μl of SDS sample buffer. Of these final samples, 17 μl was loaded and electrophoresed on an SDS-10% polyacrylamide gel (44). Protein expression levels were measured with phosphorimager

data of the relevant band compared to an internal control and corrected for the methionine content of each protein.

Southern hybridization. Total cellular DNA was isolated with the Puregene kit (Gentra Systems Inc.). DNA separated on agarose gels was transferred onto a nylon membrane (Amersham Inc.) with a vacuum transfer apparatus (Tyler Research Instruments) as follows. The gel was soaked in denaturation solution (0.25 M HCl) for 8 min, in denaturation solution (0.5 M NaOH, 1.5 M NaCl) for 45 min, and in neutralizing solution (0.5 M Tris-HCl [pH 7.5], 3 M NaCl) for another 45 min. Transfer was done in the presence of $20\times$ SSC (per liter: 175.3 g of NaCl, 88.2 g of sodium citrate, pH 7.0) for 1 h. After the transfer, DNA was fixed on the membrane by baking it at 120°C for 30 min. DNA probes were labeled with nonradioactive digoxigenin with the Genius nonradioactive nucleic acid labeling and detection system (Boehringer Mannheim). The *srgA* probe was labeled with a thermal cycling reaction method as suggested in the Genius kit. The primers used for this reaction (5'-GGAATTCACCATGAATTATGCCCCG G-3' and 3'-CGACCGGCCATTGGGACTACGTC-5') amplified the coding region of *srgA*. The product of the thermal cycling reaction was purified with the QIAquick PCR purification kit (Qiagen Inc.) and quantitated according to the Genius protocol.

The plasmid-borne-fimbrial-operon probe was generated by random-primed labeling of a 5.4-kb *SacI*-*BglII* digestion product that included the coding regions for *pefBACD* or with a smaller, *pefA*-specific probe by following the procedures recommended in the Genius protocol. Hybridizations were performed overnight at temperatures ranging from 50°C to 75°C, depending upon the stringency required, in 20 ml of hybridization solution containing between 10 and 25 ng of probe per ml. Chemiluminescent detection was used to visualize reactive bands. When the same membrane was to be used more than once, the alkali-labile probe was stripped by rinsing it in water for 2 min and incubating it for 15 min twice at 37°C in 0.4 M NaOH-0.1% SDS and then 15 min at room temperature in the same solution (with shaking). The membrane was then washed for 10 min with $2\times$ SSC and kept moist until another hybridization was begun.

Northern hybridization. RNA was isolated from static cultures consisting of 10-ml volumes grown without aeration in 20-ml test tubes. LB medium was buffered with 2-(*N*-morpholino)ethanesulfonic acid at a 100 mM final concentration, and the pH was adjusted before sterilization by filtration (64). Once cultures reached an optical density at 600 nm (OD_{600}) of approximately 0.5, cells were harvested and RNA was isolated with Trizol (Gibco Life Technologies). The final RNA samples were resuspended in formamide and stored at -20°C. RNA electrophoresis was carried out with 30 µg of total RNA for each sample. Electrophoresis was carried out in formaldehyde-containing 1.2% agarose gels and transferred to nylon membranes according to the protocol described by Fourney et al. (22). Hybridization was performed with digoxigenin-labeled probes by following the manufacturer's protocols (Genius nonradioactive nucleic acid labeling and detection kit).

Cellular fractionation procedure. For cellular localization studies with cloned SrgA, cells were grown overnight in LB with 200 µg of ampicillin at 37°C with shaking. Five milliliters of overnight culture was added to 1 liter of LB with 2% glycerol the following morning. Cells were incubated at 37°C with shaking until the OD_{600} was approximately 0.6. The cultures were split in two, either arabinose or glucose was added to a final concentration of 0.01% (vol/vol), and the incubation was continued for 3 more h. Following incubation, the cells were centrifuged at $5,000\times g$ for 5 min at 4°C. The cells were subsequently resuspended in 0.25 culture volume of cold 50 mM Tris-HCl-2 mM EDTA, pH 8.0. Cells were centrifuged as described above, and 1 g of cells was resuspended in 40 ml of cold 0.033 M Tris-HCl, pH 7.1. Forty milliliters of 40% (wt/vol) sucrose in 0.033 M Tris-HCl, pH 7.1, was then added to each gram of resuspended cells. Cells were then incubated at 25°C for 30 min with gentle shaking. Following centrifugation for 10 min at $6,000\times g$ at 4°C, the cells were quickly resuspended in cold 0.5 mM MgCl₂ and incubated on ice for 10 min with shaking. Following a final centrifugation at $6,000\times g$ for 10 min, the supernatant containing the released periplasmic proteins was retained and stored at -20°C.

