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Salmonella enterica Serotype Typhimurium Fimbrial Proteins Serve as Antigens during Infection of Mice

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The *Salmonella enterica* serotype Typhimurium genome contains 13 operons with homology to fimbrial gene sequences. Here we investigated the role of 11 serotype Typhimurium fimbrial proteins, including FimA, AgfA (CsgA), BcfA, StbA, SthA, LpfA, PefA, StdA, StcA, StiA, and StfA, as antigens during the infection of genetically resistant mice (CBA). Upon the growth of serotype Typhimurium in standard laboratory broth culture, only the expression of FimA could be detected by Western blot analysis. The infection of mice with serotype Typhimurium grown in broth culture, followed by at least one subsequent infection, resulted in seroconversion of animals to FimA, AgfA, BcfA, StbA, SthA, LpfA, PefA, StdA, StcA, StiA, and StfA positivity. Most animals seroconverted to only a subset of these fimbrial antigens. The immunization of mice with glutathione *S*-transferase (GST)-FimA, GST-AgfA, GST-BcfA, GST-StbA, GST-SthA, GST-LpfA, GST-PefA, GST-StdA, GST-StcA, GST-StiA, and GST-StfA fusion proteins resulted in reduced fecal shedding of serotype Typhimurium during a challenge compared to that by a control group immunized with purified GST protein. Collectively, these data suggest that the expression of serotype Typhimurium fimbrial antigens is induced during the infection of mice.

Salmonella serotypes are the leading cause of food-borne infections with a lethal outcome in the United States (18). *Salmonella enterica* serotype Typhimurium is the organism most frequently associated with *Salmonella*-induced diarrhea in humans (22). The intestinal carriage of *Salmonella* serotypes by apparently healthy animals arriving at slaughter is responsible for the introduction of the pathogen into the derived food products (5, 7, 16, 24, 29). Fecal contamination of the environment is an important factor in the transmission of *Salmonella* serotypes among farm animals (13, 17, 31, 32). Although the presence of *Salmonella* serotypes in the intestines and feces of farm animals is of importance for food safety, the underlying mechanisms are poorly understood.

Fimbrial adhesins are potential candidates for mediating attachment to intestinal surfaces (12). Whole-genome sequencing has revealed the presence of 13 operons containing open reading frames with homology to fimbrial gene sequences in the serotype Typhimurium genome (15). Only two of these operons, *fim* and *agf* (*csg*), have been shown by electron microscopy to mediate the expression of fimbrial filaments on the surfaces of serotype Typhimurium cells grown in broth or on agar plates, respectively (6, 10). An expression analysis of putative major fimbrial subunits from 11 serotype Typhimurium fimbrial operons by flow cytometry showed that only FimA is detectable on the bacterial surface after growth of the organism in Luria-Bertani (LB) broth (11). However, the expression of nine fimbrial operons was detected by flow cytometry on the surfaces of serotype Typhimurium cells recovered 8 h after infection from bovine ligated ileal loops (11). These data suggest that the majority of serotype Typhimurium fimbrial antigens are poorly expressed in vitro, while their expression is induced in the host environment.

Each fimbrial filament on the bacterial surface is composed of approximately 500 to 3,000 copies of a major fimbrial subunit protein. The presence of large numbers of major fimbrial subunits on the bacterial cell surface suggests that these antigens may represent good targets for the host immune response. To further investigate whether putative fimbrial operons identified by whole-genome sequencing are expressed in vivo, we studied the role of 11 putative major subunit proteins as antigens during serotype Typhimurium infections of mice.

MATERIALS AND METHODS

Bacterial strains and culture conditions. Serotype Typhimurium strain AJB3, a nalidixic acid-resistant derivative of serotype Typhimurium wild-type strain SR11 (25), has been described previously (2). *Escherichia coli* strain DH5α has been described previously (8). *E. coli* strain BL21(DE3) Star was obtained from Invitrogen. Unless stated otherwise, strains were cultured either aerobically or statically at 37°C in LB broth (10 g/liter tryptone, 5 g/liter yeast extract, 5 g/liter NaCl). LB-pH 5.1 broth was prepared as described previously by buffering LB broth with 100 mM 2-(*N*-morpholino)ethanesulfonic acid (MES) and adjusting the pH to 5.1 with acetic acid (19). LB-NaCl broth contained 10 g/liter tryptone, 5 g/liter yeast extract, and 300 mM NaCl. Colonization factor antigen (CFA) broth contained 1% Casamino Acids (Difco), 0.15% yeast extract (Difco), 0.005% $MgSO_4$, 0.0005% $MnCl_2$, 12 mM Na_2HPO_4, and 5 mM KH_2PO_4 (4). Brain heart infusion (BHI) broth contained calf brain infusion (12.5 g/liter), beef heart infusion (5 g/liter), proteose peptone (10 g/liter), NaCl (5 g/liter), glucose (2 g/liter), and Na_2HPO_4 (2.5 g/liter). LB agar plates contained 15 g/liter agar (Difco). If appropriate, antibiotics were added at the following concentrations: carbenicillin, 100 mg/liter; chloramphenicol, 30 mg/liter; kanamycin, 60 mg/liter; or nalidixic acid, 50 mg/liter.

His-tag fusion protein construction, expression, and purification. Plasmids pSW5-50, pAH96, pAH99, pAH100, pAH97, pAH101, pAH103, pAH109, pAH102, pAH107, and pAH111, encoding glutathione *S*-transferase (GST) fusion proteins with serotype Typhimurium major fimbrial subunits (AgfA, BcfA, StbA, SthA, LpfA, FimA, PefA, StdA, StcA, StiA, and StfA, respectively), were described previously (11). The purification of GST fusion proteins was per-

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TABLE 1. Oligonucleotide primers used to construct plasmids encoding six-histidine-tagged fusion proteins

Primer	Sequence (5′-3′)	Plasmid with fragment in pET101-D/TOPO	Plasmid with fragment in pQE30
agfA-HisF	CACCATGAAACTTTTAAAAGTGGCAG	pAH132-13	
agfA-HisR	ATACTGGTTAGCCGTGGCGTTG		
pETFimA1	CACCATGTCGATAATAATTCAAACG	pAH128-1	
pETFimA2	TTCGTATTTCATGATAAAGGTG		
stdA1 (histag)	CGCGGATCCGCCGATACTACACCCACAGC		pSR11
stdA2(histag)	TCCCCCGGGCGACTTCAGGACGGAAAATGTC		
pETStcA1	CACCATGAAACGTTCACTTATTGC	pAH122	
pETStcA2	AGTAACAACCGTTAAAGTCAGT		
pETStiA1	CACCATGAAACTCTCCTTAAA	pAH123	
pETStiA2	ATATTGCAGATAGAATGTTGCGG		

