Salmonella typhimurium exhibits a distinct tropism for mouse enterocytes that is linked to their expression of type 1 fimbriae. The distinct binding traits of Salmonella type 1 fimbriae is also reflected in their binding to selected mannosylated proteins and in their ability to promote secondary bacterial aggregation on enterocyte surfaces. The determinant of binding in Salmonella type 1 fimbriae is a 35-kDa structurally distinct fimbrial subunit, FimHs, because inactivation of fimHs abolished binding activity in the resulting mutant without any apparent effect on fimbrial expression. Surprisingly, when expressed in the absence of other fimbrial components and as a translational fusion protein with MalE, FimH failed to demonstrate any specific binding tropism and bound equally to all cells and mannosylated proteins tested. To determine if the binding specificity of Salmonella type 1 fimbriae was determined by the fimbrial shaft that is intimately associated with FimHs, we replaced the amino-terminal half of FimHs with the corresponding sequence from Escherichia coli FimH (FimHe) that contains the receptor binding domain of Salmonella escherichia coli fimbriae. Apparently, the quaternary constraints imposed by the fimbrial shaft on the adhesin determine the distinct binding traits of S. typhimurium type 1 fimbriae.
Salmonella Type 1 Fimbriae-mediated Tropism for Enterocytes

Madison et al. (13) demonstrated subtle but distinct differences in binding specificity between recombinant type 1 fimbriae of E. coli and K. pneumoniae. More recently, it has been reported that there is heterogeneity in binding to different mannosylated glycoproteins among type 1 fimbriae of different E. coli strains (14). These studies have also provided some clues to the underlying molecular basis for the heterogeneity in type 1 fimbrial binding. Whereas Sokurenko et al. (14) have linked heterogeneity in the binding among type 1 fimbriae of E. coli to differences in the covalent structure in their adhesin, FimH, Madison et al. (13) have implicated the fimbrial shaft in defining the fine binding specificity between E. coli and K. pneumoniae type 1 fimbriae. In view of the tropism exhibited by Salmonella for gut tissue, the association of type 1 fimbrial expression with virulent strains of Salmonella, and the heterogeneity in binding specificity among type 1 fimbriae of Salmonella and other enterobacteria, we hypothesized that the type 1 fimbriae of S. typhimurium may play a critical role in modulating bacterial tropism to the gut of the host. By virtue of their distal location on the bacterium and their affinity for specific mannosylated residues, they could determine which tissues are ultimately invaded by Salmonella.

We have previously identified the corresponding manno-binding adhesins on E. coli, K. pneumoniae, Serratia marcescens, and Enterobacter cloacae, and we have shown that their sizes ranged between 27 and 29 kDa and that they are antigenically conserved (15). Moreover, the gene encoding the adhesin is invariably at the distal end of the fim gene cluster. The complete DNA sequence of the Salmonella fim gene cluster has been determined by Clegg and Swenson (25) (GenBank™ accession number L19338), and it revealed some striking differences in composition and location of genes with that of other enteric fim gene clusters. Based on predicted amino acid sequences of the products of the Salmonella fim gene cluster, it was not possible to identify the fimbrial adhesin. Nevertheless, a gene located in the middle of the fim cluster was named fimH, because it exhibited limited homology with the adhesin of S fimbrae of E. coli. Thus, in addition to testing the contribution of Salmonella type 1 fimbriae to tissue tropism, we were interested in identifying and characterizing the putative adhesin.

Here, we demonstrate that Salmonella type 1 fimbriae mediate bacterial tropism for mouse gut epithelial cells (enterocytes) because, unlike type 1 fimbriae of E. coli, Salmonella type 1 fimbriae promoted strong bacterial binding to enterocytes but not to bladder epithelial cells. The distinct binding traits of Salmonella type 1 fimbriae were also reflected in their selective binding to particular mannosylated proteins and in mediating secondary aggregation of bacteria on the surface of enterocytes. Inactivation of a gene (fimH) encoding a 35-kDa structurally distinct protein (fimH) located in the middle of the fim cluster resulted in loss of binding activity without any loss of fimbrial expression. Surprisingly, when FimH was expressed in the absence of other fimbrial proteins, the adhesin bound equally to enterocytes and bladder cells as well as to all mannosylated proteins tested. To see if the fimbrial shaft was responsible for the distinct binding specificity exhibited by the fimbriae, we replaced the amino-terminal half of the FimH with the corresponding sequence from FimHs, which contained the receptor binding domain of FimHs. The resulting hybrid fimbriae expressed a chimeric adhesin (FimH/E) on a shaft comprised of Salmonella fimbrial protein, and its binding traits resembled that exhibited by Salmonella fimbrae. Our findings suggest that although the adhesin is the structurally distinct FimH, the tropism exhibited by S. typhimurium type 1 fimbrae is determined by the fimbrial filament on which the adhesin is presented.

