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Fibronectin Binding to the *Salmonella enterica* Serotype Typhimurium ShdA Autotransporter Protein Is Inhibited by a Monoclonal Antibody Recognizing the A3 Repeat

Robert A. Kingsley,[1]* Daad Abi Ghanem,[2] Nahum Puebla-Osorio,[2] A. Marijke Keestra,[1] Luc Berghman,[2] and Andreas J. Bäumler[1]

Department of Medical Microbiology and Immunology, College of Medicine, College Station, Texas 77843-1114,[1] *and Departments of Poultry Science and Veterinary Pathobiology, Texas A&M University, College Station, Texas 77843-2472*[2]

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ShdA is a large outer membrane protein of the autotransporter family whose passenger domain binds the extracellular matrix proteins fibronectin and collagen I, possibly by mimicking the host ligand heparin. The ShdA passenger domain consists of ~1,500 amino acid residues that can be divided into two regions based on features of the primary amino acid sequence: an N-terminal nonrepeat region followed by a repeat region composed of two types of imperfect direct amino acid repeats, called type A and type B. The repeat region bound bovine fibronectin with an affinity similar to that for the complete ShdA passenger domain, while the nonrepeat region exhibited comparatively low fibronectin-binding activity. A number of fusion proteins containing truncated fragments of the repeat region did not bind bovine fibronectin. However, binding of the passenger domain to fibronectin was inhibited in the presence of immune serum raised to one truncated fragment of the repeat region that contained repeats A2, B8, A3, and B9. Furthermore, a monoclonal antibody that specifically recognized an epitope in a recombinant protein containing the A3 repeat inhibited binding of ShdA to fibronectin.

Nontyphoidal *Salmonella* serotypes are the most frequent cause of food-borne illness with a lethal outcome in the United States (28). The serotype currently isolated most frequently from human cases of nontyphoidal salmonellosis in the United States is *Salmonella enterica* serotype Typhimurium (30). Infections commonly result from animal-to-human transmission, primarily through food products derived from livestock or domestic fowl (30). One of the main risk factors for introducing *Salmonella* serotypes into the human food supply is their presence in the intestines of healthy food animals. In the United States, between 1 and 6% of farm animals test positive for intestinal carriage of *Salmonella* serotypes (6–11, 27, 37). Stress during transport and long periods with intermittent feeding increase the spread of *Salmonella* among livestock and domestic fowl prior to slaughter (5, 7, 13, 18, 19, 27, 29, 31, 32). As a result, *Salmonella* serotypes can on average be isolated from >10% of apparently healthy animals prior to slaughter (7, 27, 37). Intestinal carriage or chronic infection of mesenteric lymph nodes may result in contamination of equipment surfaces or workers' hands at processing plants, leading to contamination of carcasses and processed foods (14, 29, 31, 32). These considerations indicate the prime importance of intestinal carriage of *Salmonella* serotypes in healthy livestock and domestic fowl for food safety in the United States. However, little is known about the mechanisms that allow *Salmonella* serotypes to persist in the intestines of apparently healthy animals. Elucidation of the underlying molecular mechanisms of intestinal persistence is needed to devise intervention strategies aimed at decreasing the prevalence of *Salmonella* serotypes at the preharvest level. We have previously described the identification of the ShdA outer surface protein of serotype Typhimurium, the first salmonella-specific factor involved in persistent intestinal carriage in the murine model of infection.

A serotype Typhimurium strain harboring a mutation in *shdA* is shed with the feces at reduced numbers and for a shorter period of time than its isogenic parent in a mouse model of intestinal persistence (20, 23). The recovery of the *shdA* mutant in reduced numbers from the feces of mice correlates with its reduced ability to colonize the murine cecum, the organ that serves as the main reservoir of luminal serotype Typhimurium in this animal model (20). Immunohistochemical analysis of cecal tissue from infected mice demonstrates that serotype Typhimurium colonizes the cecal mucosa on the epithelial brush border and at areas of epithelial erosion where the extracellular matrix is exposed to the intestinal lumen (22). ShdA is a large outer membrane protein of serotype Typhimurium that binds the extracellular matrix protein fibronectin (22). The carboxy-terminal region of ShdA (residues 1560 to 2036) shows homology with the C-terminal domains of outer membrane proteins of the autotransporter family (16, 17), including AIDA of diffuse adhering *Escherichia coli* (1, 2) and IcsA (VirG) of *Shigella flexneri* (4, 12, 25). The C-terminal domains of AIDA and IcsA are predicted to form beta barrels in the outer membrane through which an N-terminal passenger domain is transported to the bacterial surface (1, 34, 35). However, the N-terminal passenger domains of AIDA and IcsA show no sequence homology with ShdA. Flow cytometric analysis demonstrates that the surface of serotype Typhimurium can be labeled with antiserum raised against the N-

* Corresponding author. Present address: The Wellcome Trust Sanger Institute, The Wellcome Trust Genome Campus, Hinxton, Cambridgeshire, CB10 1SA, United Kingdom. Phone: 44 (0)1223 495381. Fax: 44 (0)1223 494919. E-mail: rak@sanger.ac.uk.

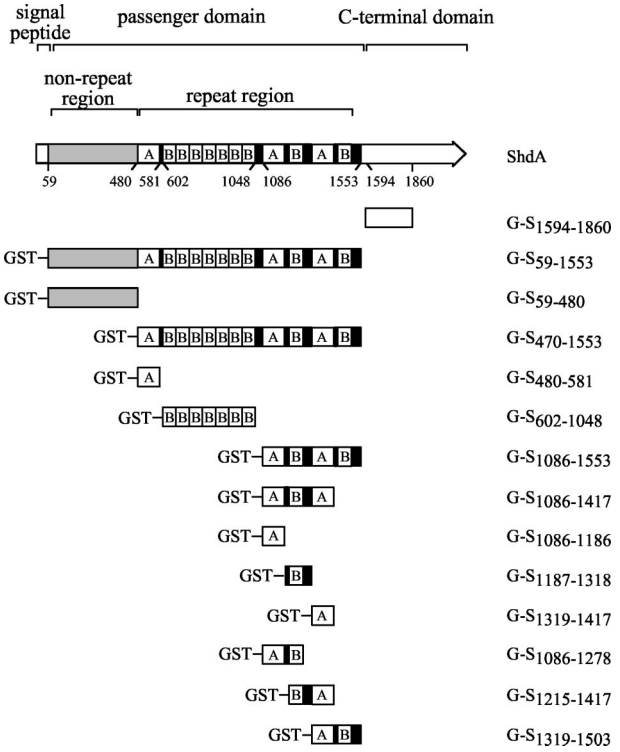

FIG. 1. Schematic representation of the ShdA primary sequence and GST-ShdA fusion proteins used in this study. The full-length ShdA protein is shown as an arrow, and GST fusion proteins are shown as bars. The signal peptide and the C-terminal domain are predicted by sequence homology with other members of the autotransporter family (23). The passenger domain is composed of a nonrepeated region (shaded bar) and a repeat region containing three type A repeats (A), nine type B repeats (B), and intervening nonrepeated sequence (solid bar).