formed as described previously (11). An antiserum against each fusion protein raised in rabbits has been described previously (11). Amino-terminal six-histidine-tagged major fimbrial subunit fusion proteins were constructed by cloning the BamHI/SmaI fragments from pAH96, pAH99, pAH100, pAH97, and pAH111 into pQE30 (QIAGEN), giving rise to plasmids pSR1 (His-BcfA), pSR3 (His-StbA), pSR4 (His-SthA), pSR6 (His-LpfA), and pSR9 (His-StfA), respectively. The BamHI/SalI fragment from pAH103 was cloned into pQE30, giving pSR7 (His-PefA). Primers stdA1histag and stdA2histag (Table 1) were used to amplify a region of the *stdA* gene corresponding to the major subunit without the amino-terminal signal sequence, which was cloned into pCR2.1 TOPO (Invitrogen), giving pSR100-1. A BamHII/SmaI fragment of pSR100-1 was cloned into pQE30, giving pSR11 (His-StdA). Carboxy-terminal histidine-tagged major fimbrial subunit fusion proteins were constructed by amplifying *fimA*, *stiA*, *stcA*, and *agfA* with the primers pET-FimA-1 and -2, pET-StiA-1 and -2, pET-StcA-1 and -2, and AgfA-HisF and -R, respectively (Table 1). PCR products were cloned directly into the pET101/D-TOPO directional cloning vector (Invitrogen), giving pAH128 (FimA-His), pAH122 (StcA-His), pAH123 (StiA-His), and pAH132-13 (AgfA-His), respectively. *E. coli* DH5α was transformed with pSR1, pSR3, pSR4, pSR6, pSR7, pSR9, or pSR11 (Table 2). BL21(DE3) Star (Invitrogen) was transformed with pAH122, pAH123, pAH128, or pAH132-13 (Table 2). Log-phase cultures of these transformants were induced to express the six-His-tagged fusion proteins with the addition of 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG; Sigma) at 37°C (pAH132-13 was induced at 30°C to increase protein solubility). The six-His-tagged fusion proteins encoded by plasmids pSR4, pSR6, pSR7, pSR9, pAH122, pAH123, and pAH128 were purified with a HisTrap kit (Amersham Pharmacia) under native conditions per the manufacturer's protocol. The fusion proteins encoded by plasmids pSR1, pSR3, pSR11, and pAH132-13 were purified under denaturing conditions according to instructions by the manufacturer using Ni-nitrilotriacetic acid (Ni-NTA) agarose (QIAGEN).

Animal experiments. Throughout this study, 8- to 10-week-old female BALB/c mice or 6- to 8-week-old female CBA/J mice were used (Jackson Laboratory).

To study seroconversion, fecal and blood samples were collected from naïve CBA/J mice prior to infection. A group of six mice was infected with serotype Typhimurium (AJB3) grown to stationary phase in static LB broth at 37°C by oral gavage with 2×10^9 CFU/animal. On days 16 and 33 after infection, mice were given a dose of 3.8×10^9 CFU/animal by oral gavage. Fecal samples were collected on day 45 after initial infection. Blood samples were collected into heparinized capillary tubes (VWR) from the lateral saphenous vein punctured with a 22G1 needle (Becton-Dickinson) on day 45 after initial infection. In a second experiment, a group of three mice was inoculated with serotype Typhimurium on days 0, 14, and 73 with doses of 3×10^9, 3.9×10^9, and 2×10^9CFU/animal, respectively, as described above. Blood and fecal samples were collected 10 and 14 days after the first and second challenges and 17 days after the final challenge. All blood samples were centrifuged at room temperature for 5 min at 8,000 rpm, and sera were removed, diluted 1:5 in phosphate-buffered saline (PBS) plus 0.1% NaN_3, and stored at −80°C. All fecal samples were treated as described previously (28). Briefly, samples were frozen at −80°C immediately after collection. Upon use, they were thawed on ice and resuspended in 1 ml PBS, pH 7.4, plus 0.1% NaN_3 plus 50 μl protease inhibitor (Calbiochem) per 100 mg feces. Samples were vortexed and then dispersed by microtip sonication at 50% duty for six 10-second bursts on ice, and particulate matter was pelleted at 4,300 rpm for 5 min at 4°C. Supernatants were removed and stored at −80°C until further use.

To study the effect of vaccination with fimbrial proteins on intestinal colonization, groups of CBA/J mice were immunized intraperitoneally with 0.1 ml of a 1:1 emulsion of either purified GST (control group; $n = 11$), GST-BcfA ($n = 5$), GST-StdA ($n = 5$), or GST-major fimbrial subunit proteins (fimbrial cocktail group; $n = 17$) and Titer-Max Gold (Sigma) as described below. On day 1, the fimbrial cocktail group received 2 to 8 μg/animal of GST-AgfA, GST-BcfA, GST-LpfA, GST-PefA, and GST-StdA. On day 15, each mouse in the fimbrial cocktail group received 4 to 10 μg of GST-FimA, GST-StbA, GST-StcA, GST-StfA, GST-SthA, and GST-StiA. On day 29, mice in the fimbrial cocktail group received a booster immunization containing the first set of proteins, and 14 days later, each animal received 5 to 10 μg of the second group of GST-fimbrial fusion proteins. The other groups were vaccinated on the same days with 20 μg of GST, GST-BcfA, or GST-StdA per mouse. Sera from mice in all four groups were collected on day 54 and used in a Western blot against GST and the six-histidine-tagged fimbrial fusion proteins at a 1:500 dilution to determine if serum immunoglobulin (Ig) antibodies had been raised against fimbrial major subunits or GST. The fimbrial cocktail group received a booster immunization on day 69 that contained 13.5 μg/animal of GST-AgfA, 12 μg/animal of GST-FimA, and 14 μg/animal of GST-StiA. Serum samples from the fimbrial cocktail mice were collected on day 85 and were analyzed for responses to FimA, StiA, and AgfA by enzyme-linked immunosorbent assays (ELISAs). On day 98, the groups were inoculated by oral gavage with 1×10^9 CFU of serotype Typhimurium AJB3 grown statically overnight at 37°C. Fecal samples from each mouse were collected at the indicated time points, homogenized in 1 ml PBS, and plated on LB plates containing nalidixic acid. On day 28 after serotype Typhimurium infection, the cecum, Peyer's patches, spleen, liver, mesenteric lymph nodes, and gall bladder were collected and homogenized in PBS. Dilutions of homogenized fecal and organ samples were plated on LB plates containing nalidixic acid. All data were converted logarithmically prior to the calculation of averages and statistical analysis. Student's t test was used to determine whether the average log CFU/mg feces for the control group was significantly different from that for the fimbrial cocktail-immunized group on each day postchallenge. P values of <0.05 were considered significant.

To look at fimbrial expression in the ceca of mice infected with serotype Typhimurium, 8- to 10-week-old female BALB/c mice ($n = 3$; see Table 5) were inoculated with approximately 1×10^9 CFU of serotype Typhimurium strain SR11. After 6 days, the cecum was collected from each mouse and fixed in 10% formalin. Tissues were embedded in paraffin, and 0.5-μm sections were prepared for immunofluorescence microscopy.

TABLE 2. Plasmids encoding six-histidine-tagged fusions to major fimbrial subunits

Fusion protein	Plasmid
His-BcfA	pSR1
His-StbA	pSR3
His-SthA	pSR4
His-LpfA	pSR6
His-StfA	pSR9
His-PefA	pSR7
His-StdA	pSR11
FimA-His	pAH128
StcA-His	pAH122
StiA-His	pAH123
AgfA-His	pAH132-13

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Immunofluorescence microscopy. Antisera specific for GST-BcfA, GST-LpfA, and GST-StcA have been described previously (11). Anti-GST antibodies were removed by preadsorbing each serum with *E. coli* DH5α(pGEX 4T-2) using a previously described protocol (9). A *Salmonella* O group B (factors 1, 4, 5, 12, and 27) (Becton Dickinson)-specific antiserum was used to detect the O antigen of serotype Typhimurium lipopolysaccharide (LPS). All antisera were filtered (0.2-μm pore size) and loaded on a recombinant protein G-agarose column (Invitrogen) according to the manufacturer's instructions. Purified antibodies were eluted from the protein G column in 2 ml of 0.1 M glycine hydrochloride (pH 2.6), and a pH of 7 was obtained by the addition of 1 M Tris-HCl, pH 8. The antibodies were concentrated, and the glycine buffer was exchanged for PBS, pH 7.4, in Centricon centrifugal filter devices with a molecular mass cutoff of 10 kDa (Millipore). These solutions (0.5 ml) were then labeled with either an Alexa Fluor 594 protein labeling kit (for the *Salmonella* O group B antiserum) or an Alexa Fluor 488 protein labeling kit (for the anti-GST-BcfA, anti-GST-LpfA, and anti-GST-StcA antisera) according to the manufacturer's instructions (Molecular Probes).