EXPERIMENTAL PROCEDURES

Bacterial Strains, Culture, and Plasmids

The bacterial strains and plasmids used in this work are described in Table I. All strains were cultured in brain heart infusion broth or in Luria broth (BBL Microbiology Systems, Cockeysville, MD).

Culture of Mouse Gut and Bladder Epithelial Cells

The immortalized mouse proximal small intestinal epithelial cell line SI-H10 was established from LFABP-21/tsA58 transgenic mice (16). These cells represent one of the first gut epithelial cell lines of mouse origin to be used to demonstrate adherence of enteric pathogens. These SI-H10 cells were cultured in Dulbecco's modified Eagle's medium (BioWhittaker, Walkersville, MD) containing 20% fetal calf serum (HyClone, Logan, UT) and 50 units of penicillin/50 µg of streptomycin/ml (Life Technologies, Inc.) at 39 °C in a humidified atmosphere of 95% air, 5% CO2. Immortalized mouse bladder epithelial cells MM45T.BL were obtained from American Type Culture Collection (ATCC, Rockville, MD). These cells were cultured in Dulbecco's modified Eagle's medium with 10% heat-inactivated fetal calf serum at 37 °C in a humidified atmosphere of 95% air, 5% CO2.

3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyldtetrazolium Bromide (MTT)1 Bacterial Adherence Assay

This assay is based on the principle that live bacteria convert MTT intracellularly to purple formazan in direct proportion to viable bacterial cell number (17). The dye MTT (Sigma) is a yellow tetrazolium salt. MTT is reduced by dehydrogenases (expressed intracellularly by the bacteria) to form purple formazan with maximum absorbance at 570 nm. The crystals of formazan that are produced could be observed microscopically in individual bacterial cells and could be quantitated in a multwell scanning spectrophotometer (enzyme-linked immunosorbent assay reader). This MTT bacterial adherence assay is a new, simple, inexpensive, and rapid colorimetric assay that we have developed in our laboratory to study the adherence of live, metabolically active bacteria. All the bacteria used in this study processed the MTT equally and produced the same amount of formazan. Increasing concentrations of bacteria produced corresponding increases in formazan as measured by A570. Dead bacteria and paraformaldehyde-fixed enterocytes or bladder epithelial cells do not react with MTT.

Cultures of bacteria were grown in static flasks at 37 °C in LB until bacteria were in mid-log phase growth. Bacteria were harvested and washed twice in phosphate-buffered saline (PBS) by centrifugation and resuspended in PBS. The suspensions were adjusted to 1×108 bacteria/ml (1 OD), and the suspensions were kept on ice until used.

Enterocytes (SI-H10 cells) or bladder epithelial cells (MM45T.BL cells) at a concentration of 0.15×106/ml were seeded in a 96-well tissue culture plate and allowed to grow in enriched medium (at 39 °C in the case of SI-H10 cells or 37 °C in the case of MM45T.BL cells) with 5% CO2. Under these conditions, most of the cells attached to the plastic culture plate and allowed to grow in enriched medium (at 39 °C in the case of SI-H10 cells or 37 °C in the case of MM45T.BL cells). These SI-H10 cells were cultured in Dulbecco's modified Eagle's medium (BioWhittaker, Walkersville, MD) containing 20% fetal calf serum (HyClone, Logan, UT) and 50 units of penicillin/50 µg of streptomycin/ml (Life Technologies, Inc.) at 39 °C in a humidified atmosphere of 95% air, 5% CO2.

*Note:* The abbreviations used are: MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyldtetrazolium Bromide; PBS, phosphate-buffered saline; BSA, bovine serum albumin; PAPM-BSA, p-amino-phenyl-a-o-mannopyranoside-BSA; HRP, horseradish peroxidase; PCR, polymerase chain reaction; RT; room temperature; PAGE, polyacrylamide gel electrophoresis; amM, α-methyl-o-mannopyranoside; kb, kilobase pair; bp, base pair.

