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Thin, Aggregative Fimbriae Mediate Binding of *Salmonella enteritidis* to Fibronectin

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The binding of human fibronectin and Congo red by an autoaggregative *Salmonella enteritidis* strain was found to be dependent on its ability to produce thin, aggregative fimbriae, named SEF 17 (for *Salmonella enteritidis* fimbriae with an apparent fimbrin molecular mass of 17 kDa). Two other fimbrial types produced by *S. enteritidis*, SEF 14 and SEF 21, were not responsible for the aggregative phenotype or for fibronectin binding. SEF 17-negative Tn*phoA* mutants which retained the ability to produce SEF 14 and SEF 21 were unable to bind human fibronectin or Congo red and lost the ability to autoaggregate. Only purified SEF 17 but not purified SEF 14 or SEF 21 bound fibronectin in a solid-phase binding assay. Furthermore, only SEF 17 was able to inhibit fibronectin binding to *S. enteritidis* whole cells in a direct competition enzyme-linked immunosorbent assay. These results indicate that SEF 17 are the fimbriae responsible for binding fibronectin by this enteropathogen.

One strategy utilized by bacterial pathogens to colonize host cell surfaces involves bacterial adhesive appendages called fimbriae which bind glycoprotein or glycolipid receptors on epithelial cells (17, 18, 35). Fimbriae are primarily composed of polymerized fimbrin protein monomers and in some cases have been shown to bind eukaryotic cell surfaces directly or via fimbria-associated adhesin proteins (2, 18, 35). Considerable interest has also focused on the ability of many bacterial pathogens to bind glycoproteins of the extracellular matrix, including fibronectin, laminin, and various collagens, since these bacterium-host interactions are potentially relevant in pathogenesis (18). Although diverse bacterial surface components such as LamB maltoporin of *Escherichia coli* (37), surface layer (S-layer) of the fish pathogen *Aeromonas salmonicida* (10), YOP1 outer membrane protein of *Yersinia* spp. (12), and lipoteichoic acid of *Streptococcus* spp. (8) have been identified as receptors for certain tissue matrix glycoproteins, fimbriae figure prominently in their ability to bind fibronectin (20, 26) as well as type IV or type V collagen (34, 38).

Fimbriae of nontyphoid *Salmonella* spp. are apparently adhesive organelles possibly required for adherence and colonization of intestinal epithelial cells by these enteropathogens in the initial stages of gastroenteritis. Among the various fimbriae produced by *Salmonella* spp. (1, 7, 9, 11, 15, 24, 36), only the role of type 1 fimbriae of *Salmonella typhimurium* in pathogenesis has been extensively explored. Although type 1 fimbriated bacteria have been shown to bind eukaryotic cell lines, freshly isolated enterocytes, and intestinal epithelial tissue, the role of these fimbriae in pathogenesis remains controversial (13, 22, 27). The role of other fimbriae in salmonellosis is unexplored except for recent evidence which implicates type 3 fimbriae of *S. typhimurium* and *Salmonella enteritidis* in binding of these strains to type V collagen (34). In addition, SEF 14 fimbriae of *S. enteritidis* 3b have been proposed as fibronectin receptors (3). However, the recent discovery that *S. enteritidis* 3b also produces SEF 17 and SEF 21 (type 1 fimbriae) in addition to SEF 14 (7, 24) raised the possibility that other fimbriae of this strain may be involved in interactions with basement membrane proteins.

This article reports the characterization of Tn*phoA* mutants unable to produce SEF 17 and the discovery that these thin, aggregative fimbriae mediate the binding of *S. enteritidis* to fibronectin and are responsible for a distinctive aggregative (rough) colonial morphology, autoaggregation, and the ability to bind Congo red.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Table 1 lists the various bacterial strains used in this study. *S. enteritidis* was routinely grown at 20 or 37°C on T medium (7), Trypticase soy agar (TSA) (Becton Dickinson Co., Cockeysville, Md.), Luria agar (LB) (29), or colonization factor agar (CFA) (14) containing 12 mM Na_2HPO_4 and 5 mM KH_2PO_4. Where noted, Congo red (Sigma Chemical Co., St. Louis, Mo.) was added to the medium at a concentration of 100 μg/ml.

Tn*phoA* mutagenesis. Tn*phoA* mutants of *S. enteritidis* 3b were generated by mating the *S. enteritidis* transductant 3b *hisG*::Tn*10* or a spontaneous rifampin-resistant 3b strain with *E. coli* SM10 carrying plasmid pRT733 (23). Tn*phoA* mutants of *S. enteritidis* were selected on solid CFA medium containing 5-bromo-4-chloro-3-indolyl phosphate (50 μg/ml) and kanamycin (50 μg/ml) in addition to tetracycline (20 μg/ml) or rifampin (100 μg/ml) as required. Colonies expressing alkaline phosphatase were isolated, grown in static liquid CFA, and used to prepare glycerol stock cultures.

P22 transductions. Phage stocks prepared after growth of transducing phage P22 *int3* HT 12/4 on various Tn*phoA* mutants were used to infect 3b cells at multiplicities of infection ranging from 0.01 to 5. Transductants were selected on LB plates containing kanamycin (30 μg/ml) and were rendered phage free by passage on Green plates (6). Transductants were screened for rifampin sensitivity, Congo

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TABLE 1. Bacterial strains[a]