The remaining pellet (containing cytoplasmic, inner membrane, and outer membrane proteins) was resuspended in 4 ml of cold 20 mM Tris-HCl, pH 7.5. Peptidoglycan was digested with lysozyme at a final concentration of 100 µg/ml for 15 min at 30°C, then the cells were sonicated on ice at a power level of 5 at 50% duty for three pulses of 5 s. One and a half milliliters of lysate was then centrifuged at $14,000\times g$ for 10 min. Soluble cytoplasmic proteins were obtained by transferring 100 µl of the resulting supernatant to a fresh tube and mixing with 100 µl of $2\times$ sample buffer (100 mM dithiothreitol, 2% SDS, 80 mM Tris-HCl [pH 6.8], 0.006% bromophenol blue, 15% glycerol). Insoluble fractions were obtained by washing the pellet twice in 750 µl of 20 mM Tris-HCl, pH 7.5, then resuspending in 1.5 ml of 1% SDS with heating and vigorous mixing. From this solution, a 100-µl sample was taken and mixed with an equal volume of $2\times$

sample buffer. Soluble and insoluble fractions were heated for 3 min at 65°C and then stored at -20°C until sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis.

Western immunoblotting. Immunoblotting was carried out as previously described by Finnen et al. (21) with either commercially available antihemagglutinin (Boehringer-Mannheim) to detect SrgA, polyclonal antiserum raised against purified *E. coli* DsbA, which cross-reacts with *S. enterica* serovar Typhimurium DsbA (68), or polyclonal antiserum to *S. enterica* serovar Typhimurium PefA (kindly provided by B. Ahmer). Oxidized and reduced states of PefA were examined by comparing the gel mobilities of PefA in samples with and without dithiothreitol.

Electron microscopy. Single colonies of the eight transformed strains were inoculated into 5 ml of LB broth with the appropriate antibiotics and incubated overnight at 37°C with moderate shaking. Cultures were collected by centrifugation and washed once with 1 ml of 30 mM Tris-HCl, pH 10.3. Cells were suspended in 200 µl of the same solution and placed on ice. A 200-mesh carbon-coated grid was floated on a drop of the bacterial suspension for 30 s, and excess liquid was removed with a paper wick. The grid was then placed on a drop of 1% uranyl acetate for 15 to 20 s. Excess stain was removed, and the samples were examined with a JEOL 100S transmission electron microscope.

Assays. Motility assays were carried out on LB plates containing 0.3% agar. Two microliters of an exponential-phase culture was stabbed into the agar, and the plates were incubated at 30°C for 8 h. The diameters of the colonies radiating out from the stab point were measured. β -Galactosidase assays were carried out as described by Miller (49). Alkaline phosphatase assays were done as described by Manoil (47).

RESULTS

Protein and genetic conservation of SrgA. SrgA was first identified as an open reading frame from sequences deposited in GenBank during a search for amino acid sequences similar to *E. coli* DsbA. A 14-kb region of an *S. enterica* serovar Typhimurium virulence plasmid that bears genes for fimbrial biosynthesis had been sequenced by Friedrich et al. (25). This length of DNA also included several open reading frames with no apparent homology to previously characterized proteins of fimbrial systems. One of these open reading frames (ORF8 [25]) was homologous to DsbA and was named SrgA after being identified as a potential target of *S. enterica* serovar Typhimurium SdiA (*sdiA*-regulated gene A) (1).

The *S. enterica* serovar Typhimurium DsbA and SrgA proteins are 37% identical, with an additional 21% being similar. The region around the active-site cysteines is the most well conserved (Fig. 1) and is also the region best conserved among other oxidoreductases in the thioredoxin superfamily, such as TrxA and protein disulfide isomerase (19). A comparison with other prokaryotic DsbA homologues showed that the SrgA region is less similar to DsbA than either Por from *H. influenzae* or TcpG from *V. cholerae*. Sequence analysis also identified SrgA homologues in *S. enterica* serovar Typhi and *S. enterica* serovar Enteritidis (57). Although these authors considered these two proteins homologues of DsbA, these proteins are much more similar to SrgA than they are to DsbA from either *E. coli* or *S. enterica* serovar Typhimurium (71). The *S. enterica* serovar Typhi homologue, Dlt, is encoded on the chromosome (*S. enterica* serovar Typhi strains generally do not carry large virulence plasmids), while the *S. enterica* serovar Enteritidis homologue is located on a large virulence plasmid. In either case, these genes are located just downstream of operons that code for *S. enterica* serovar Enteritidis fimbrial proteins (SE fimbriae), which are very similar to those of the plasmid-borne fimbria operon.