Sections of the ceca of BALB/c mice collected 6 days after infection with serotype Typhimurium were deparaffinized by standard procedures (incubation at 50°C for 20 min and three washes for 5 min each time in xylene, followed by 5-min washes in 95% ethanol, 70% ethanol, and distilled H_2O). Sections were then blocked in 5% nonfat dried milk plus 0.05% Tween 20 in PBS for 1 h. A 1:50 dilution of Alexa Fluor 594-labeled *Salmonella* O group B antiserum, a 1:50 dilution of Alexa Fluor 488-labeled antifimbrial antiserum (anti-GST-BcfA, anti-GST-LpfA, or anti-GST-StcA), and a 1:1,000 dilution of Hoechst stain (0.5 mg/ml) were added in 1 ml of blocking buffer to each slide. Slides were incubated for 1 h at room temperature in the dark before they were washed three times for 5 min each time in blocking buffer and three times for 5 min each time in PBS, pH 7.4. Excess liquid was removed from around the tissues, and SlowFade antifade solution (Molecular Probes) was applied to each slide before the coverslip was added and sealed with clear polish. The slides were incubated in the dark overnight at room temperature before analysis.

Western blot analysis. An analysis of serum samples by Western blotting was performed by separating 0.5 μg of affinity chromatography-purified GST (control) and 0.5 μg of each affinity chromatography-purified His-tagged fusion protein by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on a 12% gel. After electrophoresis, the proteins were transferred to Immobilon-P membranes (Millipore) using a Trans-Blot SD semidry electrophoretic transfer cell (Bio-Rad) according to the manufacturer's instructions. Blots were blocked in PBS containing 5% nonfat dry milk, 0.05% Tween 20, and 0.02% NaN_3 (blocking buffer) at room temperature for 1 h or overnight at 4°C. Each blot was incubated with a 1:500 dilution of serum or a 1:25 dilution of a fecal preparation taken from a particular mouse either before or after the challenge with serotype Typhimurium. All samples were diluted in blocking buffer and incubated at room temperature for 2 to 3 h, with agitation. Blots were washed three times for 5 min each time with blocking buffer. Blots detecting serum IgG antibodies were transferred to a solution with a 1:20,000 dilution of goat anti-mouse IgG (Fab specific)–alkaline phosphatase conjugate (Sigma) in blocking buffer and were incubated at room temperature for 1 h with agitation. Blots detecting fecal IgA antibodies were transferred to a solution with a 1:20,000 dilution of goat anti-mouse IgA–biotin (Amersham) in blocking buffer for 1 h at room temperature, with agitation. These blots were then washed three times for 5 min each time in blocking buffer and transferred to a 1:10,000 dilution of streptavidin-peroxidase polymer conjugate (Sigma) in blocking buffer for 1 h at room temperature, with agitation. All blots were then washed three times for 5 min each time with blocking buffer and three times with PBS. Signals were generated using Immun-star substrate (Bio-Rad) or the ECL Plus detection substrate (Amersham).

For a Western blot analysis of serotype Typhimurium grown overnight statically in LB broth, LB broth buffered to pH 5.1, LB-NaCl broth, BHI broth, or CFA broth, cultures were harvested by centrifugation. Whole-cell lysates were prepared by previously established protocols (4), including boiling in SDS-PAGE sample buffer containing 0.2 M glycine-HCl (pH 1.5), 0.5% deoxycholate, 2% SDS, or 5% β-mercaptoethanol for 10 min following electrophoresis. Alternatively, bacterial pellets were treated briefly with 90% formic acid and frozen, the acid was removed with a Speed Vac concentrator for 1 h, and the pellets were resuspended in SDS-PAGE sample buffer. Cells were boiled for 10 min and analyzed by electrophoresis.

ELISA to detect antibodies to LPS. The detection of antibodies against serotype Typhimurium LPS was based on a previously described protocol (28). In brief, LPS from serotype Typhimurium prepared by phenol extraction (Sigma) was resuspended in coating buffer (0.1 M sodium carbonate, 1.0 M NaCl, pH 9.6), and 96-well Polysorb ELISA plates (Nunc) were coated with 5 μg of antigen per well and incubated overnight at 37°C. The plates were washed four times with distilled water and patted dry before being blocked for 1 h at 37°C in blocking buffer (PBS plus 1% Tween 20). The plates were washed as before, and mouse serum (0.05 ml/well) was then added to the antigen-coated plates in duplicate twofold serial dilutions with blocking buffer as the diluent and incubated on a rocker overnight at 4°C. Plates were washed 10 times in washing buffer (PBS plus 0.05% Tween 20), and binding of the mouse serum was detected by using goat anti-mouse IgG–alkaline phosphatase conjugate diluted 1:1,000 in blocking buffer. Plates were incubated with secondary antibodies for 1 h at 37°C and washed 10 times with washing buffer. Detection was performed with 0.05 ml of *p*-nitrophenylphosphate (1 mg/ml; Sigma) diluted in glycine buffer (0.1 M glycine, 1 mM $MgCl_2$, 1 mM $ZnCl_2$, pH 10.4). Plates were developed at 37°C for 1 h, and the reaction was stopped by the addition of 0.05 ml of 0.5 M EDTA. The alkaline phosphatase activity in each well was determined by measuring the absorbance at 410 nm, and the highest serum dilution giving a reading above the background level was determined. Antibody titers are reported as fold increases in immune serum over naïve serum.

TABLE 3. Optimal binding conditions for each of the six-histidine-tagged major fimbrial subunit proteins in Ni-NTA HisSorb ELISA plates (Qiagen)

Plasmid with six-His-tagged major subunit	Amt of protein (μg/well)	Binding temp (°C)	pH
pSR1	12	37	7.5
pAH128	8	4	7.5
pSR3	10	37	7.5
pSR4	10	4	7.5
pSR6	6	37 or 4	7.5
pSR7	13	37	7.5
pAH122	8	4	7.5
pSR9	12	4	7.5
pSR11	12	4	7.5
pAH123	11	4	7.5
pAH132-13	12	37	7.5

HisSorb ELISA to detect seroconversion. Ni-NTA HisSorb ELISAs were performed per the manufacturer's instructions (QIAGEN). In brief, the saturation conditions for binding to Ni-NTA-coated wells were determined for each six-His-tagged fusion protein by applying increasing concentrations of protein to the HisSorb plates and incubating them in PBS plus 0.2% bovine serum albumin (BSA) with pH values ranging from 7.0 to 8.0 at 37°C or 4°C (Table 3). Subsequent binding studies were performed at saturation. To determine the concentration of seroconversion antibodies to the fimbrial major subunits from a particular mouse, the fusion proteins were loaded onto the plates at the indicated concentrations in a total volume of 0.2 ml of PBS plus 0.2% BSA, pH 7.5 (PBS-BSA), and allowed to incubate at either 37°C or with rocking at 4°C overnight. The plates were then washed four times with PBS plus 0.05% Tween 20 (PBS-Tween) and dried. Naive or immune samples diluted in PBS-BSA were then added to the wells and diluted in twofold serial dilutions. Plates were allowed to incubate overnight at 4°C with agitation. After four washes with PBS-Tween, 0.2 ml of a 1:10,000 dilution of secondary antibody (goat anti-mouse IgG [Fab specific], goat anti-mouse IgG [γ chain specific], or goat anti-mouse IgA [α chain specific], all conjugated to alkaline phosphatase) diluted in PBS-BSA was added to all wells and incubated at room temperature with agitation for 2 h. After the plates were washed four times, 0.05 ml of 1-mg/ml *p*-nitrophenylphosphate (Sigma) was added to each well, and the plates were incubated at 37°C for 1 h. The colorimetric reaction was stopped by adding 0.05 ml 0.5 M EDTA to each well. The alkaline phosphatase activity in each well was determined by measuring the absorbance at 410 nm, and the highest serum dilution giving a reading above the background level was determined. Antibody titers are reported as fold increases in immune serum over naïve serum.