terminal domain of ShdA (residues 59 to 1553), suggesting that this part of the protein is surface localized (22). Expression of ShdA in serotype Typhimurium increases binding of fibronectin to the bacterial surface (22). The passenger domain of ShdA binds in a heparin-sensitive mechanism to fibronectin at the Hep-2 domain via interaction with at least one cationic residue present on the surface of the ^{13}FnIII repeat module that is also the binding site for the anionic polysaccharide heparin. ShdA also bound to a second heparin-binding protein, collagen I, by a heparin-sensitive mechanism, suggesting that ShdA binding activity may represent a form of molecular mimicry of heparin binding (21). Collectively, these data suggest that ShdA-mediated binding of the extracellular matrix may be a mechanism for persistent intestinal carriage of serotype Typhimurium. To further study the molecular mechanism of ShdA-mediated host-pathogen interaction, we investigated the functional regions of the ShdA passenger domain and identified a region that contains the primary fibronectin-binding site.

MATERIALS AND METHODS

Construction and expression of recombinant proteins in *E. coli*. The expression and purification of a glutathione *S*-transferase (GST)-ShdA fusion protein containing residues 59 to 1553 of ShdA (G-S$_{59\text{-}1553}$) have been described previously (22). Plasmids for expression of GST fusion proteins with smaller segments of ShdA (Fig. 1) were constructed by PCR amplification of the corresponding regions in the *shdA* gene and subsequent cloning into the pGEX4T/1 vector restriction enzymes EcoRI and SalI (Amersham Pharmacia). For expression of fusion proteins, we used *E. coli* strain BL21 (Invitrogen). This strain, containing the recombinant plasmids, was grown in Luria-Bertani broth supplemented with 100 mg of carbenicillin/liter to mid-log phase at 37°C with shaking, and expression was induced by the addition of IPTG (isopropyl-β-D-thiogalactopyranoside) (Sigma, St. Louis, Mo.) to a final concentration of 1 mM. After incubation at 37°C with shaking for 4 h, the cells were harvested by centrifugation at 10,000 × *g* for 20 min. The pellet was resuspended in 15 ml of phosphate-buffered saline (PBS), pH 7.4, and 5 ml of protease inhibitor cocktail (Sigma) was added. The cells were lysed using a French pressure cell at 12,000 lb/in^2, and the insoluble fraction was separated by centrifugation. Fusion proteins were purified from the soluble fraction by affinity chromatography using glutathione Sepharose (Amersham Pharmacia) according to the manufacturer's instructions. The fusion proteins were eluted with 10 mM reduced glutathione (Sigma) in 50 mM Tris-HCl, pH 8.5.

Solid-phase binding assay. Binding of GST-ShdA fusion proteins to fibronectin-coated wells was determined using an enzyme-linked immunosorbent assay (ELISA). Wells of a 96-well plate (Maxisorp; Nunc, Roskilde, Denmark) were coated with 50 μl of bovine fibronectin in 100 mM Tris-HCl, pH 8, per well for 12 h at 4°C at the concentrations indicated below. Additional binding sites were blocked by incubation of 200 μl of block buffer (2% [wt/vol] nonfat milk, 0.02% [vol/vol] Tween 20 in PBS, pH 7.4) per well for 1 h. The block buffer was removed, and the wells were washed three times with distilled H_2O. The appropriate amount of test ligand in a volume of 50 μl of PBS, pH 7.4, was added in the presence or absence of inhibitor, and the plate was incubated for 90 min at room temperature. The wells were washed three times with distilled H_2O. Bound ligand (G-S$_{59\text{–}1553}$, G-S$_{59\text{–}480}$, G-S$_{480\text{–}581}$, G-S$_{602\text{–}1048}$, G-S$_{1086\text{–}1553}$, or G-S$_{470\text{–}1553}$) was detected using a 1/1,000 dilution of goat anti-GST antiserum (Amersham Pharmacia) as the primary antibody and a 1/5,000 dilution of monoclonal anti-goat immunoglobulin G alkaline phosphatase conjugate as a secondary antibody. Alkaline phosphatase activity associated with each well was detected by the addition of 1 mg of *p*-nitrophenyl phosphate/ml in 100 mM glycine, 2 mM $MgCl_2$, and 1 mM $ZnCl_2$. Alternatively, biotinylated protein ligand was detected with 1/1,000 streptavidin-horseradish peroxidase polymer conjugate (Sigma) in PBS, pH 7.4, and 0.04% Tween 20. The horseradish peroxidase activity in each well was detected by the addition of 50 μl of 0.4-mg/ml *o*-phenylenediamine dihydrochloride- 0.4-mg/ml urea hydrogen peroxide in 50 mM phosphate citrate buffer. Protein was biotinylated using sulfo-succinimidyl-6-(biotinamido) hexanoate (Pierce, Rockford, Ill.) according to the manufacturer's instructions. Briefly, a 20-fold molar excess of sulfo-succinimidyl-6-(biotinamido) hexanoate was incubated with a 1.5-mg/ml solution of G-S$_{59\text{–}1553}$ or G-S$_{470\text{–}1553}$ in PBS, pH 7.4, at room temperature for 30 min. The remaining unreacted sulfo-succinimidyl-6-(biotinamido) hexanoate was removed by dialysis against 1 liter of PBS, pH 7.4, for 10 h at 4°C. The biotinylated protein was stored at −20°C for 1 month.

Production of immune serum. Antiserum was raised in 6-lb female New Zealand White rabbits. A prebleed (5 ml) was taken, followed by the primary immunization with a 1:1 mixture of fusion protein (0.5 mg) and Titer-Max Gold (Sigma) adjuvant (total volume, 1 ml) injected subcutaneously at >10 different locations. This procedure was repeated after 14 and 28 days. After a further 2 weeks, the rabbits were anesthetized with a Ketamine, Xylaxine, and Acepromazine cocktail and exsanguinated by cardiac puncture. The serum was prepared by standard methodology and preadsorbed with GST-glutathione Sepharose five times.