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SALMONELLA TYPHIMURIUM AND HUMAN ENTEROCYTES.

Salmonella Type 1 Fimbriae-mediated Tropism for Enterocytes

Salmonella enterica serovar Typhimurium (4), an important foodborne pathogen, contains type 1 fimbriae (1), which are involved in bacterial adherence to host cells. This study aimed to characterize the role of Salmonella type 1 fimbriae in enterocyte tropism using a novel in vitro model system.

MATERIALS AND METHODS

Bacterial Strains and Cultivation

Salmonella typhimurium (strains 5959 and ORN103) and E. coli (strains DH5α and ORN103) were used in this study. Cultures were grown on LB agar plates or in LB broth at 37 °C.

Enterocyte Tropism Assay

Primary human enterocytes were isolated from human ileum and cultured on a plastic substrate. Enterocyte cultures were infected with either Salmonella typhimurium or E. coli, and the internalization of bacteria was determined by a standard microscopic technique.

RESULTS

Infection of Enterocytes by Salmonella typhimurium

Salmonella typhimurium (strain 5959) was able to efficiently infect primary human enterocytes. The infection was found to be dependent on type 1 fimbriae, as the fimbriae-negative strain (strain ORN103) showed significantly reduced infection rates.

DISCUSSION

The findings of this study suggest that type 1 fimbriae play a critical role in the tropism of Salmonella typhimurium for human enterocytes. This information is crucial for understanding the pathogenesis of Salmonella infections and could have implications for the development of new therapeutic strategies.

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Salmonella Type 1 Fimbriae-mediated Tropism for Enterocytes

*NruI/HpaI* fragment was sequenced. The sequence analysis confirmed the presence of the two introduced restriction sites as well as the integrity of the sequence of the entire PCR product. This clone was named pKT306.

**Replacement of NruI/HpaI fimHS Fragment with NruI/HpaI fimHE Fragment**—In the first of three subcloning steps, pKT306 was specifically digested with *Nru*I and *Hpa*I. The 488-bp fragment was gel-purified and cloned into the 3.6-kb fragment of pKT305, which had also been digested with *Nru*I and *Hpa*I and gel-purified. This construction was screened by *Nru*I and *Hpa*I digest for the presence of a 488-bp fragment. This plasmid was named pKT307. Additional restriction enzyme analysis also confirmed the presence of a *Kpn*I site which was introduced from the *E. coli* sequence. The second step in the reconstruction involved subcloning 6727-bp *Pst*I fragment from pISF101 into the *Pst*I site of pUC21 (This recombinant plasmid was named pKT308) and replacing the *Aar*II to *Bsu*36I fragment in pKT308 with the comparable fragment from pKT307 which contains the *fimH* sequences. The plasmid pKT308 was initially linearized with *Bsu*36I and then partially digested with *Aar*II. The 8.9-kb fragment was gel-purified. The plasmid pKT307 was digested to completion with *Aar*II and *Bsu*36I, and the 975-bp fragment was gel-purified and cloned into the 8.9-kb fragment generated in the partial digest. The resultant clones were screened by *Aar*II/*Bsu*36I digests and *NruI/HpaI* digest. One clone with the appropriate phenotype was chosen and named pKT309. The final subcloning step involved subcloning the *chimeric fimHS*-bearing operon in pACYC184 required digesting pKT309 with *Pst*I and gel purifying the 6.7-kb fragment. This fragment was cloned into the 9-kb *Pst*I fragment from pISP101. Transformants were screened by *Pst*I digestion for the presence of two fragments, approximately 9 and 6.5 kb. There is a unique *Kpn*I site in the *E. coli* sequence in the region cloned into the *S. typhimurium* fim operon. There are no *Kpn*I sites in the pACYC184 vector or in the *S. typhimurium* fim operon. This construct was named pKT310.

**Assays for Fimbriation and Isolation of Type 1 Fimbriae**

Bacterial cell expression of type 1 fimbriae was confirmed by one or more of the following tests: αM-sensitive agglutination of guinea pig erythrocytes and yeast (*Candida albicans*) cells, bacterial agglutination by anti-FimA*α* antisera, and direct binding of fimbriate-specific antibodies in enzyme-linked immunosorbent assays (19). Type 1 fimbriate strains were isolated and purified by following the methods of Dodd and Eisenstein (20).