ELISAs to detect serum antibody titers from vaccinated mice. HisSorb ELISAs to determine the antibody titers of fimbrial cocktail- or GST-immunized mice were performed basically as described above. ELISA plates were coated with a six-His-tagged major fimbrial subunit fusion protein, and serum taken from each mouse in the cocktail group was loaded in the plates in duplicate twofold serial dilutions. The titers from these sera were compared to titers from a pooled sample of sera from the control group (GST-immunized mice) loaded in the same plate in duplicate twofold serial dilutions. Serum Ig titers were detected with a 1:5,000 dilution of goat anti-mouse IgG (Fab specific)–alkaline

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phosphatase conjugate (Sigma). The alkaline phosphatase activity in each well was determined as described above. The absorbance at 410 nm for the serum at a titer of 320 among the pooled samples from the control group was used as the background cutoff absorbance for comparison to samples from the fimbrial cocktail-immunized group. The first well with an absorbance above background levels was considered positive. Each titer is reported as the fold increase over the titer of the pooled control group samples.

RESULTS

In vitro expression of serotype Typhimurium fimbrial proteins detected by Western blotting. We have recently shown that a serotype Typhimurium wild-type strain (AJB3) grown to stationary phase in static LB broth at 37°C expresses FimA on its surface, while no surface expression of AgfA, BcfA, StbA, SthA, LpfA, PefA, StdA, StcA, StiA, or StfA is detectable by flow cytometry (11). To further investigate whether the expression of fimbrial subunits was detectable after in vitro growth in broth, we performed a Western blot analysis with whole-cell lysates of serotype Typhimurium using rabbit serum specific for FimA, AgfA, BcfA, StbA, SthA, LpfA, PefA, StdA, StcA, StiA, or StfA. Whole-cell lysates were prepared by three protocols that were previously described for the disassembly of fimbriae (4). Static growth at 37°C of serotype Typhimurium strain AJB3 in LB broth (pH 7) resulted in the expression of FimA, while neither AgfA, BcfA, StbA, SthA, LpfA, PefA, StdA, StcA, StiA, nor StfA was detectable by Western blotting (data not shown).

An analysis of serotype Typhimurium grown in LB broth buffered to pH 5.1 or in CFA broth by flow cytometry using rabbit serum against AgfA, BcfA, StbA, SthA, LpfA, PefA, StdA, StcA, StiA, or StfA and an anti-rabbit–fluorescein isothiocyanate conjugate showed that in each case, none or only a small fraction of cells (<5%) showed fluorescence above background levels (11). To determine whether the low level of fimbrial expression in LB broth buffered to pH 5.1 or in CFA broth can be detected by Western blotting, serotype Typhimurium cultures grown under these conditions were analyzed as described above. The growth of serotype Typhimurium in LB broth buffered to pH 5.1 or in CFA broth resulted in the expression of FimA, while neither AgfA, BcfA, StbA, SthA, LpfA, PefA, StdA, StcA, StiA, nor StfA was detectable by Western blotting (data not shown). Similarly, FimA was the only fimbrial protein detected by Western blotting in serotype Typhimurium cultures grown in BHI broth or LB-NaCl broth (data not shown).

Seroconversion to fimbrial proteins during serotype Typhimurium infection of mice detected by Western blotting. To determine whether serotype Typhimurium fimbriae serve as antigens in vivo, the immune response to the major fimbrial subunits was analyzed after the infection of genetically resistant mice (CBA/J mice) with serotype Typhimurium (AJB3) grown to stationary phase in static LB broth at 37°C. Since the bacterial culture used to inoculate mice only expressed FimA (as shown by Western blotting and flow cytometry), we reasoned that seroconversion to other fimbrial proteins observed during infection would provide indirect evidence of their in vivo expression. Mice were inoculated with serotype Typhimurium strain AJB3 intragastrically on days 0, 16, and 33, and serum and fecal samples were collected prior to infection and on day 45 after infection.

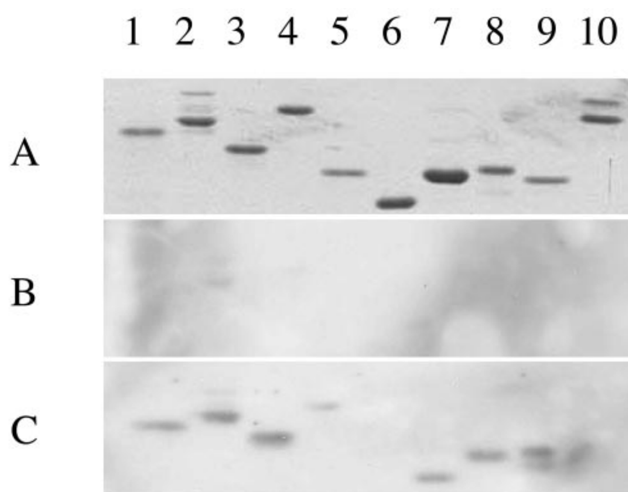

FIG. 1. Detection of fecal IgA against fimbrial antigens by Western blotting. (A) SDS-PAGE gel of purified His-tagged fusion proteins stained with Coomassie blue. (B) Western blot of an SDS-PAGE gel loaded with the same proteins as the gel in the top panel, detected with IgA extracted from fecal samples collected from a naïve mouse. (C) Fecal samples from the same animal were collected on day 45 after infection with serotype Typhimurium grown in static LB broth. The panel shows a Western blot (loaded identically to the panels above) detected with IgA extracted from a fecal sample collected 45 days after infection. Lane 1, HisBcfA; lane 2, FimA-His; lane 3, His-StbA; lane 4, His-SthA; lane 5, His-LpfA; lane 6, His-PefA; lane 7, StcA-His; lane 8, His-StfA; lane 9, His-StdA; lane 10, StiA-His.

To investigate seroconversion, we generated plasmids encoding six-histidine-tagged fusions with major fimbrial subunits, including His-BcfA, His-StbA, His-SthA, His-LpfA, His-StfA, His-PefA, His-StdA, FimA-His, StcA-His, and StiA-His. These fusion proteins were affinity chromatography purified and separated by SDS-PAGE (Fig. 1). Serum and fecal samples were investigated for the presence of antibodies (serum Ig or fecal IgA) directed against fimbriae by Western blotting with the His-BcfA, His-StbA, His-SthA, His-LpfA, His-StfA, His-PefA, His-StdA, FimA-His, StcA-His, and StiA-His fusion proteins (Fig. 1 and Table 4).