Strain	Phenotype	Source or reference
3b	*S. enteritidis* 27655 wild type	T. Wadström
3b-122	Tn*10* insertion mutant of 3b	15
3b-122-34	Tn*10* excision mutant of 122	15
3b Rif^r	Spontaneous Rif^r mutant of 3b	This study
Tn*phoA* 1-9	Tn*phoA* mutant of 3b, Rif^r	This study
Tn*phoA* 2-2	Tn*phoA* mutant of 3b, Rif^r	This study
Tn*phoA* 2-7	Tn*phoA* mutant of 3b, Rif^r	This study
Tn*phoA* 3-1	Tn*phoA* mutant of 3b, Rif^r	This study
Tn*phoA* 2-2a	Tn*phoA* transduced from 2-2 to 3b, Rif^s	This study
Tn*phoA* 2-2f	Tn*phoA* transduced from 2-2 to 3b, Rif^s	This study
Tn*phoA* 2-7f	Tn*phoA* transduced from 2-7 to 3b, Rif^s	This study
Tn*phoA* 3-1c	Tn*phoA* transduced from 3-1 to 3b, Rif^s	This study
3b-*hisG*::Tn*10*	*hisG*::Tn*10* transduced from *S. typhimurium* MA766 to 3b	This study
Tn*phoA* 3	Tn*phoA* mutant of 3b, *hisG*::Tn*10*	This study
Tn*phoA* 7	Tn*phoA* mutant of 3b, *hisG*::Tn*10*	This study
Tn*phoA* 9	Tn*phoA* mutant of 3b, *hisG*::Tn*10*	This study
E. coli SM10 pRT733		23

[a] Rif^r, rifampin resistant; Rif^s, rifampin sensitive.

red binding, and the production of the fimbriae SEF 14, SEF 17, and SEF 21 by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting (immunoblotting).

Electrophoresis. SDS-PAGE was performed by the method of Laemmli (21). Purified, aggregative SEF 17 fimbriae were pretreated with 90% formic acid prior to electrophoresis as previously described (7). Lipopolysaccharide (LPS) was analyzed by SDS-PAGE following digestion of bacterial cells suspended in SDS-PAGE sample buffer with 0.5 mg of proteinase K (Boehringer Mannheim, Laval, Canada) per ml.

Western blot analysis. Whole-cell lysates of *S. enteritidis* were routinely screened by Western blot analysis for the presence of the three fimbrial types. SEF 14 and SEF 21 fimbriae were solubilized from whole cells of *S. enteritidis* by digestion in SDS-PAGE sample buffer supplemented with 0.2 M glycine, pH 2 (100°C, 10 min). This cell extract was clarified by centrifugation (14,300 × *g*, 5 min, 25°C) and used directly for SDS-PAGE analysis. The insoluble, glycine-extracted cell material containing SEF 17 was washed twice with distilled water and then briefly exposed to 90% formic acid and lyophilized. The lyophilized, formic acid-digested material was resuspended in SDS-PAGE sample buffer just prior to electrophoresis. Samples separated by SDS-PAGE were electrophoretically transferred to nitrocellulose and screened with rabbit immune serum to SEF 14, SEF 21, and SEF 17 prepared as previously described (7, 24). Visualization of immunoreactive proteins on Western blots was accomplished by the use of goat anti-rabbit immunoglobulin G (IgG)–alkaline phosphatase conjugates (Caltag Laboratories, San Francisco, Calif.); proteins were detected by dye precipitation using the substrate 5-bromo-4-chloro-3-indolyl phosphate with nitroblue tetrazolium as an enhancer (7). Alternatively, proteins that were immunoreactive with the first antibody were incubated with biotinylated goat anti-rabbit IgG followed by streptavidin-horseradish peroxidase conjugate (Caltag Laboratories) and detected by chemiluminescence with a reagent composed of luminol, 4-iodophenol, and H_2O_2. Rabbit immune serum to bacterial alkaline phosphatase was kindly provided by F. Nano of the Department of Biochemistry and Microbiology at the University of Victoria.

DNA amplification. DNA destined for polymerase chain reaction (PCR) analysis was obtained from approximately 20 mg (wet weight) of *S. enteritidis* cells boiled in 1.0 ml of distilled H_2O for 5 min. The DNA-containing supernatant was cleared of cell debris by centrifugation (14,300 × *g*, 5 min, 25°C). The custom primers used to amplify the DNA were obtained from the Regional DNA Synthesis Laboratory (University of Calgary). One primer was a mixed biased 17-bp oligomer (GT[T/A/C]GT[T/A/C]CC[T/A/C]CA[A/G]T GGGG) designed from the N-terminal AgfA sequence (7) (see Fig. 3B). The PCR primer for the opposite strand was an 18-bp oligomer (AAAACGGGAAAGGTTCCG) derived from the IS*50*$_L$ junction fragment in Tn*phoA*-generated alkaline phosphatase gene fusions (23) (see Fig. 3B). PCR amplification was performed with 10-μl samples sealed in glass capillary tubes by using an air-driven thermocycler (Idaho Technology, Boise, Idaho) for 30 cycles of denaturation (95°C, 5 s), annealing (49°C, 5 s), and elongation (74°C, 1 min). The amplified DNA products were separated by electrophoresis on 5% polyacrylamide gels in Tris-acetate-EDTA buffer, and the DNA products were visualized by UV illumination following ethidium bromide staining of the gel (30).

Tissue matrix protein binding assays. Bacteria were harvested and resuspended in buffer to an optical density at 540 nm of 1.0. An aliquot of cells (100 μl) was placed in a tube to which approximately 20,000 cpm of ^{125}I-labelled tissue matrix protein (collagen I, II, or IV; laminin; vitronectin; or fibronectin) was added. After incubation, the cells were removed by centrifugation and the supernatant was transferred to a fresh tube. The cell pellet was washed twice with 1.0 ml of phosphate-buffered saline, and the pellet-associated ^{125}I-labelled tissue matrix protein was quantified. The level of binding was determined by calculating the amount of cell-associated ^{125}I as a percentage of the total ^{125}I-labelled protein added to the vial.