Southern hybridization analysis with a probe specific for the

FIG. 1. Comparison of amino acid sequences of SrgA. The stars indicate identical residues between SrgA and DsbA (row &) or between all of the sequences (row all). The A and B domain labeling is based on the crystal structure determination for *E. coli* DsbA (48), and the B domain is inserted into the A domain in this linear representation. The α -helical (dashed) and β -sheet (dotted) regions are boxed according to Guddat et al. (29). The α -helix region containing the active site is represented in bold. Por is the disulfide oxidoreductase from *Haemophilus influenzae* (69), and TcpG is the disulfide oxidoreductase from *Vibrio cholerae* (54).

srgA coding region was carried out on several *Salmonella* serovars to determine if *srgA* was present in additional *Salmonella* strains. Table 2 lists the strains that were tested, the sizes of the plasmids, the frequency of the strains carrying a large virulence plasmid, and whether or not the *srgA* probe hybridized to total DNA isolated from these strains. At least two strains of each serovar representing different electrophoretic types within each serovar were tested (8, 9). Hybridization was also carried out with a probe specific for the major plasmid-borne fimbrial subunit (*pefA*) in order to determine if *srgA* distribution matched *pef* operon distribution.

Of the 11 serovars tested, at least one of the strains in seven of the serovars reacted with the *srgA* probe. All of these serovars are known to carry virulence plasmids except for *S. enterica* serovar Derby. Many of the strains reacted with the *srgA* probe at high stringency, indicating that *srgA* is highly conserved in these serovars. The *pef* fimbrial genes were detected in four of the serovars tested. The presence of the plasmid-borne fimbrial operon correlated with the presence of *srgA*, but the *srgA* gene was detected only in strains of three additional serovars, *S. enterica* serovar Dublin, *S. enterica* serovar Heidelberg, and *S. enterica* serovar Pullorum.

Cellular localization of SrgA. Analysis of the amino acid sequence of SrgA showed that it contained a putative signal sequence cleavage/modification signal characteristic of a membrane-embedded lipoprotein (76). Fractionation studies were carried out to determine if SrgA was membrane associated or a soluble periplasmic protein similar to DsbA. Expression of cloned SrgA was carried out under inducing and noninducing conditions, and the resulting cells were separated into periplasmic, soluble cytoplasmic, and insoluble (membrane proteins)

fractions. The results of the fractionation procedure, presented in Fig. 2, clearly show that SrgA is associated primarily with the soluble periplasmic fraction, with no SrgA protein being detected in the membrane fraction.

Complementation analysis of disulfide oxidoreductase activity with *srgA*. To determine if SrgA had disulfide oxidoreductase activity, a clone containing the entire plasmid-encoded fimbria coding region with and without a *TnphoA* insertion in *srgA* was compared for its ability to complement a *dsbA* null mutant of *E. coli*. The strain used for the complementation studies (NLM117) contained *dsbA*::Km and encoded the MalF-LacZ102 fusion protein. Previous studies (3, 4) had shown that this fusion protein produced active β -galactosidase only when the Dsb system was nonfunctional. Complementation by the *srgA*-containing vectors was easily assayed by observing the color of colonies on plates containing 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-Gal). The vector alone and the *srgA*::*TnphoA*-containing plasmid did not complement the *dsbA* null mutant, but the *srgA*⁺ strain did, suggesting that, at least in multiple copy, *srgA* was able to substitute for *dsbA* (data not shown).

In *E. coli*, the oxidizing potential of DsbA is regenerated by the inner membrane protein DsbB (31). In order to test if the activity of SrgA was also DsbB dependent, the *srgA*-containing plasmid pLMN108 was introduced into a *dsbA*::Km *dsbB*::Tet strain (NLM131) and compared to a *dsbA*::Km strain with pLMN108 for the ability of *srgA* to restore Dsb activity on X-Gal-containing plates. SrgA was not able to complement a *dsbA* null mutant in the absence of *dsbB*, suggesting that SrgA activity was dependent upon the presence of DsbB.