Fecal samples from four mice collected prior to infection and on day 45 after infection were analyzed by Western blotting for the presence of fecal IgA recognizing fimbrial fusion proteins. Fecal pellets collected on day 45 after infection contained IgA antibodies to 1 (mouse no. 2), 7 (mouse no. 6), or 8 (mouse no. 1 and 3) of the 10 fusion proteins tested (Table 4). In contrast, no IgA antibodies recognizing fimbrial proteins were detected in fecal pellets collected prior to infection. A representative Western blot of a fecal IgA response is shown in Fig. 1. Naïve sera and sera collected on day 45 after infection from five mice were investigated for antibodies against fimbrial proteins. Sera collected on day 45 after infection contained Ig against 1 (mouse no. 4 and 5), 2 (mouse no. 6), 3 (mouse no. 2), or 7 (mouse no. 3) of the 10 fusion proteins tested (Table 4). No antibodies recognizing fimbrial proteins were detected in naïve sera from the same animals. Collectively, these data suggested that all major fimbrial subunits studied in this experiment (i.e., BcfA, StbA, SthA, LpfA, StfA, PefA, StdA, FimA, StcA, and StiA) served as antigens during the infection

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TABLE 4. Seroconversion to fimbrial antigens, as determined by Western blotting

Animal no.	Response measured	Seroconversion (+) to fimbrial protein									
		BcfA	FimA	StbA	SthA	LpfA	PefA	StcA	StfA	StdA	StiA
1	Fecal IgA	+	+	+	+	−	+	+	+	−	+
2	Fecal IgA	−	+	−	−	−	−	−	−	−	−
	Serum Ig	+	+	−	+	−	−	−	−	−	−
3	Fecal IgA	+	+	+	+	−	+	+	+	−	+
	Serum Ig	+	+	−	−	+	−	+	+	+	+
4	Serum Ig	−	−	−	−	−	−	−	−	+	−
5	Serum Ig	−	−	−	−	−	−	−	−	+	−
6	Fecal IgA	−	+	+	−	−	+	+	+	+	+
	Serum Ig	+	+	−	−	−	−	−	−	−	−

of mice (Table 4). However, none of the serum or fecal samples from infected animals contained antibodies against all 10 fimbrial proteins investigated. Instead, each animal developed serum Ig or fecal IgA responses to only a subset of these fimbrial antigens.

To quantify the antibody responses to fimbrial proteins, we analyzed serum Ig titers by ELISA. Three CBA mice were infected with serotype Typhimurium and then challenged with serotype Typhimurium 24 days and 73 days after the initial infection. The His-BcfA, His-StbA, His-SthA, His-LpfA, His-StfA, His-PefA, His-StdA, FimA-His, StcA-His, AgfA-His, and StiA-His fusion proteins were adsorbed to Ni-NTA-coated wells, and the serum Ig titers were determined using serial twofold dilutions of naïve sera and immune sera collected from mice. As a control, we determined the Ig titers to purified serotype Typhimurium LPS by ELISA. Titers against LPS continuously increased (naïve titer $<$ titer 10 days after infection $<$ titer 28 days after infection $<$ titer 90 days after infection) during the duration of the experiment. An eightfold or more increase in anti-LPS Ig titers was observed in sera from all animals collected 28 days after infection compared to naïve serum samples (Table 5). An eightfold or more increase in the Ig titer in immune serum compared to that in naïve serum was detected for AgfA, BcfA, and StdA for mouse 7, for BcfA, StcA, and SthA for mouse 8, and for BcfA, FimA, and PefA for mouse 9. For two animals (mouse no. 7 and 8), antibody titers against fimbrial proteins peaked at the last time point (90 days after infection), while the greatest titers were obtained 28 days after infection for the remaining animal (mouse no. 9) (Table 5). Generally, serum conversion to LPS (8-fold to 512-fold increase in titer) was stronger than that observed to fimbrial proteins.

Immunization of mice with multiple fimbrial antigens. To investigate how an immune response against all of the fimbrial antigens would affect intestinal colonization by serotype Typhimurium, we immunized CBA mice either with a purified GST protein (control group) or with a cocktail consisting of 11 GST fusions to fimbrial subunits (cocktail group). In a pilot experiment in which CBA mice were given all 11 GST-fimbrial fusion proteins, we noted that animals only developed an immune response to a subset of these antigens, as assessed by Western blotting (data not shown). The vaccination procedure was thus modified, and animals in the cocktail group received an injection of a mixture consisting of GST-AgfA, GST-BcfA, GST-LpfA, GST-PefA, and GST-StdA, followed 15 days later by an injection of a mixture consisting of GST-FimA, GST-

TABLE 5. Seroconversion to fimbrial antigens, as determined by ELISA

Antigen	Fold increase in immune serum titer over naïve serum titer on indicated day p.i.[a]								
	Mouse 7			Mouse 8			Mouse 9		
	10	28	90	10	28	90	10	28	90
AgfA	3	10	8	ND	ND	4	1	3	2
BcfA	ND	ND	8	ND	ND	8	1	12	4
FimA	ND	ND	4	ND	ND	2	1	16	8
LpfA	ND	ND	4	ND	ND	2	1	5	3
PefA	ND	ND	2	ND	ND	2	1	12	3
StbA	ND	ND	1	ND	ND	1	1	5	2
StcA	ND	ND	4	3	10	20	1	3	1
StdA	3	5	8	ND	ND	4	2	3	4
StfA	ND	ND	4	ND	ND	2	2	6	4
SthA	ND	ND	4	3	5	10	1	4	2
StiA	ND	ND	4	ND	ND	2	1	3	2
LPS	2	288	512	2	12	128	1	8	64

[a] ND, not determined.

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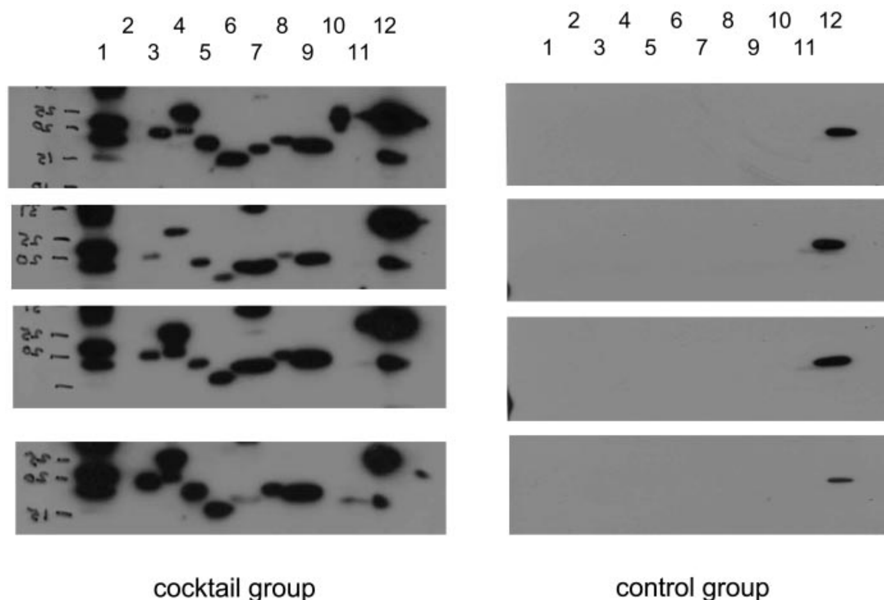

FIG. 2. Response to fimbrial antigens after vaccination with GST (control group) or with a cocktail containing 11 GST-fimbrial fusion proteins (cocktail group), as detected by Western blotting. Mice in the cocktail group received two injections with a mixture of GST-AgfA, GST-BcfA, GST-LpfA, GST-PefA, and GST-StdA and two injections with a mixture of GST-FimA, GST-StbA, GST-StcA, GST-StfA, GST-SthA, and GST-StiA. Mice in the control group received two injections with purified GST protein. The left panel shows Western blots of serum samples collected after vaccination from four animals in the cocktail group. The right panel shows Western blots of serum samples collected after vaccination from four animals in the control group. Lanes 1, HisBcfA; lanes 2, FimA-His; lanes 3, His-StbA; lanes 4, His-SthA; lanes 5, His-LpfA; lanes 6, His-PefA; lanes 7, StcA-His; lanes 8, His-StfA; lanes 9, His-StdA; lanes 10, StiA-His; lanes 11, AgfA-His; lanes 12, GST.