Solid-phase binding assay. Bacteria grown on T medium at 37°C for 24 h were harvested into Tris-buffered saline (TBS) (10 mM Tris, 0.9% NaCl, pH 7.5) and then gently homogenized in a Teflon-glass tissue grinder to obtain a uniform suspension. The cell suspension was adjusted to an optical density at 650 nm of 0.4. Purified SEF 17 fimbriae were resuspended in TBS at a concentration of 10 μg/ml. A 100-μl

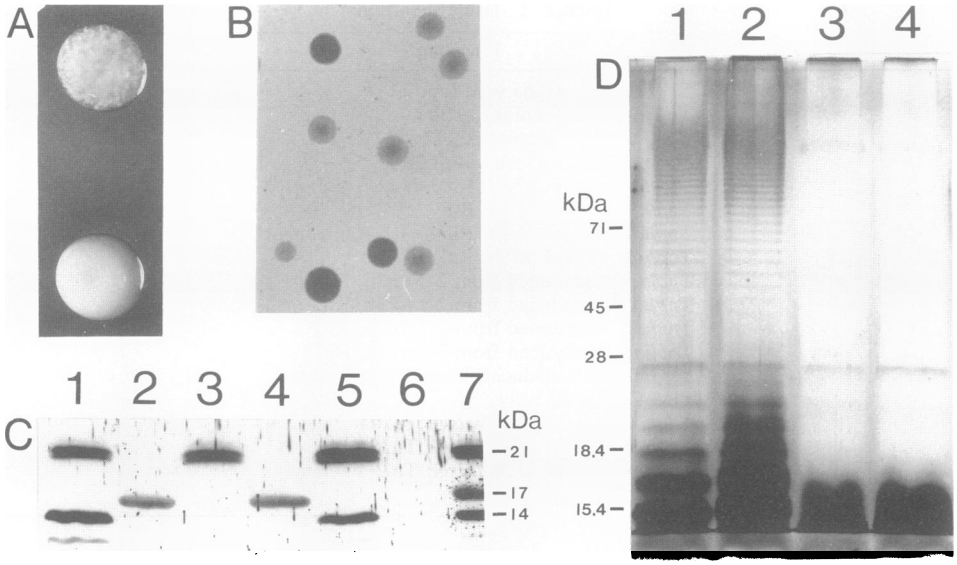

FIG. 1. Phenotypic characterization of *S. enteritidis* 3b (Agf$^+$) and SEF 17-deficient Tn*phoA* (Agf$^-$) mutants. (A) *S. enteritidis* resuspended from solid T medium in a drop of 10 mM Tris-HCl, pH 8.0. Top, *S. enteritidis* 3b (Agf$^+$); bottom, Tn*phoA* 2-2a (Agf$^-$). (B) *S. enteritidis* colonies grown on Congo red medium. *S. enteritidis* 3b (Agf$^+$) grows as dark (red) colonies, and Tn*phoA* 2-2a (Agf$^-$) grows as light (pink) colonies. (C) Western blot of SDS-PAGE sample buffer-glycine extracts of whole cells (lanes 1, 3, and 5) or of SDS-PAGE sample buffer-glycine-insoluble material from whole cells treated with formic acid (lanes 2, 4, and 6). An anti-SEF 14, anti-SEF 21, and anti-SEF 17 immune serum mixture was used as the first antibody followed by goat anti-rabbit IgG–alkaline phosphatase conjugate as described in Materials and Methods. Lanes: 1 and 2, *S. enteritidis* 3b (Agf$^+$ Sef$^+$ Fim$^+$); 3 and 4, 3b-122 (Agf$^+$ Sef$^-$ Fim$^+$); 5 and 6, Tn*phoA* 2-2a (Agf$^-$ Sef$^+$ Fim$^+$); 7, a mixture of purified SEF 14, SEF 17, and SEF 21. (D) SDS-PAGE of proteinase K-digested *S. enteritidis* stained with silver to visualize LPS. Lanes: 1, *S. enteritidis* 3b (Agf$^+$); 2, Tn*phoA* 2-2a (Agf$^-$); 3, Tn*phoA* 7 (Agf$^-$); 4, Tn*phoA* 9 (Agf$^+$).

volume of resuspended cells, purified fimbriae, or TBS alone (negative control) was added to wells of a 96-well enzyme-linked immunosorbent assay (ELISA) plate (Costar, Cambridge, Mass.). After an overnight incubation at 4°C, the wells were rinsed with 250 μl of TBS supplemented with 0.2% (wt/vol) bovine serum albumin (TBS-BSA) and then blocked for 2 h at room temperature with 200 μl of TBS containing 2% BSA. After the blocking step, the wells were washed with 250 μl of TBS-BSA three times. Each well then received 100 μl of human fibronectin (Sigma Chemical Co.). The concentration of fibronectin was varied from 0 to 100 μg/ml in order to generate binding isotherms. After incubation of the plate at room temperature for 2 h, the wells were washed five times with TBS-BSA. Then 100 μl of rabbit anti-fibronectin IgG (E-Y Laboratories, San Mateo, Calif.) diluted to 1 in 2,000 with TBS-BSA was added to every well. The plates were incubated for an additional 2 h at room temperature and washed five times. Then 100 μl of goat anti-rabbit IgG conjugated to horseradish peroxidase (Bio-Rad, Mississauga, Ontario, Canada) diluted 1,000-fold in TBS-BSA was added to each well. After 2 h, the wells were washed five times and 100 μl of a substrate solution consisting of 10 mM citric acid, pH 4.2; 0.03% H_2O_2; and 1 mM 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) was added to each well. The A_{405} of each well was determined after 30 min of incubation at room temperature. Values were corrected for nonspecific binding of fibronectin to the wells, which accounted for less than 10% of the test value.