MAb development. Production of monoclonal antibody (MAb) specific to G-S$_{470\text{-}1553}$ was performed essentially as described previously (3). For primary screening of monoclonal hybridoma supernatants, ELISA plates were coated overnight with 5 μg of G-S$_{470\text{-}1553}$ Tris-HCl (pH 8)/ml, and upon incubation of the hybridoma supernatants, mouse anti-ShdA was detected with a peroxidase-conjugated secondary antibody and H_2O_2-tetramethylbenzidine as the substrate system. Immunopositive culture wells were then submitted to a secondary screening to test for the MAb's capacity to block interaction of G-S$_{470\text{-}1553}$ with fibronectin. To this end, ELISA plates were coated with fibronectin (5 μg of Tris-HCl [pH 8]/ml). The plates were washed with distilled H_2O and then incubated with a mixture of the MAb and purified G-S$_{470\text{-}1553}$. Binding of G-S$_{470\text{-}1553}$ to fibronectin-coated wells was detected with polyclonal rabbit anti-ShdA (α-ShdA) antiserum and a peroxidase-conjugated secondary antibody as described above. The hybridomas that had lower photometric signals in this secondary screening than in a control binding assay containing no MAb were expanded, cloned by limiting dilution, and stored in liquid nitrogen for further use. Two MAbs that produced the lowest photometric signals in the binding assay were designated 3-9D 9F and 4-10A 11B, and a third that had no detectable

inhibitory activity was designated 4-10G 8G. For mass production of MAb 3-9D 9F, we used a membrane-based high-density cell culture technology (CELLine 1000; Integra Biosciences, Ijamsville, Md.).

RESULTS

Time dependence and affinity of ShdA binding to fibronectin. Binding of the passenger domain of ShdA (G-$S_{59\text{-}1553}$) (Fig. 1) to fibronectin-coated wells was described previously using ELISA (22). G-$S_{59\text{-}1553}$ had a predicted molecular mass of 178 kDa but migrated with an apparent molecular mass of >250 kDa in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). A discrepancy between the predicted molecular mass and the apparent molecular mass is common for proteins containing acidic repeated sequences, including FnbpA, a fibronectin-binding protein of *Staphylococcus aureus* (26). The predicted passenger domain of ShdA is also highly acidic, containing 188 (~12%) acidic residues (glutamate and aspartate) out of 1,498 total residues. To further characterize fibronectin-binding activity, we first determined the time dependency of binding using an ELISA. G-$S_{59\text{-}1553}$ was added to 96-well plates coated with 1 nmol of bovine fibronectin. After incubation for different time intervals, the wells were washed, and the amount of well-associated G-$S_{59\text{-}1553}$ was detected with α-GST antiserum using ELISA. The amount of bound G-$S_{59\text{-}1553}$ increased in a time-dependent manner for the first 60 min. Incubation of fibronectin and G-$S_{59\text{-}1553}$ for >60 min did not increase the amount of G-$S_{59\text{-}1553}$ bound to wells, indicating that binding equilibrium had been reached (data not shown). In all subsequent binding assays, we therefore incubated test peptides in fibronectin-coated wells for 60 min.

We next determined the concentration required for half-maximal binding at equilibrium of G-$S_{59\text{-}1553}$ to bovine plasma fibronectin by using an ELISA. Increasing concentrations of G-$S_{59\text{-}1553}$ were added to 96-well plates coated with 0.25 μg of bovine plasma fibronectin and incubated for 60 min. The amount of well-associated G-$S_{59\text{-}1553}$ was detected with α-GST antiserum using ELISA (Fig. 2A). Saturation of binding was reached at a G-$S_{59\text{-}1553}$ concentration of $\sim 3 \times 10^{-7}$ M, and half-maximal binding occurred when the fusion protein was added to wells at a concentration of $\sim 1.2 \times 10^{-7}$ M.

The ShdA repeat region contains the primary fibronectin-binding site. The ShdA passenger domain (residues 59 to 1553) of serotype Typhimurium consists of two regions, an N-terminal region (residues 59 to 470; nonrepeat region) followed by a second region (residues 470 to 1553; repeat region) composed almost entirely of two types of imperfect and direct amino acid repeats that we have designated repeat type A and repeat type B (Fig. 1). The type A repeat consists of an ~100-amino-acid motif that is repeated three times. The type B repeat consists of a 60-amino-acid motif that is repeated nine times (23). To investigate whether either of these regions exhibits binding in the absence of the other, we constructed two GST fusion proteins consisting of residues 59 to 480 (G-$S_{59\text{-}480}$) and 470 to 1553 (G-$S_{470\text{-}1553}$) (Fig. 1). While G-$S_{59\text{-}480}$ migrates in SDS-PAGE at approximately its predicted molecular mass of 70 kDa, G-$S_{470\text{-}1553}$ migrated at ~230 kDa, considerably higher than its predicted molecular mass of 136 kDa. This is likely due to the presence of acidic repeats in this region. The fibronectin-binding properties of these fusion proteins were compared by ELISA to those of GST and a GST fusion containing the entire ShdA passenger domain (G-$S_{59\text{-}1553}$) (Fig. 3A). Binding of the nonrepeat region (G-$S_{59\text{-}480}$) to fibronectin-coated wells was greater than that of GST but less than that of the entire ShdA passenger domain (G-$S_{59\text{-}1553}$). In contrast, the repeat region (G-$S_{470\text{-}1553}$) bound to immobilized fibronectin at a level comparable to that of the ShdA passenger domain (G-$S_{59\text{-}1553}$) under these conditions.

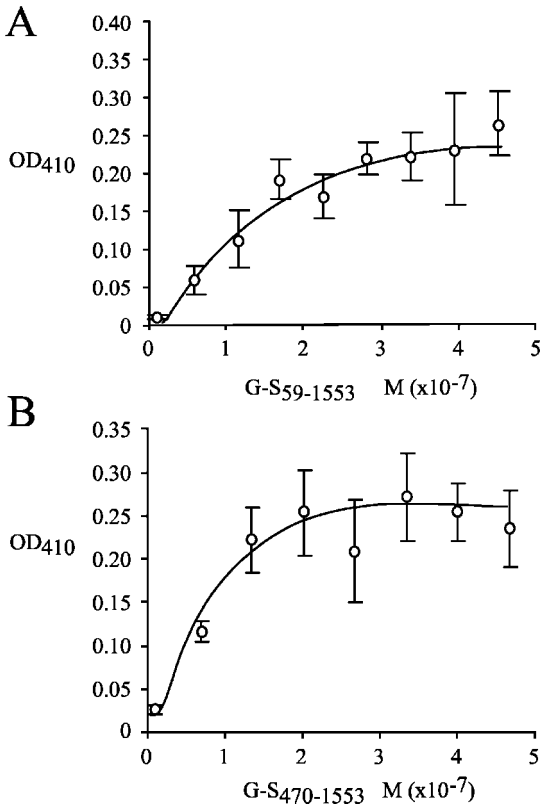

FIG. 2. Binding kinetics of ShdA interaction with fibronectin. Binding of G-$S_{59\text{-}1553}$ or G-$S_{470\text{-}1553}$ to fibronectin (0.25 μg)-coated 96-well plates was determined by ELISA using α-GST serum. G-$S_{59\text{-}1553}$ (A) and G-$S_{470\text{-}1553}$ (B) interactions with fibronectin were determined by incubating increasing concentrations of ligand in fibronectin-coated wells. After washes, the amount of well-associated GST fusion protein was determined using α-GST antibody in an ELISA. The mean optical densities at 410 nm (OD_{410}) from three identical wells ± standard errors are plotted.