TABLE 2. Distribution of *srgA* and the plasmid-borne fimbrial operon among *Salmonella* serovars

Serovar and strain ^a	Plasmid size ^b (kb)	Prevalence (% of strains examined)	<i>spv</i> region present ^c	<i>srgA</i> present	Fimbrial operon present
Typhimurium	90	89	+		
SARA1				+	+
SARA2				+	+
SARB66				+	+
SL1344				+	+
Choleraesuis	50	100	+		
SARB4				+	+
SARB6				+	+
SARB5				—	—
SARB7				—	—
Derby	60	ND ^d			
SARB9				+	+
SARB10				+	+
SARB11				—	—
Dublin	75	89	+		
SARB12				+	—
SARB13				+	—
SARB14				+	—
Enteritidis	54	87	+		
SARB16				+	+
SARB17				+	—
SARB18				+	+
SARB19				+	—
Heidelberg	95	38	—		
SARB23				+	—
SARB24				—	—
Montevideo	125	ND			
SARB30				—	—
SARB31				—	—
Newport	70	20	—		
SARB36				—	—
SARB37				—	—
SARB38				—	—
Panama	30	22	—		
SARB39				—	—
SARB40				—	—
SARB41				—	—
Pullorum	89	ND	+		
SARB51				—	—
SARB52				+	—
Saintpaul					
SARA22	ND	ND	ND	—	—

^a Strain designations determined by Boyd et al. (9) and Beltran et al. (8) in reference collections of *Salmonella* serovars.

^b Compilation of data (32, 34, 56).

^c Compilation of data (32, 56).

^d ND, not determined.

SrgA oxidizes a component of plasmid-encoded fimbriae. It was previously thought that expression of *srgA* did not affect plasmid-encoded fimbriae, but those studies were carried out in a *dsbA*⁺ *E. coli* background strain (25), where *E. coli* DsbA could provide oxidizing potential, masking a *srgA* phenotype. To determine if oxidoreductase activity was necessary for plasmid-encoded fimbrial gene expression and to determine if SrgA could provide that oxidoreductase activity, a set of eight strains that differed in their genotypes at *dsbA*, *srgA*, and the plasmid-borne fimbrial operon were constructed in an *E. coli* background strain that expresses no native fimbriae. The plasmid-borne fimbria genes were carried on a multicopy plasmid. Cells were examined by electron microscopy following negative

FIG. 2. SDS-PAGE Coomassie-stained gel (A) and Western immunoblot (B) showing the location of SrgA. Strain NLM2198/pLMN107 was grown in the absence (—) and presence (+) of arabinose induction of SrgA. Cells were harvested and fractionated as described in Materials and Methods. WC, whole-cell lysate; P, periplasmic fraction; C, soluble cytoplasmic fraction; M, membrane fraction. Molecular size markers are indicated to the left of the panels.

staining, allowing the visualization of fimbriae as a densely staining region at the cell surface (Fig. 3).

In the absence of both SrgA and DsbA (MC1061 *dsbA*::Km p30Tn2.7; Fig. 3D), no visible fimbriae were produced, whereas strains MC1061 and MC1061 *dsbA*::Km expressed large amounts of plasmid-encoded fimbriae in the presence of plasmid p30 (Fig. 3A and B), suggesting that disulfide oxidoreductase activity is necessary for plasmid-encoded fimbria expression and that either SrgA or DsbA could provide that activity. Fimbriae were also present in MC1061 p30Tn2.7, as originally observed (25).

SrgA partially complements *dsbA*-mediated flagellum defect. Using the same strains, we also compared the activities of DsbA and SrgA on flagellum production by electron microscopy, where flagella are visible as undulating threads in the background (Fig. 3). It had been established previously that the P ring protein of the *E. coli* flagellar apparatus contains an essential disulfide bond that is oxidized by the *dsb* system (14). Strain MC1061 is normally motile, but its production or assembly of flagella was eliminated by introduction of the *dsbA*::Km gene disruption (Fig. 3G versus H). Production of flagella was restored to strain MC1061 *dsbA*::Km by the presence of plasmid p16-1 carrying *dsbA*⁺ (Fig. 3H versus F). Strain MC1061 carrying plasmid p30 also expressed flagella.