StbA, GST-StcA, GST-StfA, GST-SthA, and GST-StiA. This vaccination was repeated, and fecal IgA and serum Ig were collected 11 days after the last immunization. Antibodies to fimbrial antigens were detected by Western blotting using the His-BcfA, His-StbA, His-SthA, His-LpfA, His-StfA, His-PefA, His-StdA, FimA-His, StcA-His, AgfA-His, and StiA-His fusion proteins, while anti-GST antibodies were detected using purified GST. All mice in the control group had antibodies to GST but not to fimbrial antigens, as determined by Western blots using a 1:200 dilution of serum (Fig. 2). Mice in the cocktail group had responses to BcfA, StbA, SthA, LpfA, StfA, PefA, StdA, and StcA, but in most animals in the cocktail group, responses to AgfA, FimA, and StiA were low or absent (Fig. 2). The cocktail group thus received a final boost consisting of GST-AgfA, GST-FimA, and GST-StiA. A final blood sample was collected from animals 16 days after the last booster immunization.

To quantify the antibody responses to fimbrial proteins in the cocktail group, we analyzed serum Ig titers from samples collected after immunization for 10 mice from each group. The His-BcfA, His-StbA, His-SthA, His-LpfA, His-StfA, His-PefA, His-StdA, FimA-His, StcA-His, AgfA-His, and StiA-His fusion proteins were adsorbed to Ni-NTA-coated wells, and the serum Ig titers were determined by ELISA as described above. The average increases in antibody titers in the cocktail group compared to those in the control group are shown in Table 6. The serum Ig response to FimA remained poor despite repeated immunizations. However, the responses to the other fimbrial antigens were, on average, increased compared to those in mice infected with serotype Typhimurium (Tables 5 and 6).

Twenty-nine days after the last immunization, both groups of mice were challenged with serotype Typhimurium. Fecal samples were collected over a 28-day period to determine the number of bacteria shed with the feces. Mice in the cocktail group shed significantly lower numbers ($P < 0.05$) of serotype Typhimurium with their feces than did mice in the control group on days 5, 7, 9, 15, and 20 after infection (Fig. 3A). By day 28, both groups were colonized by similar numbers of bacteria. When bacteria were recovered from organs on day 28 after infection, there was also no difference in the colonization

TABLE 6. Titers of antibodies to fimbrial antigens elicited by vaccination

Antigen	Average fold increase in serum Ig titer for mice in cocktail group compared to that for mice in control group
BcfA[a]	60
FimA[b]	2
StbA[a]	5
SthA[a]	8
LpfA[a]	26
PefA[a]	5
StcA[a]	5
StfA[a]	10
StdA[a]	53
StiA[b]	5
AgfA[b]	5

[a] Titers were measured in samples collected 54 days after the first vaccination (i.e., after the second GST-FimA, GST-StbA, GST-StcA, GST-StfA, GST-SthA, and GST-StiA injection).

[b] Titers were measured in samples collected 85 days after the first vaccination (i.e., after the booster injection with GST-AgfA, GST-StiA, and GST-FimA).

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FIG. 3. (A) Bacteria recovered from feces of mice vaccinated either with GST (squares) or with a cocktail containing 11 GST-fimbrial fusion proteins (circles) and then challenged with serotype Typhimurium. Data represent geometric means ± standard errors. (B) Bacteria recovered from organs 28 days after infection of mice vaccinated either with GST (control) or with a cocktail containing 11 GST-fimbrial fusion proteins (cocktail). The circles represent data for individual animals.

of the cecum ($P > 0.05$) (Fig. 3B). However, the animals in the control group carried higher numbers of bacteria in the spleen, on average, than did animals in the cocktail group ($P < 0.05$).

To determine whether immunization with those fimbrial antigens that had elicited high antibody titers (i.e., BcfA and StdA [Table 6]) would reduce fecal shedding when used individually for vaccination, groups of five mice were immunized with GST-BcfA, GST-StdA, or GST and subsequently challenged with serotype Typhimurium as described above. Mice in the GST-BcfA and GST-StdA vaccine groups shed similar numbers of serotype Typhimurium with their feces to those shed by mice in the cocktail group (data not shown).

Detection of fimbrial expression in the murine cecum by fluorescence microscopy. To directly analyze the in vivo expression of selected fimbrial antigens, BALB/c mice were inoculated with serotype Typhimurium, and ceca were collected 6 days after infection. Serotype Typhimurium was detected by fluorescence microscopy with Alexa Fluor 594-labeled anti-O antiserum (red fluorescence), and the expression of fimbrial proteins (BcfA, StcA, and LpfA) was detected with Alexa Fluor 488-labeled antiserum (green fluorescence). Colocalization of red and green fluorescence provided further evidence of the in vivo expression of BcfA, StcA, and LpfA in the ceca of mice (Fig. 4).

DISCUSSION

The elaboration of type 1 fimbriae on the surfaces of serotype Typhimurium cells grown in broth culture was first described by electron microscopic observation in 1958 (6). The biosynthesis of type 1 fimbriae is mediated by the *fimAICDHF* operon of serotype Typhimurium (3, 14, 23). A distinct fimbrial type, termed thin curled fimbriae, was subsequently described by electron microscopic observation of the surfaces of serotype

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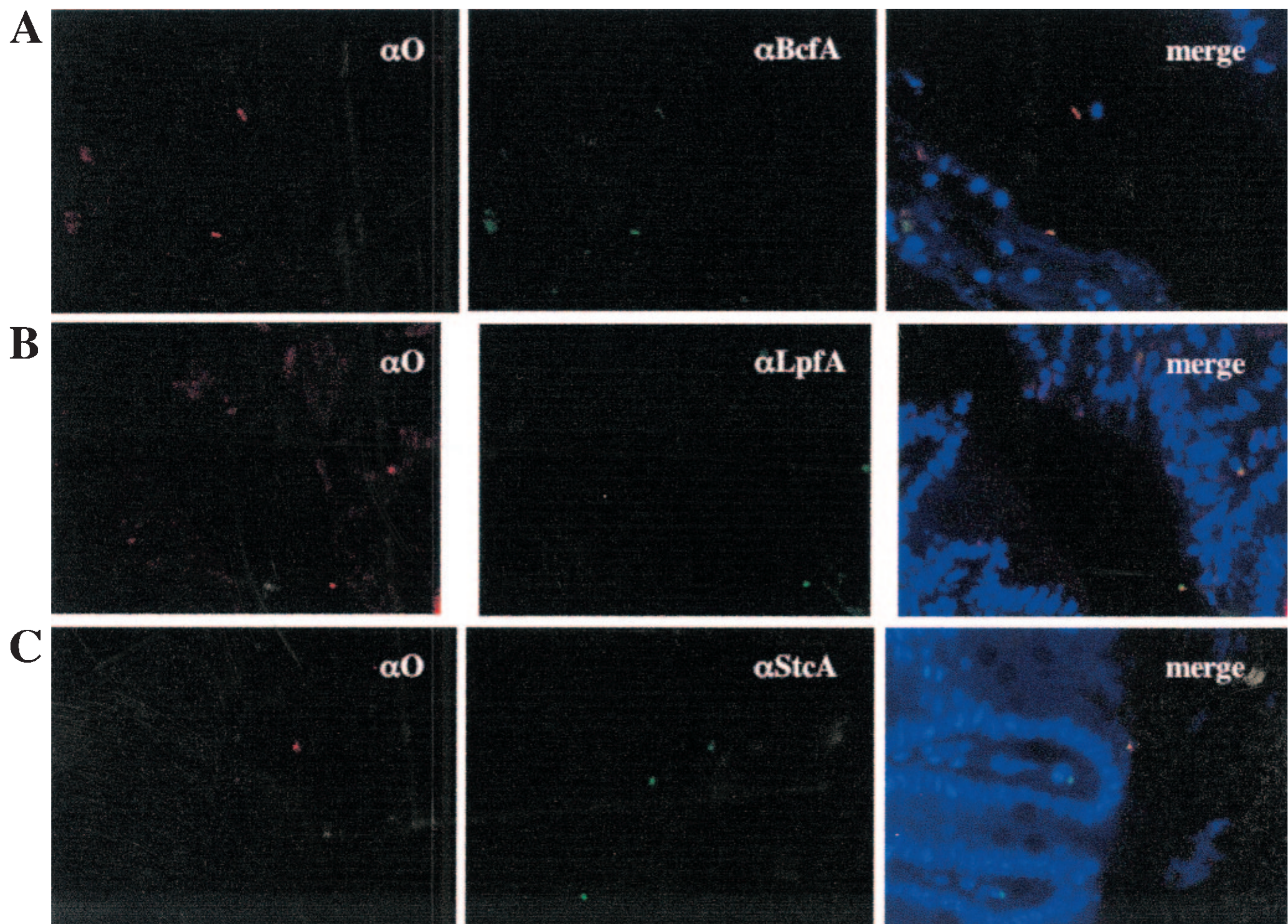