To determine the specificity of fibronectin binding by ShdA, we tested the abilities of GST and GST fusion proteins containing the nonrepeat region (G-$S_{59–480}$) or the repeat region (G-$S_{470–1553}$) to inhibit binding of a biotinylated GST fusion containing the ShdA passenger domain (biotin-G-$S_{59–1553}$) to bovine fibronectin in a solid-phase binding assay (Fig. 4A). A biotinylated GST fusion to the ShdA passenger domain (biotin-G-$S_{59–1553}$) (33 nM) was incubated in 96-well plates coated with bovine plasma fibronectin in the presence of increasing concentrations (0.6, 6, 60, and 600 nM) of either GST, G-$S_{59–1553}$, G-$S_{59–480}$, or G-$S_{470–1553}$. The GST fusion protein containing the ShdA passenger domain (G-$S_{59–1553}$) inhibited binding of biotin- G-$S_{59–1553}$ to fibronectin at a concentration

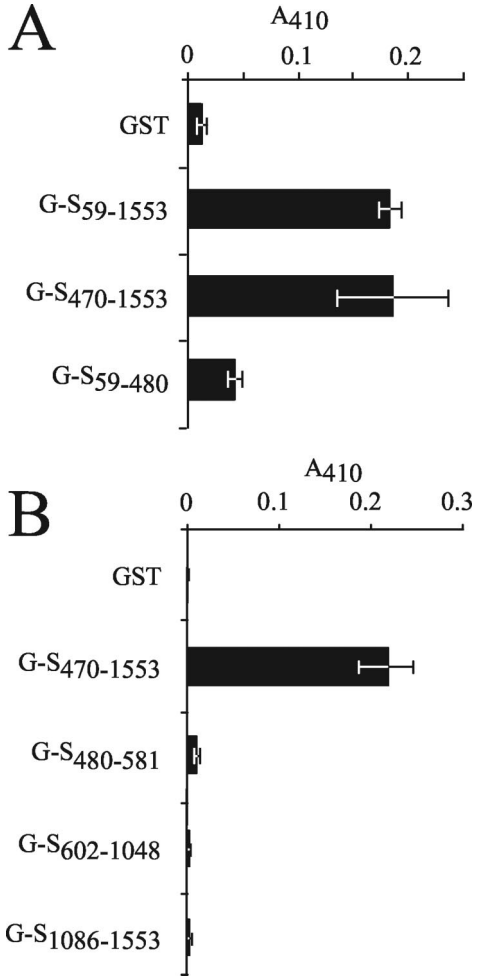

FIG. 3. Binding of ShdA passenger domain and truncated recombinant peptides. Thirty-three nanomolar G-$S_{59\text{-}1553}$, G-$S_{59\text{-}480}$, G-$S_{470\text{-}1553}$, or GST (A) and G-$S_{59\text{-}480}$, G-$S_{480\text{-}581}$, G-$S_{602\text{-}1048}$, G-$S_{1086\text{-}1553}$, or GST (B) were incubated in wells coated with fibronectin (0.25 μg). After washes, the amount of well-associated GST fusion protein was determined using α-GST antibody in an ELISA. The mean A_{410} values from six identical wells ± standard errors are plotted.

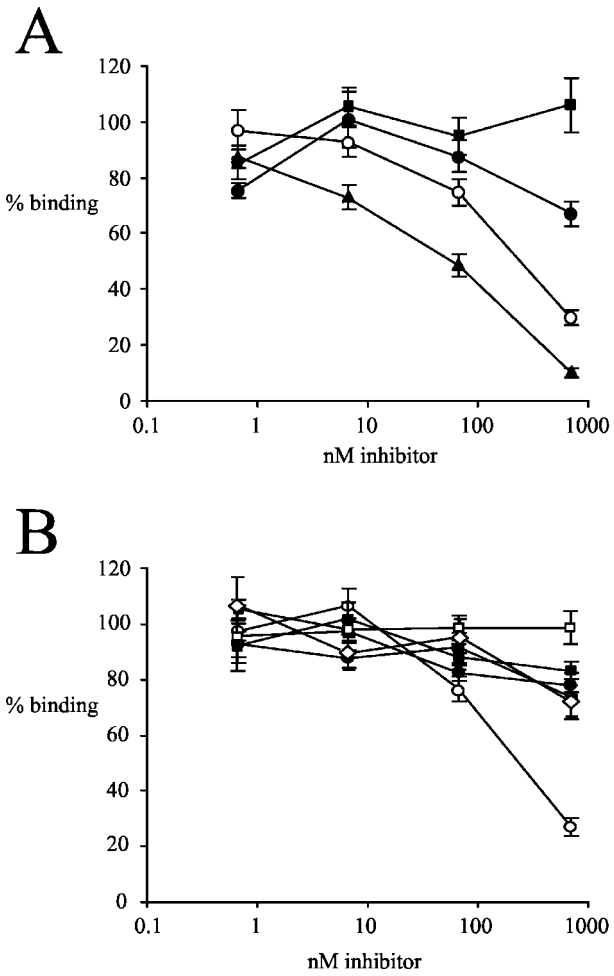

FIG. 4. Inhibition of biotin- G-$S_{59\text{–}1553}$ or biotin- G-$S_{470\text{–}1553}$ binding to fibronectin by unlabeled ShdA passenger domain or truncated peptides. (A) Thirty-three nanomolar biotinylated G-$S_{59\text{–}1553}$ was incubated in wells coated with fibronectin (0.25 μg) with increasing amounts of G-$S_{59\text{–}1553}$ (solid triangles), GST (solid squares), G-$S_{59\text{–}480}$ (solid circles), or G-$S_{470\text{–}1553}$ (open circles). (B) Thirty-three nanomolar biotinylated G-$S_{470\text{–}1553}$ was incubated in wells coated with fibronectin (0.25 μg) with increasing amounts of G-$S_{480\text{–}581}$ (solid triangles); G-$S_{602\text{–}1048}$ (open squares); G-$S_{1086\text{–}1553}$ (solid circles); G-$S_{470\text{–}1553}$ (open circles); a combination of G-$S_{480\text{–}581}$, G-$S_{602\text{–}1048}$, and G-$S_{1086\text{–}1553}$ (diamonds); or GST (solid squares). In each case, biotin associated with each well following washing was determined by ELISA using streptavidin conjugated with horseradish peroxidase. The mean A_{410} values from six identical wells ± standard errors, expressed as percentages of values for control wells lacking added test peptides, are plotted.

of 6 nM and inhibited binding by ~50% at 60 nM. The GST fusion protein containing the repeat region (G-$S_{470\text{–}1553}$) inhibited binding at a concentration of ≥60 nM, and the GST fusion containing the nonrepeat region (G-$S_{59\text{–}480}$) exhibited weak inhibition at a concentration of 600 nM (Fig. 4A). No inhibitory activity of GST was detectable up to 600 nM.