**Extraction of Periplasmic Contents and Purification of MalE/ FimH* Fusion Protein**

Once the expression of MalE/FimH* fusion was confirmed by immunoblotting, we induced cultures with 0.3 mM isopropyl-β-D-thiogalactopyranoside (IPTG) to induce the large amounts of the fusion proteins in the periplasmic fraction (19). Periplasmic fluid was extracted from 2-liter culture suspensions as described previously (19) and passed over a Sepharose-amylose affinity column. The fusion protein was bound to the column via its affinity tag (the MalE portion). Bound fusion protein was subsequently eluted by the addition of 10 mM maltose and the eluate was subjected to SDS-PAGE (19).

**SDS-PAGE, Immunoblotting, and Densitometry**

Purified FimH* wild type, FimH* mutant, and FimH* hybrid fimbrial preparations (100-μl volume each) were boiled for 5 min in 100 μl of 0.125 N HCl. 100 μl of 0.1 N sodium hydroxide (NaOH) was added to this mixture followed by the addition of 100 μl of 4× SDS-PAGE sample buffer and boiling for 8 min. These fimbrial samples were subjected to SDS-PAGE on a 15% slab gel by the method of Laemmli (21). Purified MalE/FimH* fusion protein was subjected to SDS-PAGE on a 10% slab gel. The proteins were subsequently electrophoretically transferred onto nitrocellulose membranes. The immunostaining of specific protein was performed essentially as described previously (19) using antiserum raised against FimA*α* or FimH*α* or MalE. Densitometric scans of protein bands were performed optically with the Eagle Eye II still video system (Stratagene, La Jolla, CA).

**Overlay Assays Employing Mouse Enterocytes, Bladder Epithelial Cells, and Mannosylated Glycopeptides (PAPM-BSA and HRP)**

Purified fusion protein comprising of MalE and FimH* (MalE/ FimH*) and MalE were subjected to SDS-PAGE and transferred onto nitrocellulose membranes. After blocking in 3% bovine serum albumin (BSA) in PBS for 1 h, the nitrocellulose blot was overlaid with 5 × 10^6 biotinylated mouse enterocytes or bladder cells in the presence and absence of 100 mM αmM (19). The mouse cells were biotinylated as described previously (19). After 1 h of incubation at 22 °C, the blot was rinsed several times with PBS, and the bound cells were probed with alkaline phosphatase-conjugated avidin (Sigma) followed by the substrate 4-chloro-1-naphthol. The blots were examined for a binding reaction from other enterobacterial type 1 fimbriae, we compared enterocyte binding of the recombinant bacteria expressing type 1 fimbriae of *E. coli* with those expressing *Salmonella* fimH*α*. We observed that the number of adherent bacteria expressing *Salmonella* type 1 fimbriae was considerably more than the number of adherent bacteria expressing *E. coli* fimbriae (Fig. 1A). Since *Salmonella* do not typically infect the urinary tract, whereas *E. coli* is a frequent pathogen, we compared the binding to mouse bladder epithelial cells of bacteria expressing *Salmonella* and *E. coli* type 1 fimbriae (Fig. 1B). Whereas *E. coli* type 1 fimbriae mediated high levels of bacterial binding to bladder cells and lower levels of binding to enterocytes (Fig. 1A and B), bacteria expressing *Salmonella* type 1 fimbriae bound only enterocytes. Therefore, *Salmonella* and, perhaps, *E. coli* exhibit distinct binding tropisms in vitro reflecting their colonization sites in vivo (Fig. 1, A and B).

**Characterization of the Binding Properties of *S. typhimurium* Type 1 Fimbriae—Heterogeneity in binding among different type 1 fimbriated bacteria may be manifested in differential...
binding to selected mannosylated glycoproteins (14, 24). To confirm the distinct binding properties of *Salmonella* and *E. coli* type 1 fimbriae, we compared their ability to bind two mannosylated glycoproteins, horseradish peroxidase (HRP) and *p*-amino-phenyl-α-D-mannopyranoside-BSA (PAPM-BSA). Fimbrial binding to the glycoproteins resulted in bacterial agglutination and sedimentation that can be readily monitored by a fall in the absorbance values of the mixture. Whereas bacteria expressing *E. coli* or *Salmonella* type 1 fimbriae bound and were aggregated by PAPM-BSA, only bacteria expressing *E. coli* type 1 fimbriae bound and were aggregated by HRP (Fig. 2, A and B). Thus, compared with *E. coli* type 1 fimbriae, *Salmonella* fimbriae exhibited a more selective binding reaction implying that its mannose-binding adhesin possesses a distinct recognition motif from that on *E. coli* type 1 fimbriae.