Fimbria-mediated inhibition of fibronectin binding to cells. To test the abilities of purified SEF 14, SEF 17, and SEF 21 to inhibit the binding of fibronectin to *S. enteritidis* cells, a direct competition assay between these three purified fimbrial preparations and fibronectin was used. An equal volume of fibronectin was added to an equal volume of various concentrations of a given fimbrial preparation for a final fibronectin concentration of 100 μg/ml. These fibronectin-fimbria solutions were then used in place of fibronectin in the solid-phase binding assay. Fibronectin bound to whole cells on ELISA plates was then assayed as described above.

RESULTS

Description of Tn*phoA* mutants unable to produce SEF 17. *S. enteritidis* strains defective in the production of exported proteins were generated by Tn*phoA* mutagenesis (23). Routine screening of several of the Tn*phoA* mutants revealed several isolates whose colonial morphologies deviated significantly from that of wild-type *S. enteritidis* 3b. Typically, *S. enteritidis* 3b grown on T medium forms aggregative, rough colonies which adhere to the agar and maintain their shape even when dislodged and pushed along the agar surface. The bacteria from such colonies are extremely autoaggregative and difficult to resuspend in liquid. Conversely, several of the mutants lacked this aggregative colonial morphology, forming instead mucoid colonies which resuspended readily in liquid (Fig. 1A and Table 2). Wild-type *S. enteritidis* 3b colonies grown on T medium containing Congo red bound this hydrophobic dye, whereas the nonaggregative mutants did not (Fig. 1B). *S. enteritidis* 3b grown in static CFA broth for 48 to 72 h normally produced a surface pellicle, whereas the 25 nonaggregative mutants tested did not (Table 2).

Electron microscopic observation of negatively stained, nonaggregative mutants of *S. enteritidis* isolated from T medium indicated that they did not produce characteristic SEF 17 fimbriae associated normally with wild-type 3b cells (data not shown). Moreover, Western blot analysis confirmed that these mutants did not produce AgfA, the fimbrin

TABLE 2. Representative phenotypes of Tn*phoA* mutants of *S. enteritidis* 3b

Strain	Morphology[a]	CR binding[b]	Pellicle[c]	Aggregation[d]	Fimbrin produced[e]			LPS[f]
					SEF 14 (SefA)	SEF 17 (AgfA)	SEF 21 (FimA)	
3b	Ag	+	+	+	+	+	+	+
3b-122	Ag	+	+	+	−	+	+	+
3b-122-34	Ag	+	+	+	−	+	+	+
3b Rif^r	Ag	+	+	+	+	+	+	+
Tn*phoA* 1-9	Ag	+	+	+	+	+	+	+
Tn*phoA* 2-2	NAg	−	−	−	+	−	+	+
Tn*phoA* 2-7	NAg	−	−	−	+	−	+	+
Tn*phoA* 3-1	NAg	−	−	−	+	−	+	+
Tn*phoA* 2-2a	NAg	−	−	−	+	−	+	+
Tn*phoA* 2-2f	Ag	+	+	+	+	+	+	+
Tn*phoA* 2-7f	NAg	−	−	−	+	−	+	+
Tn*phoA* 3-1c	NAg	−	−	−	+	−	+	+
3b-*hisG*::Tn*10*	Ag	+	+	+	+	+	+	+/−[g]
Tn*phoA* 3	NAg	−	−	−	+	−	+	−
Tn*phoA* 7	NAg	−	−	−	+	−	+	−
Tn*phoA* 9	Ag	+	+	+	+	+	+	−

[a] Colonial morphology of colonies on T medium. Ag, aggregative (rough); NAg, nonaggregative (mucoid).
[b] Congo red (CR) binding by colonies grown on T medium. +, red colony; −, pale pink colony.
[c] Pellicle production after 48 to 72 h of growth on liquid static medium. +, solid pellicle; −, no pellicle.
[d] Observed aggregation of cells resuspended from T medium into buffer. +, cells remain in clumps and settle quickly; −, cells do not clump or settle quickly.
[e] The presence of the fimbrin subunits SefA, FimA, and AgfA from SEF 14, SEF 21, and SEF 17, respectively, were determined by Western blot analysis of whole cells as described in Materials and Methods.
[f] The presence of LPS O chain was determined by SDS-PAGE analysis of proteinase K-digested whole cells as described in Materials and Methods.
[g] LPS O-chain-positive and LPS O-chain-negative transductants present in culture.

subunits (or unprocessed prefimbrin) to SEF 17 (Fig. 1C and Table 2), although they still produced SefA and FimA fimbrins of SEF 14 and SEF 21, respectively, when grown in CFA broth (Fig. 1C and Table 2) or CFA agar. Like wild-type *S. enteritidis* 3b, these SEF 17-deficient mutants produced little or no SEF 14 and SEF 21 when grown on solid TSA or T medium. These results implicated SEF 17 as being responsible for the aggregative nature of *S. enteritidis* 3b. Alterations in O-chain LPS did not cause the aggregative phenotype since mutants which lacked normal amounts of O-chain LPS retained the distinctive aggregative colonial morphology unless they also lacked the ability to produce SEF 17 (Table 2). Of 60 independent Tn*phoA* mutant strains surveyed, only those strains which lacked the ability to produce SEF 17 also exhibited the mucoid morphology, a nonaggregative phenotype, and an inability to bind Congo red (Table 2).

Characterization of Tn*phoA* mutants. The *S. enteritidis* Tn*phoA* mutants that were unable to produce the AgfA fimbrin of SEF 17 were further analyzed to determine whether any of the AgfA$^-$ phenotypes arose from insertional inactivation of *agfA*. Western blot analysis indicated that the Tn*phoA* mutants analyzed comprised three groups. Two mutants, Tn*phoA* 2-2a and Tn*phoA* 2-7f, carry Tn*phoA* within *agfA* since both produced fusion proteins, of approximately 55 to 70 kDa, which reacted with immune sera raised to either bacterial alkaline phosphatase or SEF 17 (Fig. 2). The second group included Tn*phoA* 3-1c and Tn*phoA* 7, which produced fusion proteins reactive only with immune serum to bacterial alkaline phosphatase, suggesting that if Tn*phoA* was inserted in *agfA*, the fusion protein did not carry an epitope recognized by the immune serum to SEF 17. Alternatively, the transposon may have been inserted in an unidentified gene required for SEF 17 fimbriation (Fig. 2). Finally, Tn*phoA* 3 apparently did not produce an alkaline phosphatase fusion protein (Fig. 2).