To estimate the affinity of fibronectin binding to the ShdA repeat region, we determined by ELISA the concentration required for half-maximum binding at equilibrium for fibronectin binding of G-$S_{470\text{-}1553}$. Increasing concentrations of the GST fusion protein containing the repeat region (G-$S_{470\text{-}1553}$) were added to 96-well plates coated with 5 μg of bovine plasma fibronectin/ml, and binding was allowed to proceed for 60 min (Fig. 2B). The concentration of G-$S_{470\text{-}1553}$ at which half-maximum binding to fibronectin was observed was $\sim 0.75 \times 10^{-7}$ M. Thus, the ShdA passenger domain (Fig. 2A) and the ShdA repeat region (Fig. 2B) bound fibronectin with similar affinities. Together, these data suggested that the primary fibronectin-binding domain was contained within the repeat region (residues 470 to 1553) of the ShdA passenger domain.

Fibronectin-binding activity of truncated peptides derived from the ShdA repeat region. To further investigate binding of the ShdA repeat region, three additional GST-tagged fusion proteins were constructed that together contained all of the repeats present in the ShdA passenger domain. The first contained repeat A1 (G-$S_{480\text{-}581}$), the second contained repeats B1 through B7 (G-$S_{602\text{-}1048}$), and the third contained repeats A2, B8, A3, and B9 (G-$S_{1086\text{-}1553}$) (Fig. 1). Both G-$S_{602\text{-}1048}$ and G-$S_{1086\text{-}1553}$ migrated at apparent molecular masses greater

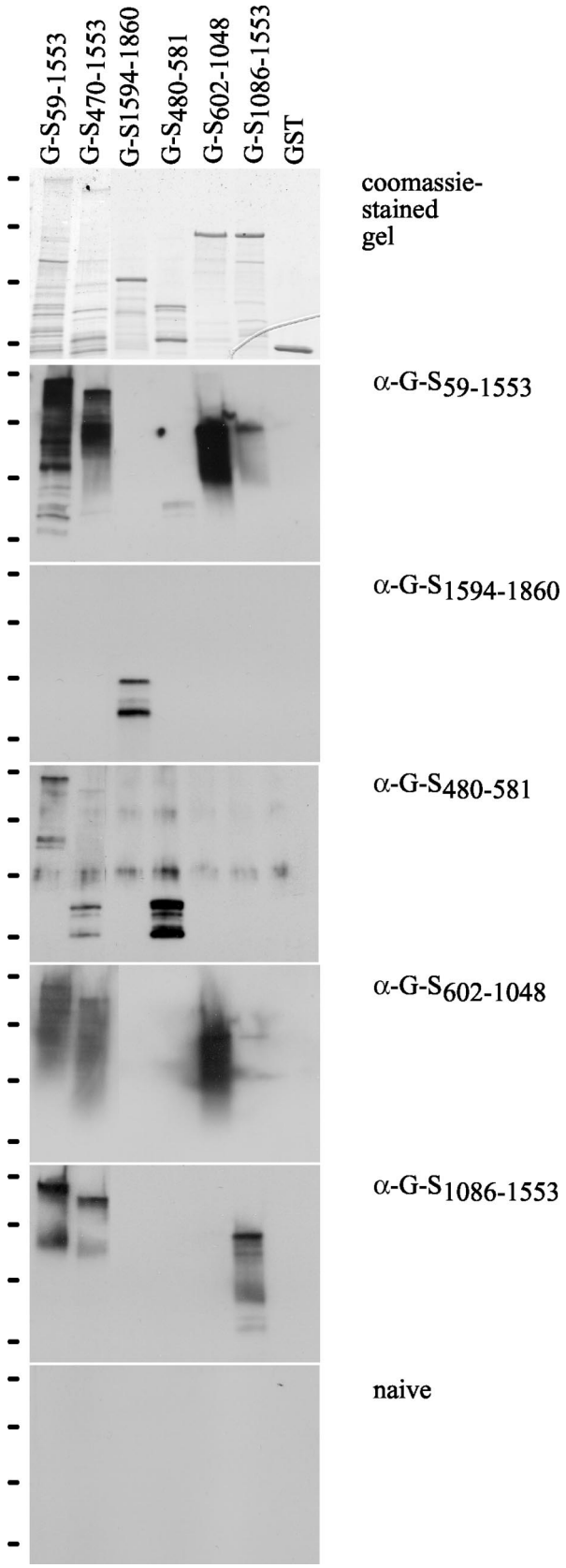

FIG. 5. Reactivities of rabbit immune sera raised against G-$S_{59-1553}$, G-$S_{1594-1860}$, G-$S_{480-581}$, G-$S_{602-1048}$, G-$S_{1086-1553}$, and naïve serum. The top panel shows a 4 to 15% gradient SDS-PAGE stained with Coomassie blue in which a total of 3 μg of purified G-$S_{59-1553}$, G-$S_{470-1553}$, G-$S_{1594-1860}$, G-$S_{480-581}$, G-$S_{602-1048}$, G-$S_{1086-1553}$ or GST/lane was separated. The other panels are Western blots of a 4 to 15% gradient polyacrylamide gel in which 0.15 μg of the recombinant proteins/lane was separated by SDS-PAGE. Western blots were detected with antisera raised against each recombinant protein or naïve serum (as indicate beside each panel) that was previously preadsorbed with purified GST-glutathione Sepharose. The positions of the 250-, 100-, 50-, and 25-kDa molecular mass standards (top to bottom) are indicated by horizontal bars on the left of each panel.

than their predicted molecular masses of 71 and 76 kDa, respectively, probably due to the presence of acidic repeats. None of these fusion proteins (G-$S_{480-581}$, G-$S_{602-1048}$, or G-$S_{1086-1553}$) bound to fibronectin-coated wells at levels comparable to that of the entire repeat region (G-$S_{470-1553}$) (Fig. 3B). While the GST fusion protein containing the A1 repeat (G-$S_{480-581}$) exhibited a small but significant increase in binding to fibronectin-coated wells compared to the GST control, fusion proteins G-$S_{602-1048}$ and G-$S_{1086-1553}$ did not exhibit greater binding than GST.