**Aggregative Bacterial Adherence Mediated by S. typhimurium Type 1 Fimbriae**—Another remarkable feature of *Salmonella* type 1 fimbriae-mediated adherence is that secondary bacterial aggregates appeared to form around already adherent bacteria on the surface of enterocytes. When the adherence of *S. typhimurium* X4252 or *E. coli* ORN103(pSH2101) to live SI-H10 enterocytes was examined by light microscopy, the bacteria appeared to be associated with the epithelial cells in distinct aggregates. The bacterial aggregates on the surface of enterocytes appeared after about 45 min of incubation, and the size of the aggregates increased with prolonged incubation (data not shown). Many of the bacteria in the aggregates seemed to be adhering to each other rather than to the enterocyte surface. Scanning electron micrographs illustrating the peculiar bacterial aggregation mediated by *Salmonella* type 1 fimbriae on the surface of the gut epithelium is shown in Fig. 3, A and C. None of the type 1 fimbriated bacteria appeared to bind each other prior to addition to the epithelial monolayer, indicating that this phenomenon is related to post-adhesion events. This phenomenon appeared to be unique to *Salmonella* type 1 fimbriae because *E. coli* ORN103(pSH2) bound enterocytes but failed to form bacterial aggregates (data not shown). None of the nonfimbriated *S. typhimurium* X4253 and *E. coli* ORN103 bound epithelial cells or formed aggregates on enterocytes (Fig. 3, B and D). This secondary adherence mediated by *Salmonella* type 1 fimbriae could potentially augment its tropic effects by amplifying the numbers of bacteria found associating with the enterocyte surface.

**Identification of the Determinant of Adhesion on S. typhimurium Type 1 Fimbriae**—We sought to identify the determinant on *S. typhimurium* type 1 fimbriae responsible for mediating specific adherence to enterocytes. The organization and make up of genes within the *S. typhimurium fim* cluster have been determined by Clegg and Swenson (25). However, because of the dissimilarities in the size, number, and location of various genes in the *Salmonella fim* cluster and other enterobacterial *fim* clusters, it was not possible to readily identify the determinant of binding. For example, the regulatory genes of
Salmonella (fimZ, fimY, and fimW) are located at the 3' end of the gene cluster, whereas the E. coli type 1 fimbrial operon regulatory genes (fimB and fimE) are located at the 5' end of the gene cluster (Fig. 4A). Moreover, the DNA sequence of the S. typhimurium fim locus has recently been determined by Clegg and colleagues (GenBank accession number L19338 (25)), and none of the predicted protein sequences exhibited any homology with the type 1 fimbrial adhesins of E. coli or other enterobacteria. It has previously been reported that the amino-terminal region of the FimH proteins of a wide range of enteric bacteria was antigenically conserved (15). Therefore, we used an antibody directed at a synthetic peptide corresponding to residues 1–25 of FimH to probe for the Salmonella type 1 fimbrial adhesin. The fimbriae were prepared from S. typhimurium X4252, and the antisera reacted specifically with a band at 35 kDa on the Western blot (Fig. 4B). A similar band was detected when fimbriae from the clone ORN103(pISF101) was probed with FimH antisera (data not shown). Thus, despite the lack of homology in the primary structure between FimHs and any of the gene products of Salmonella fim cluster, the antibody reacted with a 35-kDa protein. Presumably, the antibody was able to recognize conserved conformational epitopes. An open reading frame encoding a 35-kDa protein is present in the S. typhimurium gene cluster. This gene is located in the middle of the fim gene cluster which is distinct from the traditional location of enterobacterial fimH at the distal 3' end of the gene cluster.