To confirm that Tn*phoA* 2-2a and Tn*phoA* 2-7f were *agfA* insertional mutants, PCR amplification of the *agfA* gene sequence postulated to reside upstream of Tn*phoA* in both mutants was attempted with a biased mixed probe designed from the N-terminal amino acid sequence of AgfA (7) and a primer derived from the IS*50*$_L$ sequence of Tn*phoA* located at the junction of Tn*phoA*-generated alkaline phosphatase gene fusions (23) (Fig. 3B). As predicted, PCR amplification of Tn*phoA* mutant DNA resulted in the production of major DNA fragments of approximately 230 and 390 bp for Tn*phoA* 2-2a and Tn*phoA* 2-7f, respectively (Fig. 3A, lanes 4 and 5). In addition, PCR amplification of DNA from Tn*phoA* 3 DNA resulted in the amplification of a DNA fragment slightly smaller than that for Tn*phoA* 2-2a (Fig. 3A, lane 7). These data suggest that Tn*phoA* 3 also harbors Tn*phoA* within *agfA* but that the fusion is out of frame, resulting in a lack of

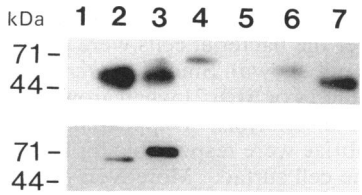

FIG. 2. Western blot analysis of alkaline phosphatase fusion proteins produced by *S. enteritidis* Tn*phoA* mutants. SDS-PAGE sample buffer-glycine-extracted *S. enteritidis* cells subjected to Western blot analysis were incubated with immune serum to alkaline phosphatase (upper panel) or to immune serum to SEF 17 (lower panel). The Western blots were incubated with goat anti-rabbit IgG-biotin followed by streptavidin-horseradish peroxidase and developed with chemiluminescent reagents as described in Materials and Methods. Lanes: 1, *S. enteritidis* 3b; 2, Tn*phoA* 2-2a; 3, Tn*phoA* 2-7f; 4, Tn*phoA* 3-1c; 5, Tn*phoA* 3; 6, Tn*phoA* 7; 7, *E. coli* alkaline phosphatase. Molecular masses of prestained protein standards (Bethesda Research Laboratories, Gaithersburg, Md.) are noted on the left.

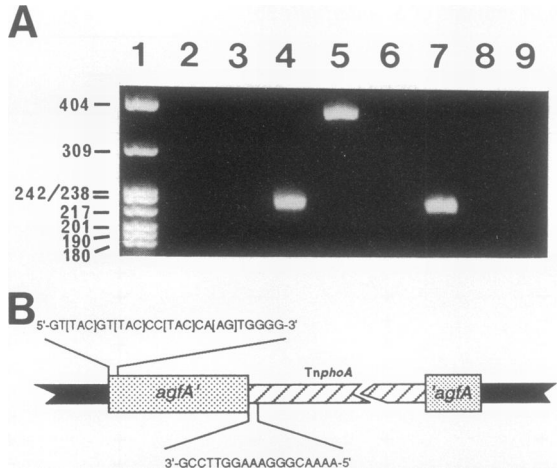

FIG. 3. (A) PCR amplification of DNA fragments from *S. enteritidis* 3b Tn*phoA* mutants harboring Tn*phoA* within *agfA*. Lanes: 1, DNA size markers generated by *Msp*I-digested pBR322; 2, no cellular DNA (PCR control); 3, *S. enteritidis* 3b; 4, Tn*phoA* 2-2a; 5, Tn*phoA* 2-7f; 6, Tn*phoA* 3-1c; 7, Tn*phoA* 3; 8, Tn*phoA* 7; 9, Tn*phoA* 2-2f. The sequences of the two PCR primers used are indicated in panel B. (B) Schematic representation of the region of *S. enteritidis* 3b Tn*phoA* 2-7f DNA containing the fusion of the *agfA* gene with Tn*phoA*. The *agfA* gene (approximately 0.48 kb, stippled box) which encodes the fimbrin for SEF 17 is interrupted by Tn*phoA* (7.65 kb, hatched box). The primer sequences which were used to generate the PCR products are indicated.

detectable anti-alkaline phosphatase immunoreactive bands (Fig. 2). The other mutants, Tn*phoA* 3-1c and Tn*phoA* 7, did not possess amplifiable DNA fragments under the conditions used (Fig. 3A). Further characterization of these two mutants is in progress to determine the location of the Tn*phoA* insertion.

The DNA sequence of the cloned 390-bp PCR fragment amplified from Tn*phoA* 2-7f was determined (sequence to be published elsewhere) and confirmed that the AgfA$^-$ phenotype of Tn*phoA* 2-7f resulted from insertional inactivation of *agfA* (Fig. 3B) since the first 12 codons of this DNA fragment were found to encode amino acids whose sequence corresponded to N-terminal amino acid residues 2 to 13 of AgfA (7).

Affinity of various tissue matrix proteins for *S. enteritidis*. ^{125}I-labelled laminin and type I, II, and IV collagens, but not vitronectin, bound to whole *S. enteritidis* cells to the same extent whether the bacterial cells were grown on TSA at 20 or 37°C (data not shown). Since *S. enteritidis* produces SEF 17 but little SEF 14 or SEF 21 when grown on TSA as judged by Western blot analysis, it appeared unlikely that these latter two fimbriae were responsible for binding these matrix proteins to the cell surface. Moreover, *S. enteritidis* cannot produce SEF 14 when grown below 30°C (24, 36), thereby ruling out the notion that these appendages might serve as the sole fibronectin receptor (3). This result was confirmed with a Tn*10* insertional mutant of *S. enteritidis* 3b, strain 3b-122 (15) (data not shown), which produced SEF 17 and SEF 21 but not SEF 14 (Fig. 1C).