In order to address the question of whether the truncated proteins were able to cooperate to bind bovine fibronectin, we tested the ability of the A1 repeat (G-$S_{480–581}$), the B1 to B7 repeats (G-$S_{602–1048}$), and the A2, B8, A3, and B9 repeats (G-$S_{1086–1553}$) to inhibit the binding of a biotinylated GST fusion containing the repeat region of the ShdA passenger domain (biotin- G-$S_{59–1553}$) to bovine fibronectin in a solid-phase binding assay (Fig. 4B) individually and in combination. The biotinylated GST fusion to the repeat region (biotin- G-$S_{470–1553}$) was incubated in 96-well plates coated with bovine plasma in the presence of increasing concentrations of G-$S_{470–1553}$, G-$S_{480–581}$, G-$S_{602–1048}$, G-$S_{1086–1553}$, or GST. The GST fusion protein containing the complete repeat region (G-$S_{470–1553}$) inhibited binding of biotin- G-$S_{470–1553}$ to fibronectin at a concentration of 60 nM. The GST fusion proteins G-$S_{480–581}$, G-$S_{602–1048}$, and G-$S_{1086–1553}$ and GST, individually or in combination, did not exhibit inhibitory activity (Fig. 4B). This suggested that either the fibronectin-binding sites within the repeat region overlap the truncated recombinant proteins (G-$S_{480–581}$, G-$S_{602–1048}$, and G-$S_{1086–1553}$) and cannot function separately or the truncated proteins do not fold correctly.

Inhibition of fibronectin binding by polyclonal immune serum. A potential limitation of measuring the binding and/or inhibitory activities of truncated peptides derived from the ShdA passenger domain is that binding activity may be affected as a result of misfolding. To overcome these potential limitations, we determined the ability of rabbit immune sera specific for the truncated peptides of ShdA to inhibit the binding of the GST fusion protein containing the ShdA passenger domain (G-$S_{59–1553}$) to bovine fibronectin in a solid-phase binding assay. As a negative control, we constructed a GST fusion containing a region of the C-terminal domain of ShdA (residues 1594 to 1860), a region thought to mediate transport of the ShdA passenger domain across the bacterial outer membrane (G-$S_{1594–1860}$) (Fig. 1) but not fibronectin binding. Rabbit immune sera were raised against the GST fusion proteins containing the ShdA passenger domain (G-$S_{59–1553}$), repeat A1 (G-$S_{480–581}$), repeats B1 to B7 (G-$S_{602–1048}$), the region con-

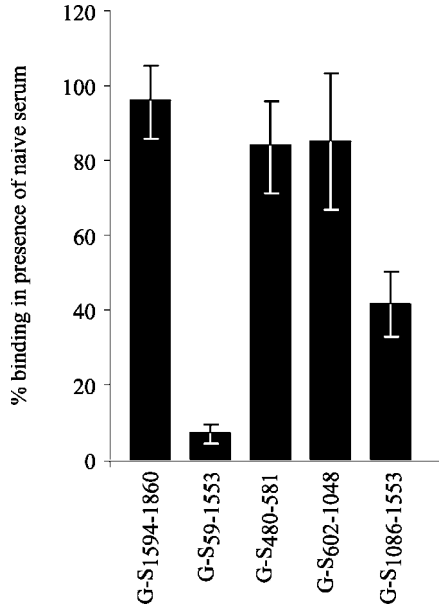

FIG. 6. Inhibition of ShdA binding to fibronectin by rabbit immune sera raised to ShdA fragments. Shown is the binding of G-$S_{59\text{-}1553}$ (33 nM) to wells coated with fibronectin (0.25 μg) in the presence of rabbit immune sera raised against the recombinant proteins G-$S_{1594\text{-}1860}$, G-$S_{59\text{-}1553}$, G-$S_{480\text{-}581}$, G-$S_{602\text{-}1048}$, and G-$S_{1086\text{-}1553}$. Each serum was preadsorbed with GST prior to the assay. After washes, the amount of well-associated $S_{59\text{-}1553}$ fusion protein was determined using α-GST antibody in an ELISA. Binding is expressed as a percentage of binding in the presence of naïve serum. The mean A_{410} values from four identical wells ± standards errors, expressed as percentages of values for control wells lacking added test peptides, are plotted. Percentages were converted logarithmically before the calculation of statistics.

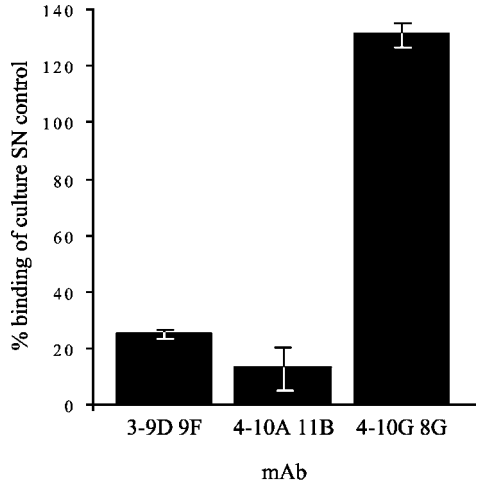

FIG. 7. Inhibitory activities of MAbs on ShdA binding to fibronectin. Shown is binding of G-$S_{59\text{-}1553}$ (33 nM) to wells coated with fibronectin (0.25 μg) in the presence of MAbs 4-10G 8G, 4-10A 11B, and 3-9D 9F. After washes, the amount of well-associated $S_{59\text{-}1553}$ fusion protein was determined using α-GST antibody in an ELISA. Binding is expressed as a percentage of binding in the absence of MAb. The mean A_{410} values from four identical wells ± standard errors, expressed as percentages of values for control wells lacking added test peptides, are plotted. SN, supernatant.

taining repeats A2, B8, A3, and B9 (G-$S_{1086–1553}$), and the C-terminal domain (G-$S_{1594–1860}$). To remove anti-GST antibodies, each serum was preadsorbed with GST bound to glutathione Sepharose resin. Following preadsorption, anti-GST antibodies were not detectable with any of the sera by Western blotting (Fig. 5). Antiserum raised against the ShdA passenger domain (α-G-$S_{59–1553}$) detected GST fusion proteins containing all regions of the ShdA passenger domain but did not detect the GST fusion protein raised against the C-terminal domain (G-$S_{1594–1860}$). Antiserum raised against the C-terminal domain fragment (G-$S_{1594–1860}$) detected only this protein. Antisera raised against G-$S_{480–581}$, G-$S_{602–1048}$, and G-$S_{1086–1553}$ each detected the passenger domain and the repeat region in addition to the protein used to immunize in each case. The abilities of these antisera to inhibit fibronectin binding by the ShdA passenger domain (G-$S_{59–1553}$) were determined by ELISA (Fig. 6). In the presence of antiserum raised against the ShdA passenger domain (α-G-$S_{59–1553}$), binding to fibronectin-coated 96-well plates was reduced to <10% of that observed in the presence of preimmune serum (Fig. 6). This indicated that antibodies in the polyclonal serum that recognized one or more epitopes of the ShdA passenger domain were able to compete with fibronectin for binding to ShdA. To further localize the positions of these epitopes, we tested the abilities of sera raised to the truncated peptides of the ShdA passenger domain to inhibit binding of the ShdA passenger domain to bovine fibronectin. Antisera raised against repeat A, repeats B1 to B7, or the C-terminal domain (α-G-$S_{1594–1860}$) did not significantly decrease binding of the ShdA passenger domain (G-$S_{59–1553}$) to bovine fibronectin. In contrast, the presence of antiserum raised to repeats A2, B8, A3, and B9 reduced binding to ~40% of that in the presence of preimmune serum, suggesting that an important component of the ligand-binding domain may be contained in this region.