If the gene encoding the 35-kDa protein was the Salmonella adhesin, we predicted that specific inactivation of this gene in the Salmonella fim cluster would result in loss of the adhesive phenotype. We introduced in-frame translation termination codons within the fimH coding region in the fim operon of pISF101. The insertion of the linker containing the in-frame stop codons within the fimHs gene was verified by DNA sequencing. The newly generated plasmid, pKT303 (also described in Table 1), was introduced into the E. coli host strain ORN103. The resultant transformant was examined for fimbrial expression by electron microscopy and found to express a comparable number of fimbriae per bacteria as E. coli ORN103(pISF101). Also, there appeared to be no obvious difference in morphology between FimHs’ mutant and wild type fimbriae (Fig. 4C). The length of the mutant fimbriae also appeared to be comparable to that of the wild type.

We confirmed biochemically that the mutant fimbriae were deficient in the 35-kDa FimHs protein. We isolated fimbriae from the mutant ORN103(pKT303) and from ORN103-(pISF101) expressing wild type fimbriae and then probed them with an antibody raised against a synthetic peptide corresponding to residues 1–25 of FimHs on a Western blot. A second Western blot was also performed with an antibody to the 21-kDa FimA subunit of Salmonella type 1 fimbriae (FimAs). As shown in Fig. 4D, a 35-kDa protein was detected in the lane containing wild type fimbriae (lane 3) but not in the lane containing mutant fimbriae (lane 4), although both lanes contained equal amounts of FimAs (see corresponding lanes 1 and 2). It is also noteworthy that comparative densitometric analyses of FimAs and FimHs bands in the wild type fimbrial preparation indicated that the ratio of the adhesin to the major subunit is 1:10. This contrasts with E. coli type 1 fimbriae where the ratio ranges from 1:100 to 1:60 (15).

We examined the ability of E. coli ORN103(pKT303) to bind mouse gut and bladder epithelial cells and the two mannosylated proteins (PAPM-BSA and HRP). We found that in contrast to ORN103(pISF101) expressing native Salmonella fimbriae, the mutant ORN103(pKT303) expressing FimHs-deficient fimbriae failed to mediate any appreciable binding to the monolayer of enterocytes or to bladder cells (Fig. 1, A and B). Moreover, bacteria expressing the mutant fimbriae were not aggregated by either PAPM-BSA or HRP (Fig. 2, A and B). Together, the data suggest that the 35-kDa FimHs protein is the determinant on Salmonella type 1 fimbriae responsible for mediating adherence. However, these data do not preclude the contribution of other fimbrial components in the binding process.

Expression of FimHs as a Translational Fusion Protein with Maltose-binding Protein (MalE) and Examination of Its Adhesive Capacity—We sought to investigate if FimHs could mediate its binding in the absence of other fimbrial proteins. We cloned fimHs but found that the recombinant gene product was highly unstable in the bacterial periplasm. We attempted to stabilize the FimHs by expressing it as a translational fusion protein with MalE as described previously for the stabilization of FimHs (19). The periplasm was isolated, and the MalE/FimH S fusion protein (Fig. 4E) was shown to bind enterocytes, we performed a cell blotting assay where the ratio ranges from 1:100 to 1:60 (15).

To determine whether the MalE/FimHs fusion protein could bind enterocytes, we performed a cell blotting assay where the ratio ranges from 1:100 to 1:60 (15). The length of the mutant fimbriae also appeared to be comparable to that of the wild type. As shown in Fig. 4D, a 35-kDa protein was detected in the lane containing wild type fimbriae (lane 3) but not in the lane containing mutant fimbriae (lane 4), although both lanes contained equal amounts of FimAs (see corresponding lanes 1 and 2). It is also noteworthy that comparative densitometric analyses of FimAs and FimHs bands in the wild type fimbrial preparation indicated that the ratio of the adhesin to the major subunit is 1:10. This contrasts with E. coli type 1 fimbriae where the ratio ranges from 1:100 to 1:60 (15).

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To determine whether the MalE/FimHs fusion protein could bind enterocytes, we performed a cell blotting assay where MalE/FimHs were immobilized onto nitrocellulose membrane and then probed with biotinylated enterocytes in the presence and absence of 100 mM. As shown in Fig. 5B, the enterocytes bound MalE/FimHs (lane 2) but not MalE (lane 1). Furthermore, this binding reaction was inhibitable by amM (lane 4). Thus, FimHs alone is capable of mediating mannos-inhibitable binding to mouse enterocytes.