Binding of fibronectin by *S. enteritidis* SEF 17. *S. enteritidis* 3b and several derivative strains were grown on T medium to favor SEF 17 production and were examined for the ability to bind fibronectin. Parental *S. enteritidis* 3b strains that produced SEF 17 also bound fibronectin, whereas SEF 17-negative Tn*phoA* mutants did not bind fibronectin (Table 3). When highly purified SEF 14, SEF 17, and SEF 21 were tested for the ability to bind fibronectin, only SEF 17 bound this tissue matrix protein (Table 3). In addition, purified SEF 17, but not purified SEF 14 or SEF 21, was able to inhibit the binding of fibronectin to whole *S. enteritidis* cells in a direct competition ELISA (Fig. 4).

TABLE 3. Constants for fibronectin binding to Tn*phoA* mutants of *S. enteritidis* and purified fimbriae

Strain or purified fimbria	Fimbriae produced			Fibronectin binding constant[a]
	SEF 14	SEF 17	SEF 21	
Strain				
3b	+	+	+	2.1 ± 0.5
3b-*hisG*::Tn*10*	+	+	+	2.8 ± 0.3
Tn*phoA* 7	+	−	+	ND
3b-122	−	+	+	2.9 ± 0.8
Tn*phoA* 2-2a	+	−	+	ND
Tn*phoA* 2-2f	+	+	+	3.3 ± 0.6
Tn*phoA* 2-7f	+	−	+	ND
Tn*phoA* 3-1c	+	−	+	ND
Fimbriae				
SEF 17				7.4 ± 0.5
SEF 14				ND
SEF 21				ND

[a] Values reported are (K_d × 10^{-5} M) ± (SD × 10^{-5} M), where SD is standard deviation. ND, binding not detected.

DISCUSSION

The results of this study clearly establish a role for SEF 17 fimbriae of *S. enteritidis* as fibronectin receptors. This confirms previous studies which established the ability of *S. enteritidis* 3b to bind fibronectin but negates the suggestion that SEF 14 fimbriae mediate fibronectin binding by *S. enteritidis* 3b (3). The SEF 14-deficient mutant, *S. enteritidis* 3b-122, bound fibronectin as efficiently as wild-type 3b, and purified SEF 14 failed to bind fibronectin or to inhibit fibronectin binding by *S. enteritidis* 3b. Moreover, Baloda (3) found that *S. enteritidis* 3b bound fibronectin after growth

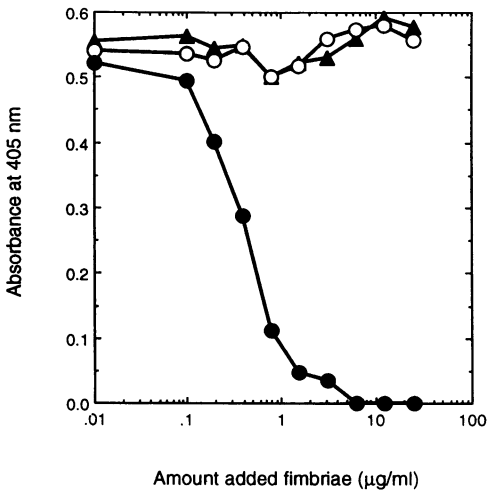

FIG. 4. Inhibition of fibronectin binding to *S. enteritidis* 3b cells by purified fimbriae. A direct competition assay was performed as described in Materials and Methods to test the abilities of highly purified SEF 14 (○), SEF 21 (▲), and SEF 17 (●) to inhibit fibronectin binding to *S. enteritidis* 3b cells.

at 37, 33, and 20°C, but this last temperature is nonpermissive for the expression of SEF 14 (24, 36), suggesting that some other cell surface component was likely responsible for the binding of fibronectin by this strain. The fibronectin binding constant for purified SEF 17 (74 μM) is of the same order of magnitude as that determined for whole cells expressing SEF 17 (21 to 33 μM). This supports the contention that the observed binding of fibronectin by *S. enteritidis* in this study is mediated by SEF 17. Biphasic binding kinetics were not observed, indicating that *S. enteritidis* 3b grown under the conditions used in this study (solid T medium at 37°C) likely possessed only a single class of fibronectin binding molecules. The binding affinity, in the micromolar range, of SEF 17 for fibronectin indicates a lower affinity than found for other bacteria, normally determined to be in the nanomolar range (10, 16).

In addition to binding fibronectin, whole *S. enteritidis* cells were observed to bind laminin and type I, II, and IV collagens. Apart from binding type II collagen (3), *S. enteritidis* has not previously been shown to bind these basement membrane components. *S. enteritidis* and *S. typhimurium* also bind collagen V via type 3 fimbriae (34). Thus, it appears that the fimbrial adhesins of *S. enteritidis* confer the ability to bind to a variety of basement membrane fibrillar proteins.

SEF 17 fimbriae, like the related aggregative GVVPQ fimbriae of *E. coli* (7a), promote the binding of the hydrophobic dye Congo red by bacteria that produce the fimbriae. Thus, Congo red binding provides a simple and rapid test for screening *Salmonella* and *E. coli* strains which harbor SEF 17 or related fimbriae. In addition, this phenotype may be indicative of virulence since Congo red binding by other important human and animal pathogens, including *Yersinia*, *Escherichia*, *Shigella*, *Vibrio*, *Neisseria*, and *Aeromonas* species, has been correlated with virulence (4, 19, 28, 32), although the virulence mechanism revealed by this test is as yet unknown.