Inhibition of fibronectin binding by ShdA-specific MAbs. A second approach, complementary to that described in the previous section, was used to identify the region of ShdA that contained the fibronectin-binding domain. MAbs specific to ShdA were tested by ELISA for the ability to inhibit ShdA interaction with fibronectin-coated wells. Hybridoma cells were generated from suspensions of splenocytes from mice hyperimmunized with a recombinant protein containing residues 470 to 1553 of ShdA by fusion with Sp2/0 myeloma cells. Culture supernatants from 18 independent hybridoma cell lines that secreted immunoglobulin G specific for G-$S_{470\text{-}1553}$ varied in their abilities to inhibit ShdA binding to fibronectin-coated wells in an ELISA (data not shown). Three MAbs, designated 4-10A 11B, 3-9D 9F, and 4-10G 8G, were characterized further. Coincubation of MAbs 4-10A 11B and 3-9D 9F resulted in reduction of ShdA binding to fibronectin-coated wells to ~6 and 25%, respectively, of binding in the absence of MAb. In contrast, coincubation of MAb 4-10G 8G did not result in reduced binding of ShdA to fibronectin-coated wells (Fig. 7). The locations of epitopes recognized by each MAb were identified by expression of recombinant N-terminal GST fusion proteins containing fragments of the ShdA repeat region in *E. coli* and subsequent separation by SDS-PAGE. Following Western transfer, the immobilized proteins were detected with each MAb or α-GST polyclonal serum (Fig. 8A). 4-10G 8G, which did not inhibit ShdA binding, recognized an

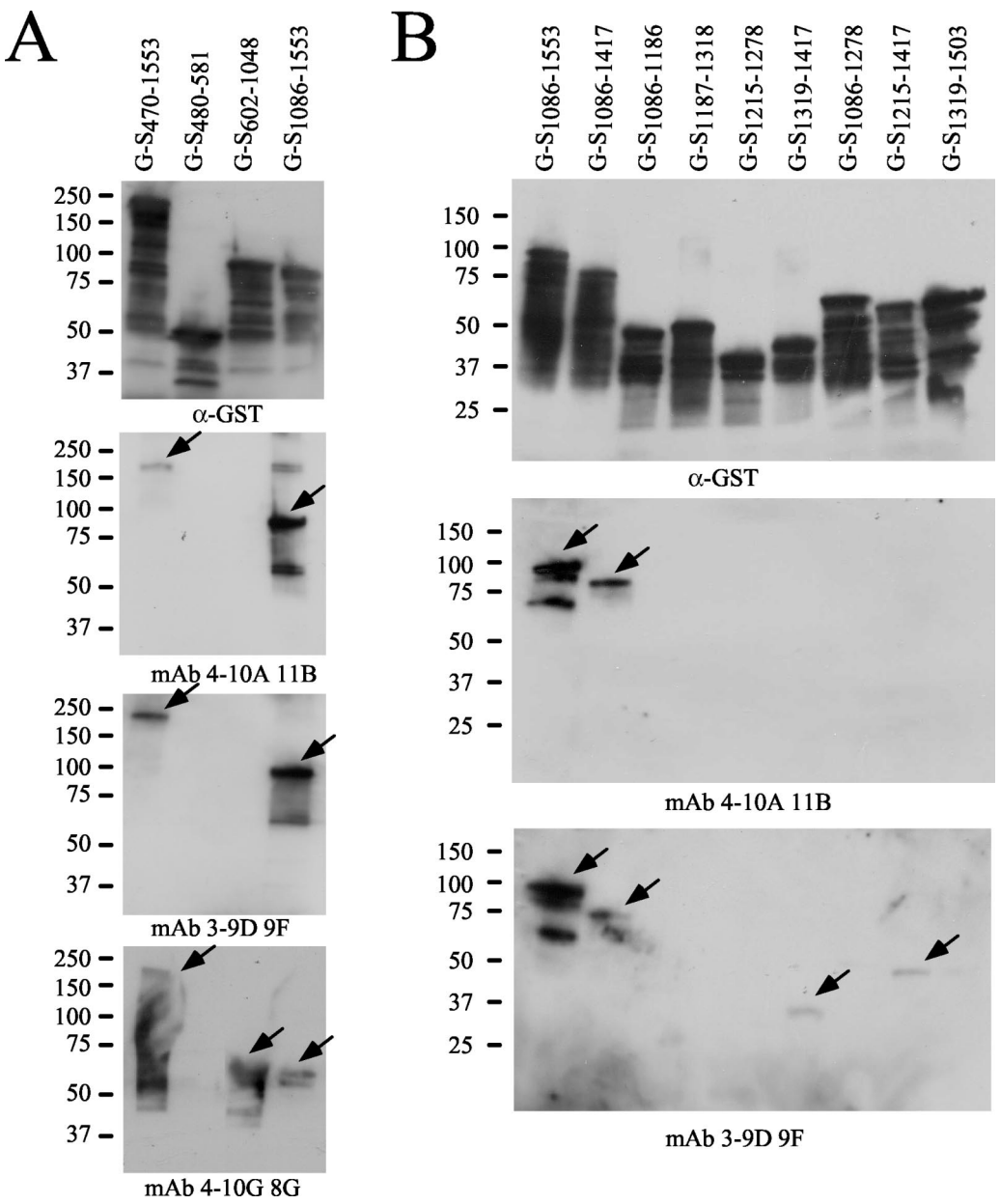

FIG. 8. Epitope mapping of MAbs. Whole-cell lysates of *E. coli* expressing the GST fusion proteins G-$S_{470-1553}$, G-$S_{480-581}$, G-$S_{602-1048}$, and G-$S_{1086-1553}$ (A) or G-$S_{1086-1553}$, G-$S_{1086-1417}$, G-$S_{1086-1186}$, G-$S_{1187-1318}$, G-$S_{1215-1278}$, G-$S_{1319-1417}$, G-$S_{1086-1278}$, G-$S_{1215-1417}$, and G-$S_{1319-1503}$ (B) were separated on 4 to 15% gradient SDS-PAGE, transferred to membranes by Western blotting, and detected with polyclonal α-GST immune serum or MAb as indicated below each panel. Positive signals are indicated by arrows.

epitope in the recombinant protein containing repeats B1 to B7 and one containing repeats A2, B8, A3, and B9 but not one containing repeat A1. In contrast, 4-10A 11B and 3-9D 9F recognized an epitope present in the recombinant protein containing repeats A2, B8, A3, and B9 but not that present in repeat A1 or repeats B1 to B7 (Fig. 8A). Further analysis with recombinant proteins containing smaller fragments from the region containing repeats A2, B8, A3, and B9 indicated that 4-10A 11B and 3-9D 9F recognize distinct epitopes (Fig. 8B). 4-10A 11B recognized an epitope in the recombinant protein containing repeats A2, B8, and A3, but smaller fragments, including ones with overlapping sequence, were not recognized. In contrast, 3-9D 9F recognized all recombinant proteins containing repeat A3. Repeat A3 is a 97-residue sequence containing 16 acidic residues.