To see if the isolated FimHs also exhibited the characteristic binding specificity exhibited by Salmonella type 1 fimbriae, we tested the ability of the immobilized protein to bind bladder cells and the two mannosylated proteins, PAPM-BSA or HRP.
The binding to bladder cells was undertaken as described for enterocytes. However, the assay for assessing binding to HRP is notable because it involves a single step that simultaneously takes advantage of the highly mannosylated nature of the glycoprotein (reflected in its ability to bind FimHS) and its intrinsic enzymatic properties (reflected in its ability to react with the Western blot substrate, 4-chloro-1-naphthol). Binding of PAPM-BSA to FimHS was assessed by the ability of the glycoprotein to block the binding of HRP to MalE/FimH S. Surprisingly, we found that FimH S bound bladder cells as well as it bound enterocytes, and it bound both mannosylated compounds equally well (Fig. 5C). This was a surprising finding and indicated that although Salmonella type 1 fimbriae appeared selective in its binding of mannosylated compounds, its FimHs adhesin did not exhibit this selective binding trait. In view of the fact that in the native fimbriae, the FimH S is intimately associated with the fimbrial shaft, it is likely that this structure may play a role in mediating some of the selective binding traits of the fimbriae.

Creation and Characterization of Hybrid S. typhimurium Fimbriae Containing Chimeric FimHes—To explore further the relative contribution of FimHs and the Salmonella fimbrial shaft to fimbrial binding specificity, we replaced a stretch comprising amino acid residues 1–173 of FimHS with a corresponding stretch of residues 1–158 from FimH E containing the receptor binding domain of FimH E (19). We reasoned that if the shaft influenced the binding specificity, then the resulting hybrid fimbriae would function as Salmonella fimbriae. Alternatively, if the hybrid fimbriae behaved more as E. coli fimbriae, then we would assume that the primary structure of FimHs was responsible for the distinct binding traits of the fimbriae.

The binding to bladder cells was undertaken as described for enterocytes. However, the assay for assessing binding to HRP is notable because it involves a single step that simultaneously takes advantage of the highly mannosylated nature of the glycoprotein (reflected in its ability to bind FimHs) and its intrinsic enzymatic properties (reflected in its ability to react with the Western blot substrate, 4-chloro-1-naphthol). Binding of PAPM-BSA to FimHs was assessed by the ability of the glycoprotein to block the binding of HRP to MalE/FimHs. Surprisingly, we found that FimHs bound bladder cells as well as it bound enterocytes, and it bound both mannosylated compounds equally well (Fig. 5C). This was a surprising finding and indicated that although Salmonella type 1 fimbriae appeared selective in its binding of mannosylated compounds, its FimHs adhesin did not exhibit this selective binding trait. In view of the fact that in the native fimbriae, the FimHs is intimately associated with the fimbrial shaft, it is likely that this structure may play a role in mediating some of the selective binding traits of the fimbriae.
Salmonella shaft still retained the binding traits of native Salmonella fimbriae. Conceivably, the fimbrial shaft has an overriding influence on the binding specificity regardless of the primary structure of the adhesin.

Several aspects regarding the morphology and composition of the hybrid fimbriae were noted. Western blot analyses of the fimbriae employing FimA- and FimH-specific antisera followed by densitometry revealed that the amount of FimH in native Salmonella fimbriae incorporated into the fimbrial filament was approximately 40% less than the level of FimH in native Salmonella fimbriae (Fig. 6A). This observation may account for why the binding of bacteria expressing FimH to the enterocytes was less than the level of FimH in native Salmonella fimbriae (Fig. 1, A and B). Transmission electron microscopy revealed that the hybrid fimbriae were aberrant, with a curly and tangled appearance throughout its entire length (Fig. 6B). Type 1 fimbriae typically are highly stable organelles that require boiling in 0.125 M hydrochloric acid (HCl) to induce complete depolymerization. When we compared the stability of the hybrid fimbriae with that of native Salmonella fimbriae, we found that the hybrids were highly unstable. Whereas native Salmonella fimbriae required HCl treatment followed by boiling in SDS-PAGE sample buffer to be depolymerized, hybrid fimbriae were completely depolymerized merely by boiling in SDS-PAGE sample buffer at pH 6.8 (Fig. 6C). Thus, the incorporation of partially heterologous FimH into the Salmonella fimbrial filament had a profound effect on both fimbrial morphology and stability.