When SrgA function was present in the absence of DsbA, flagella were produced, but in reduced quantity (Fig. 3B). Cells expressing DsbA possessed four to eight flagella per cell, whereas cells in which only SrgA was expressed possessed about one flagellum per cell. Flagellar expression was also

FIG. 3. Electron micrographs of negatively stained *E. coli* MC1061 expressing DsbA (*dsbA*⁺) (left column) or not expressing DsbA (*dsbA*::Km) (right column) with different plasmid constructs in each row as indicated. Panels A and B, *pef*⁺ *srgA*⁺; panels C and D, *pef*⁺ *srgA*; panels E and F, *pef* *srgA* *dsbA* overexpressed; panels G and H, vector-only control.

examined by measuring motility. Table 3 shows that *dsbA* null strains were nonmotile, while *dsbA* provided in *trans* and expressed by induction with 0.01% arabinose partially restored motility. Under the same induction conditions, SrgA-mediated

TABLE 3. Motility of *dsbA*- and *srgA*-complementing strains assessed by colony diameters in soft agar plates^a

Strain	Colony diameter (mm)		
	0.2% Glucose	Arabinose	
		0.01%	0.1%
NLM119	33	30	35
NLM117 + pBAD24	2	2	2
NLM117 + pLMN106	5	15	36
NLM117 + pLMN107	3	5	20
NLM117 + pLMN108	4	6	22

^a Assays were performed four times. Standard deviations were within 5% of the mean. Exponential-phase cells grown in 0.4% glycerol were stabbed into soft agar plates containing LB, 0.4% glycerol, 0.3% agar, and either glucose or arabinose. The diameters of the zones of growth were measured after 8 h of growth at 30°C.

FIG. 4. Western immunoblot analysis of SrgA dependence of oxidation status of PefA. Blot was developed with anti-PefA antibodies. Half of the PefA protein in lane 1 shifted upwards to run at the reduced location as a result of dithiothreitol diffusion from the markers in the adjacent lane (not shown). Lane 1, NLM2153/p30; lane 2, NLM2198/p30Tn2.7; lane 3, NLM2153/p30Tn2.7; lane 4, NLM2198/p30; lane 5, NLM2198/p30Tn2.7/pCB6; lane 6, NLM2198/p30Tn2.7/pCB6 with arabinose induction. The presence or absence of *dsbA* and *srgA* is indicated at the bottom of the figure (* indicates the presence of *srgA* in *trans*; oe indicates overexpression of *srgA*). The anti-PefA antibody cross-reacts with material running at the dye front of the gel (6-kDa marker). The relative positions of the 16- and 6-kDa molecular size markers are indicated.

motility (in either the hemagglutinin-tagged or native SrgA construct) remained similar to that under repressed (glucose) conditions. At 0.1% arabinose, DsbA conferred motility to a level comparable to that of the wild type, while SrgA-complemented strains were still less motile than the wild type. It was previously established that *S. enterica* serovar Typhimurium with SrgA but lacking DsbA is nonmotile (71).

Major fimbrial subunit, PefA, is oxidized by SrgA. An examination of the amino acid sequence of the PefA fimbrial subunit showed that it contained two cysteine residues at positions 36 and 80, residues conserved in fimbrial homologues such as *E. coli* FedA (38). With anti-PefA antibodies used to detect PefA, it was first established that PefA underwent a significant mobility shift upwards on SDS-PAGE gels in the presence of a reducing agent such as dithiothreitol (Fig. 4). The mobility shift was too small to correlate with a dimer-to-monomer conversion of PefA (monomer mass of approximately 17 kDa) and was therefore interpreted to indicate an intramolecular disulfide bond.

The ability of *S. enterica* serovar Typhimurium SrgA and DsbA to oxidize the PefA fimbrial subunit was then examined (Fig. 4). The plasmids p30 (*srgA*⁺) and p30Tn2.7 (*srgA*) were introduced into an *S. enterica* serovar Typhimurium strain that had been cured of the native virulence plasmid and that also carried either the *dsbA*⁺ or *dsbA* mutant allele. If neither DsbA nor SrgA was present, PefA protein was not detected with anti-PefA antibodies. In a *dsbA*⁺ *srgA* background, a very small amount of PefA could be detected running in the oxidized position (Fig. 4, lane 3), but in a *dsbA* *srgA*⁺ background, the same amount of oxidized PefA was detected as was seen in a *dsbA*⁺ *srgA*⁺ background, indicating that the necessary oxidizing activity was derived almost completely from SrgA. Because PefA was not detected when the plasmid-encoded fimbriae were expressed from p30Tn2.7, a polar effect of the Tn2.7 insertion on plasmid-encoded fimbria expression needed to be ruled out. When only *srgA* was provided in *trans* in cells containing p30Tn2.7, PefA protein was again detected (Fig. 4, lane

TABLE 4. Alkaline phosphatase activity of DsbA- and SrgA-complemented strains^a

Strain	Alkaline phosphatase activity		
	0.2% Glucose	Arabinose	
		0.01%	0.1%
NLM119	766	744	660
NLM117/ pBAD24	14	27	40
NLM117/pLMN106	17	445	940
NLM117/pLMN108	13	76	600

^a Assays were performed four times. All standard deviations were within 10% of the mean. Activity units were as for Miller assays (49).