FIG. 4. Detection of fimbrial antigens and O antigen in the ceca of mice infected with serotype Typhimurium. Magnification, ×400. The left panels show the detection of the serotype Typhimurium O antigen by fluorescence microscopy (Alexa Fluor 594 [red fluorescence]). The middle panels show the binding of anti-BcfA serum (A), anti-LpfA serum (B), or anti-StcA serum (C) to the same sections (Alexa Fluor 488 [green fluorescence]). The right panels show overlays of red fluorescence (O antigen), green fluorescence (fimbrial proteins), and blue fluorescence (Hoechst nuclear stain).

Typhimurium cells grown on agar plates (10). N-terminal sequencing of the major subunit of thin curled fimbriae, termed AgfA (CsgA), identified this structure as being orthologous to the thin aggregative fimbriae of *S. enterica* serotype Enteritidis and the curly fimbriae of *Escherichia coli* (26). Although whole-genome sequencing revealed the presence of 13 putative fimbrial operons in serotype Typhimurium, type 1 fimbriae and thin curled fimbriae are currently the only two fimbrial structures that have been visualized on the surface of this pathogen by electron microscopy (15). We have recently shown by flow cytometry with antisera against 11 major fimbrial subunits that when serotype Typhimurium is cultured in LB broth, the only fimbrial antigen detectable on its surface is FimA (11). Here we extend this finding by showing that after growth under various in vitro growth conditions (including LB broth), the only fimbrial protein detected in serotype Typhimurium by Western blotting was FimA. A transcriptional fusion to the *pef* operon is induced when serotype Typhimurium is cultured in LB broth buffered to pH 5.1 (19). An analysis of serotype Typhimurium grown in LB broth buffered to pH 5.1 by flow cytometry using rabbit anti-PefA antiserum and an anti-rabbit–fluorescein isothiocyanate conjugate showed that only a small fraction of cells (approximately 3%) have fluorescence above background levels (11). This low level of PefA expression was not detectable by the Western blot performed for this study. Likewise, a flow cytometric analysis of serotype Typhimurium for the expression of 11 fimbrial proteins upon growth in CFA broth showed that a considerable fraction of cells (approximately 15%) express FimA, while only a small fraction of cells (<5%) show fluorescence above background levels when labeled with antisera specific for other fimbrial proteins (11). Here we show that FimA is the only fimbrial protein detectable by Western blotting in serotype Typhimurium grown in CFA broth. Collectively, these data suggest that with the exception of FimA and AgfA, fimbrial proteins are either not expressed or are expressed at very low levels (i.e., below the limit of detection of Western blotting) when serotype Typhimurium is cultured under standard laboratory growth conditions.

Although it is currently not known which regulatory mechanisms prevent the in vitro expression of the majority of the 13 fimbrial operons identified by whole-genome sequencing, previous studies provided support for the idea that the expression

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of the encoded antigens is induced in vivo. Seroconversion to LpfA is observed in mice upon infection with a serotype Typhimurium *aroA* vaccine strain (20), while chickens infected with *S. enterica* serotype Enteritidis seroconvert to PefA and FimA (1, 30). Furthermore, the expression of BcfA, StbA, SthA, LpfA, PefA, StdA, StcA, StiA, and StfA is detected by flow cytometry 8 h after the infection of bovine ligated ileal loops with serotype Typhimurium (11). Here we show that an oral infection of mice with serotype Typhimurium resulted in seroconversion to all 11 fimbrial proteins studied. Since the serotype Typhimurium culture grown in LB broth and used to inoculate mice expressed only FimA (as shown by Western blotting), seroconversion suggested that the expression of AgfA, BcfA, StbA, SthA, LpfA, PefA, StdA, StcA, StiA, and StfA was induced in vivo during the course of the infection. The detection of serotype Typhimurium expressing BcfA, StcA, and LpfA in the ceca of infected mice by fluorescence microscopy provided further support for the in vivo expression of fimbrial antigens (Fig. 4).

Serotype Typhimurium infection resulted in higher antibody titers against LPS than against individual fimbrial proteins. Each individual animal only developed a response against a subset of the 11 fimbrial proteins investigated. A possible explanation for this observation may be the regulation of fimbrial protein expression by phase variation. However, the phase-variable expression of fimbrial genes has only been investigated for the *fim*, *lpf*, and *pef* operons (19, 21, 27), and it is currently not clear whether other fimbrial operons also alternate between phase-on and phase-off expression states. Alternatively, the fact that different animals developed antibodies against different subsets of fimbrial proteins may be due to genetic differences between animals. In support of this idea, differences in the responses of individual animals were also observed when mice were vaccinated with a cocktail of fimbrial proteins (Fig. 2).

The immunization of mice with mixtures of GST-AgfA, GST-BcfA, GST-StbA, GST-SthA, GST-LpfA, GST-PefA, GST-StdA, GST-StcA, GST-StiA, and GST-StfA fusion proteins resulted in serum Ig responses that were increased at least fivefold compared to those in mice immunized with GST. The only exception was GST-FimA, which did not elicit a marked increase in serum Ig titer when injected into mice in conjunction with other fimbrial fusion proteins. FimA was the only protein whose expression could be detected by Western blotting in the serotype Typhimurium culture used to challenge mice immunized with fimbrial proteins. Thus, the fact that an immune response against AgfA, BcfA, StbA, SthA, LpfA, PefA, StdA, StcA, StiA, and StfA resulted in a significant reduction of bacterial numbers shed with the feces further corroborated the notion that these serotype Typhimurium antigens are expressed during infection of mice.