SEF 17 fimbriae clearly account for the extreme autoaggregative nature of *S. enteritidis* 3b. Autoaggregation of *Salmonella* species and rough colonial morphology have often been attributed to alterations in LPS, but SEF 17 is also responsible for this phenotype, even in the absence of LPS O-antigen polysaccharide. The autoaggregation and distinctive colonial morphology of *S. enteritidis* 3b are not due to LPS modification or to the expression of SEF 14 or SEF 21. SEF 17-deficient Tn*phoA* mutants which retain the ability to produce SEF 14 and SEF 21 did not aggregate or produce heavy pellicles under the static culture conditions used. Moreover, SEF 14-deficient *S. enteritidis* 3b-122 was still autoaggregative. Type 1 fimbriae of *Salmonella* spp. and other enteric bacteria also promote pellicle production when cells are grown in static broth (25, 33), but our data indicate that these type 1 fimbriae are not the only appendages which promote pellicle production. In addition, repeated subculturing of bacterial pathogens often results in the loss of fimbria production and the associated aggregative phenotype (5). This phenomenon may also account for the fact that many *S. enteritidis* strains appear to be SEF 17 deficient upon initial screening (6a).

The results of this study show that SEF 17 fimbriae are multifunctional organelles which can be envisioned to enhance the survival of *S. enteritidis* in the intestinal milieu and facilitate the initial association of these enteropathogens with the intestinal epithelium. SEF 17-mediated aggregation may be important if autoaggregation enhances the survival of the bacteria as they encounter stomach acids, surfactants, and other biocides present in the digestive tract (31). Moreover, clumps of bacteria may ensure a sufficient and viable inoculum capable of evading phagocytosis or other host defenses. It has been hypothesized that bacterial interactions with proteins of the basement membrane may provide *S. enteritidis* and other enteropathogens with a mechanism for colonizing the intestinal epithelium, especially if the tissue has been damaged to expose basement membrane components (16, 22). SEF 17 fimbriae may play such a role by using exposed fibronectin for securing bacterial attachment. The ability of *S. enteritidis* to bind several basement membrane proteins suggests the presence of additional attachment mechanisms which may also contribute to the colonization of intestinal epithelium by this enteropathogen.

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REFERENCES

1. **Aleksic, S., and V. Aleksic.** 1979. Purification and physicochemical analysis of the fimbrial antigen in two different genera of enterobacteriaceae: *Salmonella enteritidis* and *Yersinia enterocolitica*. Zentralbl. Bakteriol. Mikrobiol. Hyg. I Abt. Orig. A **243**:177–196.
2. **Arp, L. H.** 1988. Bacterial infection of mucosal surfaces: an overview of cellular and molecular mechanisms, p. 3–27. *In* J. A. Roth (ed.), Virulence mechanisms of bacterial pathogens. American Society for Microbiology, Washington, D.C.
3. **Baloda, S. B.** 1988. Characterization of fibronectin binding to *Salmonella enteritidis* strain 27655R. FEMS Microbiol. Lett. **49**:483–488.
4. **Bhaduri, S., C. Turner-Jones, and R. V. Lachica.** 1991. Convenient agarose medium for simultaneous determination of the low-calcium response and Congo red binding by virulent strains of *Yersinia enterocolitica*. J. Clin. Microbiol. **29**:2341–2344.
5. **Blake, M. S., C. M. MacDonald, and K. P. Klugman.** 1989. Colony morphology of piliated *Neisseria meningitidis*. J. Exp. Med. **170**:1727–1736.
6. **Chan, R. K., D. Botstein, T. Watanabe, and U. Ogata.** 1972. Specialized transduction of tetracycline resistance by phage P22 in *Salmonella typhimurium*. Virology **50**:883–898.

6a. **Collinson, S. K.** Unpublished data.

7. **Collinson, S. K., L. Emödy, K.-H. Müller, T. J. Trust, and W. W. Kay.** 1991. Purification and characterization of thin, aggregative fimbriae from *Salmonella enteritidis*. J. Bacteriol. **173**:4773–4781.

7a. **Collinson, S. K., L. Emödy, T. J. Trust, and W. W. Kay.** 1992. Thin aggregative fimbriae from diarrheagenic *Escherichia coli*. J. Bacteriol. **174**:4490–4495.

8. **Courtney, H. S., W. A. Simpson, and E. H. Beachey.** 1983. Binding of streptococcal lipoteichoic acid to fatty acid-binding sites on human plasma fibronectin. J. Bacteriol. **153**:763–770.
9. **Critchton, P. B., D. E. Yakubu, D. C. Old, and S. Clegg.** 1989. Immunological and genetical relatedness of type-1 and type-2 fimbriae in salmonellas of serotype gallinarum, pullorum, and typhimurium. J. Appl. Bacteriol. **67**:283–291.
10. **Doig, P., L. Emödy, and T. J. Trust.** 1992. Binding of laminin and fibronectin by the trypsin-resistant major structural domain of the crystalline virulence surface array protein of *Aeromonas salmonicida*. J. Biol. Chem. **267**:43–49.
11. **Duguid, J. P., E. S. Anderson, and I. Campbell.** 1966. Fimbriae and adhesive properties in salmonellae. J. Pathol. Bacteriol. **92**:107–138.
12. **Emödy, L., J. Heesemann, H. Wolf-Watz, M. Skurnik, G.**

Kapperud, P. O'Toole, and T. Wadström. 1989. Binding to collagen by *Yersinia enterocolitica* and *Yersinia pseudotuberculosis*: evidence for *yopA*-mediated and chromosomally encoded mechanisms. J. Bacteriol. **171:**6674–6679.