5), eliminating the possibility of polar effects. Interestingly, significant overproduction of SrgA caused PefA to accumulate in the reduced conformation (Fig. 4, lane 6).

Comparison of SrgA and DsbA disulfide oxidoreductase activities. In order to more closely compare the activities of DsbA and SrgA, the genes for both proteins were cloned under the control of the tightly regulated *ara* promoter in the pBAD expression vector pBAD24 (33), creating pLMN106 and pLMN108, respectively. DsbA and SrgA were expressed in this tightly regulated system in order to produce equivalent amounts of both proteins. By cloning *dsbA* and *srgA* into the pBAD vector so that both clones contained the same regulatory DNA sequences upstream of their respective ATG start sites, promoter-related effects on protein expression were avoided. A comparison of the DsbA and SrgA protein levels at three different levels of arabinose induction by pulse labeling and immunoprecipitation showed that the proteins were being made in approximately equal amounts (data not shown). The stability of DsbA and SrgA was determined by pulse-chase labeling and immunoprecipitation, and these experiments showed that the proteins were both very stable, with half-lives of greater than 30 min (data not shown).

Once it had been established that DsbA and SrgA were being produced from the pBAD vector, the ability of both proteins to oxidize disulfide bonds in cellular proteins was examined. The ability of DsbA and SrgA to restore motility was assayed in a *dsbA*::Km strain as described above. The capacity of DsbA and SrgA to restore alkaline phosphatase activity in a *dsbA*::Km strain was also measured. Alkaline phosphatase contains two disulfide bonds that are necessary for phosphatase activity (16). A *dsbA* null strain produces approximately 50-fold less active alkaline phosphatase than a *dsbA*⁺ strain (4), as did NLM117/pBAD24, NLM117/pLMN106 (*dsbA*⁺), and NLM117/pLMN108 (*srgA*⁺) when grown under repressing conditions (Table 4). Under conditions of induction with 0.01% arabinose, the alkaline phosphatase activity of the *dsbA* mutant strain containing plasmid-borne *dsbA* increased to over half that of a wild-type strain, while the activity in the *dsbA* strain with plasmid-borne *srgA* was still 10-fold less than the wild-type activity. Upon induction with 0.1% arabinose, the alkaline phosphatase activity of both strains was equal to or greater than that of the wild type.

Growth conditions for PefA and *srgA* expression. In a comprehensive study by Nicholson and Low (52), it was shown that plasmid-encoded fimbria expression is regulated in a DNA methylation-dependent manner. They also found that plasmid-

FIG. 5. Comparison of growth conditions affecting expression of PefA and transcription of *srgA*. Samples were loaded in the same order for panels A and B. Panel A, Western immunoblot with anti-PefA antibodies. Molecular size standards are indicated to the left of the image. Panel B, Northern blot with *srgA* probe. RNA standards are indicated in kilonucleotides (Knt) to the left of the image. Lane 1, NLM2153 containing p30, grown in LB at pH 5.1; lane 2, NLM2217 grown in LB at pH 5.1; lane 3, NLM2217 grown in LB at pH 7.0; lane 4, NLM2217 grown in LB plus glucose at pH 5.1; lane 5, NLM2217 grown in LB plus glucose at pH 7.0; lane 6, NLM2153 grown in LB at pH 5.1.

encoded fimbriae were expressed only under nonaerated conditions in rich medium with the pH lowered to 5.1. In the present study, the expression of plasmid-encoded fimbriae was monitored under several growth conditions by examining PefA expression by Western immunoblotting, while *srgA* expression was monitored under the same growth conditions by looking for the presence or absence of *srgA* transcripts by Northern hybridization. Controls for this experiment consisted of a virulence plasmid-cured strain (NLM2153) with (positive control) or without (negative control) cloned *pefA* (p30). Wild-type *S. enterica* serovar Typhimurium containing pStSR100 (NLM2217) was used for the experimental assays.