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REFERENCES

1. **Barbour, E. K., L. H. El Jurdi, O. M. Faroo, N. J. Daghir, and M. Bouljihad.** 2000. Chronological recognition by chicken of antigenic polypeptides in Salmonella enteritidis with different plasmid profiles: relationship to infection rate. J. Vet. Med. Sci. **62:**565–570.
2. **Bäumler, A. J., R. M. Tsolis, and F. Heffron.** 1996. Contribution of fimbrial operons to attachment to and invasion of epithelial cell lines by *Salmonella typhimurium*. Infect. Immun. **64:**1862–1865.
3. **Clegg, S., and G. F. Gerlach.** 1987. Enterobacterial fimbriae. J. Bacteriol. **169:**934–938.
4. **Collinson, S. K., L. Emody, K. H. Muller, T. J. Trust, and W. W. Kay.** 1991. Purification and characterization of thin, aggregative fimbriae from *Salmonella enteritidis*. J. Bacteriol. **173:**4773–4781.
5. **Dargatz, D. A., P. J. Fedorka-Cray, S. R. Ladely, and K. E. Ferris.** 2000. Survey of Salmonella serotypes shed in feces of beef cows and their antimicrobial susceptibility patterns. J. Food Prot. **63:**1648–1653.
6. **Duguid, J. P., and R. R. Gillies.** 1958. Fimbriae and haemagglutinating activity in *Salmonella*, *Klebsiella*, *Proteus*, and *Chromobacterium*. J. Pathol. Bacteriol. **75:**519–520.
7. **Fedorka-Cray, P. J., J. T. Gray, and C. Wray.** 2000. *Salmonella* infections in pigs, p. 191–207. *In* C. Wray and A. Wray (ed.), *Salmonella* in domestic animals. CABI Publishing, New York, N.Y.
8. **Grant, S. G. N., J. Jessee, F. R. Bloom, and D. Hanahan.** 1990. Differential plasmid rescue from transgenic mouse DNAs into *Escherichia coli* methylation-restriction mutants. Proc. Natl. Acad. Sci. USA **87:**4645–4649.
9. **Gruber, A., and B. Zingales.** 1995. Alternative method to remove antibacterial antibodies from antisera used for screening of expression libraries. BioTechniques **19:**28–30.
10. **Grund, S., and A. Weber.** 1988. A new type of fimbriae on Salmonella typhimurium. Zentbl. Veterinarmed. B **35:**779–782.
11. **Humphries, A. D., M. Raffatellu, S. Winter, E. H. Weening, R. A. Kingsley, R. Droleskey, S. Zhang, J. Figueiredo, S. Khare, J. Nunes, L. G. Adams, R. M. Tsolis, and A. J. Bäumler.** 2003. The use of flow cytometry to detect expression of subunits encoded by 11 *Salmonella enterica* serotype Typhimurium fimbrial operons. Mol. Microbiol. **48:**1357–1376.
12. **Humphries, A. D., S. M. Townsend, R. A. Kingsley, T. L. Nicholson, R. M. Tsolis, and A. J. Bäumler.** 2001. Role of fimbriae as antigens and intestinal colonization factors of *Salmonella* serovars. FEMS Microbiol. Lett. **201:**121–125.
13. **Hurd, H. S., J. K. Gailey, J. D. McKean, and M. H. Rostagno.** 2001. Rapid infection in market-weight swine following exposure to a Salmonella typhimurium-contaminated environment. Am. J. Vet. Res. **62:**1194–1197.
14. **Lockman, H. A., and R. Curtiss III.** 1992. Isolation and characterization of conditional adherent and non-type 1 fimbriated *Salmonella typhimurium* mutants. Mol. Microbiol. **6:**933–945.
15. **McClelland, M., K. E. Sanderson, J. Spieth, S. W. Clifton, P. Latreille, L. Courtney, S. Porwollik, J. Ali, M. Dante, F. Du, S. Hou, D. Layman, S. Leonard, C. Nguyen, K. Scott, A. Holmes, N. Grewal, E. Mulvaney, E. Ryan, H. Sun, L. Florea, W. Miller, T. Stoneking, M. Nhan, R. Waterston, and R. K. Wilson.** 2001. Complete genome sequence of Salmonella enterica serovar Typhimurium LT2. Nature **413:**852–856.
16. **McKean, J. D., H. S. Hurd, S. Larsen, M. Rostagno, R. Griffith, and I. Wesley.** 2001. Impact of commercial pre-harvest processes on the prevalence of Salmonella enterica in cull sows. Berl. Munch. Tierarztl. Wochenschr. **114:**353–355.
17. **McLaren, I. M., and C. Wray.** 1991. Epidemiology of Salmonella typhimurium infection in calves: persistence of salmonellae on calf units. Vet. Rec. **129:**461–462.
18. **Mead, P. S., L. Slutsker, V. Dietz, L. F. McCaig, J. S. Bresee, C. Shapiro, P. M. Griffin, and R. V. Tauxe.** 1999. Food-related illness and death in the United States. Emerg. Infect. Dis. **5:**607–625.
19. **Nicholson, B., and D. Low.** 2000. DNA methylation-dependent regulation of pef expression in Salmonella typhimurium. Mol. Microbiol. **35:**728–742.
20. **Nicholson, T. L., and A. J. Bäumler.** 2001. *Salmonella enterica* serotype Typhimurium elicits cross-immunity against a *Salmonella enterica* serotype Enteritidis strain expressing LP fimbriae from the *lac* promoter. Infect. Immun. **69:**204–212.
21. **Norris, T. L., R. A. Kingsley, and A. J. Bäumler.** 1998. Expression and transcriptional control of the *Salmonella typhimurium lpf* fimbrial operon by phase variation. Mol. Microbiol. **29:**311–320.
22. **Olsen, S. J., R. Bishop, F. W. Brenner, T. H. Roels, N. Bean, R. V. Tauxe, and L. Slutsker.** 2001. The changing epidemiology of salmonella: trends in serotypes isolated from humans in the United States, 1987–1997. J. Infect. Dis. **183:**753–761.
23. **Purcell, B. K., J. Pruckler, and S. Clegg.** 1987. Nucleotide sequence of the genes encoding type 1 fimbrial subunits of *Klebsiella pneumoniae* and *Salmonella typhimurium*. J. Bacteriol. **169:**5831–5834.
24. **Schlosser, W., A. Hogue, E. Ebel, B. Rose, R. Umholtz, K. Ferris, and W. James.** 2000. Analysis of Salmonella serotypes from selected carcasses and raw ground products sampled prior to implementation of the Pathogen

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Reduction, Hazard Analysis and Critical Control Point Final Rule in the US. Int. J. Food Microbiol. **58:**107–111.
25. **Schneider, H. A., and N. D. Zinder.** 1956. Nutrition of the host and natural resistance to infection. V. An improved assay employing genetic markers in the double strain inoculation test. J. Exp. Med. **103:**207–223.
26. **Stolpe, H., S. Grund, and W. Schroder.** 1994. Purification and partial characterization of type 3 fimbriae from Salmonella typhimurium var. Copenhagen. Zentbl. Bakteriol. **281:**8–15.
27. **Tinker, J. K., and S. Clegg.** 2001. Control of FimY translation and type 1 fimbrial production by the arginine tRNA encoded by fimU in Salmonella enterica serovar Typhimurium. Mol. Microbiol. **40:**757–768.
28. **Valentine, P. J., B. P. Devore, and F. Heffron.** 1998. Identification of three highly attenuated *Salmonella typhimurium* mutants that are more immunogenic and protective in mice than a prototypical *aroA* mutant. Infect. Immun. **66:**3378–3383.
29. **Wells, S. J., P. J. Fedorka-Cray, D. A. Dargatz, K. Ferris, and A. Green.** 2001. Fecal shedding of Salmonella spp. by dairy cows on farm and at cull cow markets. J. Food Prot. **64:**3–11.
30. **Woodward, M. J., E. Allen-Vercoe, and J. S. Redstone.** 1996. Distribution, gene sequence and expression in vivo of the plasmid encoded fimbrial antigen of *Salmonella* serotype Enteritidis. Epidemiol. Infect. **117:**17–28.
31. **Wray, C., J. N. Todd, and M. Hinton.** 1987. Epidemiology of Salmonella typhimurium infection in calves: excretion of S. typhimurium in the faeces of calves in different management systems. Vet. Rec. **121:**293–296.
32. **Wray, C., N. Todd, I. M. McLaren, and Y. E. Beedell.** 1991. The epidemiology of Salmonella in calves: the role of markets and vehicles. Epidemiol. Infect. **107:**521–525.

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