DISCUSSION

The mechanisms by which *Salmonella* serotypes persist in the intestines of livestock and domestic fowl is a matter of considerable relevance to food safety and public health. The outer surface protein ShdA, an autotransporter family protein of serotype Typhimurium, is essential for long-term persistent intestinal carriage in a murine model of salmonellosis. Many

autotransporter family proteins that are essential for the virulence of a number of gram-negative bacteria are known to interact with host proteins via their extracellular localized passenger domain. This suggested to us that ShdA may also interact with host proteins and that this property may be involved in persistent intestinal carriage. We previously reported that ShdA binds extracellular matrix proteins, including fibronectin and collagen I, but it is unknown whether these binding properties are required for ShdA-mediated persistent intestinal carriage. Abrogation of ShdA interaction with fibronectin during natural infections and observation of the effect of this on persistent intestinal carriage is necessary to test the hypothesis that fibronectin-binding activity, or binding activity to other receptors mediated by the same ligand-binding domain of ShdA, is necessary for ShdA-mediated persistent intestinal carriage. As a first essential step toward addressing this question, we have investigated ShdA interaction with fibronectin in vitro to define domains containing the primary binding sites.

Here, we have investigated ShdA binding to fibronectin by a number of approaches that implicated the repeat region of the passenger domain as containing the primary ligand-binding domain. In a first approach, the repeat region of the ShdA passenger domain was implicated in fibronectin binding, since a recombinant protein containing the repeat region, but not one containing the N-terminal nonrepeat region, bound to fibronectin with similar affinity as measured by the concentration of ligand required to achieve half-maximal binding at equilibrium (~100 nM in each case). These data were supported by the observation that the repeat region, but not the nonrepeat region, was able to inhibit binding of the passenger domain to fibronectin. It was not possible to calculate the dissociation constant for the ShdA interaction with fibronectin. However, it is important to note that the reported concentration at which half-maximal binding at equilibrium was observed is likely an underestimate, since the recombinant fusion proteins used in this study were prone to degradation during expression in the *E. coli* host. This degradation could not be reduced by expression in protease-negative strains (data not shown). Since we found that the truncated peptides of the repeat region did not retain binding activity, it is likely that only the full-length protein contributed to binding in the assays reported here (21, 38).

The repeat region has an estimated molecular mass of ~100 kDa and consists of 1,073 residues, the majority of which are components of either type A or type B repeated sequence modules. Since smaller recombinant proteins containing fragments of the repeat region did not retain the ability to bind fibronectin, it seems unlikely that fibronectin binds a simple linear sequence within ShdA. Rather, it is likely that a relatively large region of ShdA, when folded correctly, is required for the ligand-binding domain to adopt the appropriate conformation. A similar situation has been described for the invasin protein of *Yersinia pseudotuberculosis*, whose binding to β1 integrins requires a minimal region consisting of 192 residues of a C-terminal domain for molecular mimicry of fibronectin binding of β1 integrins (24). However, the absence or incorrect folding of neighboring domains had no measurable effect on ligand binding of recombinant invasin using in vitro binding assays. Invasin molecular mimicry of fibronectin β1 integrin binding is dependent on three key residues that have identical separations in both fibronectin and invasin. The crystal structure model of invasin demonstrates that the three key residues of invasin (Asp811, Asp911, and Arg863), which are distantly separated on the primary amino acid sequence, are present in different protruding loops on the carboxyl-terminal domain (15). The precise conformation of the loops, and therefore the key residues, is heavily dependent on correct folding of the domain. The repeat region of ShdA may also form a single domain that when correctly folded has one or more binding sites for fibronectin.

Our second approach to study ShdA binding to fibronectin involved the use of ShdA-specific polyclonal antibodies or MAbs. The fact that only polyclonal serum raised against either the entire ShdA passenger domain or a recombinant protein containing A2, B8, A3, and B9 was capable of inhibiting ShdA binding to fibronectin was the first indication that the repeats A2, B8, A3, and B9 contained important components of the ligand-binding domain. Furthermore, of three ShdA-specific MAbs, the epitopes recognized by the two MAbs that inhibited ShdA binding (3-9D 9F and 4-10A 11B) were mapped to the A2-B8-A3 repeats, while the MAb (4-10G 8G) with no inhibitory activity was mapped to an epitope present in the B1 to B7 repeats. That 4-10A 11B was unable to recognize overlapping recombinant proteins smaller than A2-B8-A3 suggested that this MAb recognized an epitope not present in these truncated peptides, presumably due to misfolding. However, 3-9D 9F recognized all recombinant proteins containing the A3 repeat, suggesting that the sequence in this part of the correctly folded ShdA repeat region contributes an exposed surface, binding to which inhibits interaction with fibronectin either by directly competing for a binding site or by steric hindrance. ShdA binds one or more basic amino acids that form a cationic cradle in the Hep-2 domain of fibronectin via electrostatic forces (21). It therefore seems likely that acidic residues in the ShdA ligand-binding domain are involved in this interaction. The A3 repeat is composed of 97 residues, of which 16 have acidic side chains (aspartate or glutamate). Since the cationic cradle of the fibronectin Hep-2 domain forms a continuous patch of positive charge contributed from six surface-exposed basic residues, it seems likely that several acidic residues from repeat A3, and potentially other residues from elsewhere in the ShdA passenger domain, may contribute to binding.

That the repeat region alone is necessary and sufficient for maximal fibronectin binding in vitro raises a question as to the function of the nonrepeat region. The nonrepeat region consists of 421 residues with a predicted molecular mass of 42 kDa that contain no common sequence motifs and exhibit no amino acid homology with sequences in the available databases. It is possible that the nonrepeat region has a second activity unrelated to that of the fibronectin domain of the repeat region. A multidomain and multifunctional character has been described for a number of bacterial outer surface proteins. For example, the FnbpA protein of *S. aureus* has multiple fibronectin-binding motifs in an extended carboxy domain and fibrinogen-binding activity in a globular domain in the N-terminal domain (33, 36). These binding activities are completely independent, since fragments containing only one domain retain maximal binding activity for its cognate receptor (26). Another possibility is that the nonrepeat region of ShdA, while not directly

participating in fibronectin binding in vitro, may nonetheless be required for either secretion or correct presentation of the ligand-binding domain (repeat region) on the bacterial cell surface and thus may be required to facilitate interaction with fibronectin or other ligands during natural infections.

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