It was found that PefA protein could be detected only under growth conditions that also resulted in expression of *srgA* (Fig. 5). These growth conditions corresponded to statically grown cultures in LB medium at pH 5.1, as was found by Nicholson and Low (52), and it was also found that the addition of glucose to the low-pH culture medium somewhat enhanced PefA expression (Fig. 5). PefA protein and *srgA* transcripts were not found if the pH was raised to 7.0 or if growth of the cultures was carried out with gentle rotation or aeration (data not shown). Under all conditions tested, the *srgA* transcript appeared to be rapidly degraded, as detection of a full-length transcript of a minimum predicted length of a 750 nucleotides

was always accompanied by detection of smaller RNA species that hybridized to the *srgA* probe. The transcription of *pefA* was also followed by Northern hybridization experiments, and *pefA* expression consistently correlated with *srgA* expression (data not shown).

DISCUSSION

We have determined that *S. enterica* serovar Typhimurium carries a gene encoding a disulfide oxidoreductase, SrgA, on the virulence plasmid pStSR100. The region of the plasmid that contains *srgA* also bears the genes for non-type 1 fimbriae (25). SrgA was not originally identified as being important for plasmid-encoded fimbria biosynthesis, as testing for the effect of *srgA* knockouts was carried out in a *dsbA*⁺ *E. coli* strain (25), in which DsbA apparently provided sufficient disulfide-oxidizing potential in the absence of SrgA. We show here that disulfide oxidoreductase activity is necessary for surface expression of plasmid-encoded fimbriae and that that activity is provided by SrgA from *S. enterica* serovar Typhimurium.

In order to compare the properties of *S. enterica* serovar Typhimurium SrgA and *E. coli* DsbA, these genes were cloned under the control of the *ara* promoter so that they could be expressed in a highly controlled fashion. Although Siegele and Hu (62) have shown that individual cells in a culture are not induced to the same level of expression with the pBAD system with subsaturating amounts of arabinose, the cell populations compared here were induced with arabinose concentrations sufficient to induce cloned-gene expression in a majority of the cells (62). The results show that in a population of cells, SrgA activity is lower than DsbA activity on the substrates examined. This could be for two general reasons. A disulfide bond transfer must occur twice in the *E. coli*-based assay system used in order for a new disulfide bond to be inserted into the substrate. First the disulfide of SrgA must be oxidized, and then SrgA must transfer that disulfide to the substrate protein. The decrease in the efficiency of disulfide bond formation seen with SrgA on these nonnative substrates could be due to either a lowering of the rate of oxidation of SrgA (reoxidation efficiency) or a lowering of the rate of transfer of the disulfide bond from SrgA to the substrate protein (substrate specificity). It was not possible to differentiate between these two possibilities in this study.

A comparison of the amino acid sequences of SrgA and DsbA shows some interesting features (Fig. 1). The first conserved region of the two proteins is around the cysteine active site of both proteins. Fifteen of the 22 amino acids surrounding the active site are identical. However, in SrgA there are two proline residues between the cysteines in the active site, whereas the other proteins are all C-P-H-C. Grauschopf et al. (28) have shown in vitro that a DsbA active site mutated to C-P-P-C is 2,000-fold less oxidizing than a C-P-H-C active site. The experiments done here to measure the oxidizing capacity of DsbA and SrgA by measuring the restoration of alkaline phosphatase activity suggest that SrgA is less efficient at oxidizing the disulfide bonds in *E. coli* alkaline phosphatase than DsbA is. It is likely that this difference in the active sites of these two proteins at least partly contributes to their differences in activity. Alkaline phosphatase is not a natural sub-

strate for SrgA, as *S. enterica* serovar Typhimurium does not produce alkaline phosphatase.

In addition to the dissimilar active sites, other regions of SrgA also differ from DsbA. In the B domain of SrgA, there is one stretch of 26 amino acids (80 to 106) that has only three residues identical to those of DsbA. Often the amino acids that do not match those of DsbA or the other DsbA homologues are changed to arginine or lysine. Eight of the 14 positively charged residues in the B domain of SrgA are in positions that are not charged in DsbA, Por, or TcpG. It has been proposed that regions of the B domain of DsbA may be important for substrate interaction (30, 48). The different amino acid composition in this region of SrgA may be involved in determining substrate specificity. The B domain is also proposed to be important for interaction with DsbB. As results show that SrgA activity in *E. coli* is DsbB dependent, the regions of similarity between the B domains of SrgA and DsbA may be involved in interaction with DsbB. This hypothesis will be tested once *S. enterica* serovar Typhimurium DsbB is cloned and characterized.