13. **Ernst, R. K., D. M. Dombroski, and J. M. Merrick.** 1990. Anaerobiosis, type 1 fimbriae, and growth phase are factors that affect invasion of HEp-2 cells by *Salmonella typhimurium*. Infect. Immun. **58:**2014–2016.
14. **Evans, D. G., D. J. Evans, Jr., and J. W. Tjoa.** 1977. Hemagglutination of human group A erythrocytes by enterotoxigenic *Escherichia coli* isolated from adults with diarrhea: correlation with colonization factor. Infect. Immun. **18:**330–337.
15. **Feutrier, J., W. W. Kay, and T. J. Trust.** 1986. Purification and characterization of fimbriae from *Salmonella enteritidis*. J. Bacteriol. **168:**221–227.
16. **Fröman, G., L. M. Switalski, A. Faris, T. Wadström, and M. Höök.** 1984. Binding of *Escherichia coli* to fibronectin. J. Biol. Chem. **259:**14899–14905.
17. **Isaacson, R. E.** 1988. Molecular and genetic basis of adherence for enteric *Escherichia coli* in animals, p. 28–44. *In* J. A. Roth (ed.), Virulence mechanisms of bacterial pathogens. American Society for Microbiology, Washington, D.C.
18. **Isberg, R. R.** 1991. Discrimination between intracellular uptake and surface adhesion of bacterial pathogens. Science **252:**934–938.
19. **Kay, W. W., B. M. Phipps, E. E. Ishiguro, and T. J. Trust.** 1985. Porphyrin binding by the surface array virulence protein of *Aeromonas salmonicida*. J. Bacteriol. **164:**1332–1336.
20. **Kelly, N. M., J. L. Kluftinger, B. L. Pasloske, W. Paranchych, and R. E. W. Hancock.** 1989. *Pseudomonas aeruginosa* pili as ligands for nonopsonic phagocytosis by fibronectin-stimulated macrophages. Infect. Immun. **57:**3841–3845.
21. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227:**680–685.
22. **Lindquist, B. L., E. Lebenthal, P.-C. Lee, M. W. Stinson, and J. M. Merrick.** 1987. Adherence of *Salmonella typhimurium* to small-intestinal enterocytes of the rat. Infect. Immun. **55:**3044–3050.
23. **Manoil, C., and J. Beckwith.** 1985. Tn*phoA*: a transposon probe for protein export signals. Proc. Natl. Acad. Sci. USA **82:**8129–8134.
24. **Müller, K.-H., S. K. Collinson, T. J. Trust, and W. W. Kay.** 1991. Type 1 fimbriae of *Salmonella enteritidis*. J. Bacteriol. **173:**4765–4772.
25. **Old, D. C., I. Corneil, L. F. Gibson, A. D. Thomson, and J. P. Duguid.** 1968. Fimbriation, pellicle formation and the amount of growth of salmonellas in broth. J. Gen. Microbiol. **51:**1–16.
26. **Olsén, A., A. Jonsson, and S. Normark.** 1989. Fibronectin binding mediated by a novel class of surface organelles on *Escherichia coli*. Nature (London) **338:**652–655.
27. **Omioke, I., B. Lindquist, R. Abud, J. Merrick, and E. Lebenthal.** 1989. The effect of protein-energy malnutrition and refeeding on the adherence of *Salmonella typhimurium* to small intestinal mucosa and isolated enterocytes in rats. J. Nutr. **120:**404–411.
28. **Payne, S. M., and R. A. Finkelstein.** 1977. Detection and differentiation of iron-responsive avirulent mutants on Congo red agar. Infect. Immun. **18:**94–98.
29. **Rosner, J. L.** 1972. Formation, induction and curing of bacteriophage P1 lysogens. Virology **48:**679.
30. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
31. **Savage, D. C.** 1987. Factors influencing biocontrol of bacterial pathogens in the intestine. Food Technol. **41:**82–87.
32. **Stugard, C. E., P. A. Daskaleros, and S. M. Payne.** 1989. A 101-kilodalton heme-binding protein associated with Congo red binding and virulence of *Shigella flexneri* and enteroinvasive *Escherichia coli* strains. Infect. Immun. **57:**3534–3539.
33. **Swaney, L. M., Y.-P. Liu, C.-M. To, C.-C. To, K. Ippen-Ihler, and C. C. Brinton, Jr.** 1977. Isolation and characterization of *Escherichia coli* phase variants and mutants deficient in type 1 pilus production. J. Bacteriol. **130:**495–505.
34. **Tarkkanen, A.-M., B. L. Allen, B. Westerlund, H. Holthöfer, P. Kuusela, L. Risteli, S. Clegg, and T. K. Korhonen.** 1990. Type V collagen as the target for type-3 fimbriae, enterobacterial adherence organelles. Mol. Microbiol. **4:**1353–1361.
35. **Tennent, J. M., S. Hultgren, B.-I. Marklund, K. Forsman, M. Göransson, B. E. Uhlin, and S. Normark.** 1990. Genetics of adhesin expression in *Escherichia coli*, p. 79–110. *In* B. H. Iglewski and V. L. Clark (ed.), Molecular basis of bacterial pathogenesis. Academic Press, Inc., New York.
36. **Thorns, C. J., M. G. Sojka, and D. Chasey.** 1990. Detection of a novel fimbrial structure on the surface of *Salmonella enteritidis* by using a monoclonal antibody. J. Clin. Microbiol. **28:**2409–2414.
37. **Valkonen, K. H., J. Veijola, B. Dagberg, and B. E. Uhlin.** 1991. Binding of basement-membrane laminin by *Escherichia coli*. Mol. Microbiol. **5:**2133–2141.
38. **Westerlund, B., P. Kuusela, J. Risteli, L. Risteli, T. Vartio, H. Rauvala, R. Virkola, and T. K. Korhonen.** 1989. The O75X adhesin of uropathogenic *Escherichia coli* is a type IV collagen-binding protein. Mol. Microbiol. **3:**329–337.