Although SrgA and DsbA both display disulfide oxidoreductase activity, polyclonal antiserum raised against DsbA does not cross-react with SrgA (68). Although it is expected that polyclonal serum contains antibodies able to recognize conformational epitopes, these epitopes are probably not retained in proteins subjected to SDS-PAGE. Given the differences in the primary amino acid sequences of SrgA and DsbA, the structures are probably different enough that extensive linear epitope similarity does not exist. The anti-DsbA serum did, however, cross-react with a protein present in either wild-type or virulence plasmid-cured strains of *S. enterica* serovar Typhimurium, suggesting that a chromosomally encoded homologue of DsbA is present in *S. enterica* serovar Typhimurium. Further characterization of this protein has shown that it is indeed a DsbA homologue (70).

Two additional *Salmonella* disulfide oxidoreductases, one from *S. enterica* serovar Enteritidis and the other from *S. enterica* serovar Typhi, are much more similar to SrgA than DsbA, and both are located just downstream of operons that contain genes similar to those of the plasmid-borne fimbria operon, encoding SE fimbriae of *S. enterica* serovar Enteritidis (57). Although six distinct types of fimbriae have been described in *S. enterica* serovar Typhimurium and in *S. enterica* serovar Enteritidis (5) and two types in *S. enterica* serovar Typhi (57), only the plasmid-encoded fimbriae and SE fimbriae have been shown to have a disulfide oxidoreductase gene located downstream of the fimbrial operon.

In the present study, it was shown that PefA, the major structural subunit of the fimbrial shaft, contains a disulfide bond, as this protein shifts in relative mobility in the presence and absence of reducing agents on SDS-PAGE. Many of the fimbriae of *S. enterica* serovar Typhimurium and *E. coli* contain two cysteine residues, including type 1 fimbriae and type IV fimbriae (17, 78). In the case of type IV fimbriae in *E. coli*, DsbA is required to maintain the stability of the major structural subunit protein (78). SrgA plays this role in the stabilization of PefA, as demonstrated by PefA detection experiments in the presence and absence of SrgA, where it appears that PefA is rapidly degraded in the absence of SrgA-mediated

oxidation. The type IV pili of *E. coli* are also rapidly degraded in the absence of DsbA-mediated oxidation (78).

S. enterica serovar Typhimurium DsbA expression in an *srgA* mutant strain can stabilize PefA only to a very limited extent under the growth conditions tested, suggesting that SrgA is a plasmid-encoded fimbria-specific disulfide oxidoreductase. Western immunoblotting for DsbA in these studies showed that it was being expressed (data not shown). Supporting a PefA-specific role for SrgA activity is the observation that *srgA* expression is regulated coordinately with that of the plasmid-borne fimbria genes. The *pefA* gene is predicted to be part of an operon consisting of at least two genes (52) and is located approximately 6 kb upstream of *srgA* (25). It is unlikely that the *pefA* and *srgA* loci are on the same transcript, as a negative regulator (*pefI*) of plasmid-encoded fimbria expression that is not cotranscribed with *pefA* (52) is located between *pefA* and *srgA*.

The mechanism by which the coordinated regulation of *pefA* and *srgA* occurs is not currently understood. Schembri and Klemm (59) recently proposed a model for the modulation of fimbrial gene expression that relies on the thiol-disulfide status of OxyR. In their model, it is the expression of disulfide bond-requiring fimbriae that constitutes a signal transduction mechanism, whereby the need for oxidizing equivalents affects the cytoplasmic pool of glutathione via DsbB and this shifts OxyR to a predominantly reduced state. Reduced OxyR then acts as a repressor of fimbrial expression. Since OxyR is an important regulator of genes involved in the cell's oxidative stress response (12, 66, 79), it could play a role in pH-induced expression of the plasmid-encoded fimbriae and SrgA. A role for OxyR in plasmid-encoded fimbria gene expression in *Salmonella* spp. has not been reported in the literature. The plasmid-encoded fimbriae are also subject to phase variation that is mediated by Lrp, Dam, H-NS, and RpoS (52), any of which could play a role in SrgA expression as well. Studies are under way to further examine the regulation of SrgA and the plasmid-encoded fimbriae.

It is interesting that *S. enterica* serovar Typhimurium maintains two disulfide oxidoreductases, DsbA, encoded on the chromosome, and SrgA, encoded on the virulence plasmid. Initial studies with the chromosomal disulfide oxidoreductase have shown that it is a true homologue of *E. coli* DsbA (71). This work with SrgA is the first description of a substrate-specific disulfide oxidoreductase, and ongoing studies are addressing the nature of this substrate specificity.

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