**DISCUSSION**

Although it has been known for a relatively long time that S. typhimurium expresses type 1 fimbriae, the potential contribution of these organelles to infection has largely been ignored. In light of recent observations from several different laboratories on the role of E. coli and K. pneumoniae type 1 fimbriae in promoting bacterial infections and its harmful inflammatory sequelae (19, 26, 27), it is conceivable that these organelles also contribute significantly to the pathogenesis of Salmonella.

S. typhimurium invariably invades its hosts through the gut, reflecting a distinct adhesive preference for gut tissue. A battery of contiguous genes residing within SPI-1, a 40-kb pathogenicity island mapping at 63 min in the S. typhimurium chromosome, is largely responsible for mediating entry of Salmonella into epithelial cells (28). However, none of these genes appear to mediate Salmonella’s tropism for enterocytes. Indeed, many of these “invasion-associated genes” are expressed only after the bacteria has already established intimate contact with the host cell. We reasoned that the determinant conferring tropism for gut tissue is an already expressed bacterial surface moiety and is likely to be one or more of its cell-surface fimbriae. Considering their length and peritrichous orientation, these organelles could be the first bacterial components to make contact with host tissue. Fimbriae have been implicated in mediating tissue tropism in several pathogens. For example, the fimbriae on pathogenic Neisseria gonorrhoeae (29) and pyelonephritogenic E. coli (30) mediate bacterial tropism for human genital cells and kidney tissue, respectively.

Salmonella type 1 fimbriae exhibit a distinct tropism for gut tissue because it mediates high levels of bacterial binding to mouse enterocytes but not to mouse bladder cells. The binding pattern of Salmonella type 1 fimbriae contrasts with that of E. coli type 1 fimbriae which mediated moderate bacterial binding to enterocytes and even higher levels of bacterial binding to bladder cells. The binding patterns displayed by these fimbriae correlate with the natural sites of infection of their respective
bacteria in the host. *Salmonella* typically infects the gut and *E. coli* the urinary tract, but prior to infecting the urinary tract the *E. coli* were probably inhabitants of the gut. The distinct binding traits of *Salmonella* type 1 fimbriae is also evident from their ability to bind PAPM-BSA but not HRP, whereas *E. coli* type 1 fimbriae bound well to both mannosylated proteins. Conceivably, the *Salmonella* type 1 fimbriae are able to recognize a more limited range of oligomannose motifs than *E. coli* type 1 fimbriae. That *Salmonella* type 1 fimbriae exhibit a distinct binding pattern is supported by earlier work of Firon et al. (12) who suggested that the “binding pocket” on the fimbriae is sterically different from that on *E. coli* type 1 fimbriae. To explain the tropism exhibited by *Salmonella* type 1 fimbriae, we speculate that the oligomannose motifs recognized by *Salmonella* are absent or inaccessible on HRP and on bladder cells but are present on PAPM-BSA and on enterocytes. The targeting of *S. typhimurium* to the gut epithelium is clearly essential because if this does not occur the infection is abrogated. Thus, the contribution of type 1 fimbriae to the pathogenesis of *S. typhimurium* is potentially pivotal.

It is known that fimbriae-mediated bacterial binding to mucosal cells can trigger various host cell responses that cumulatively can significantly affect the course of the infectious process (27, 31). What is less well known is that the specific coupling of bacterial fimbriae with complementary receptors on host cells may activate genes within the bacteria, notably those

![FIG. 5. Cloning, expression, and purification of a stable FimH::MalE fusion protein.](image-url)
that enhance bacterial virulence in vivo (32). One consequence of Salmonella type 1 fimbria-mediated binding to enterocytes is the formation of discrete bacterial aggregates on the surface of the gut epithelial cell. The aggregates could be the combined effect of replication of attached bacteria and recruitment of proximate bacteria through their fimbriae. This phenomenon is seen with Salmonella type 1 fimbriae but not with E. coli type 1 fimbriae. However, it is distinct from bacterial aggregation mediated by the recently described thin fimbriae of S. typhimurium (16) because it was not seen in the absence of the host cell. That the Salmonella type 1 fimbrial adhesin is involved is indicated because aggregative adherence can be blocked by the addition of a D-mannose analog. The mechanism could involve binding of Salmonella fimbriae to mannosylated compounds on the surface of neighboring bacteria. Since the aggregation does not occur when bacteria are cultured alone, it appears that the expression of mannosylated compounds on the bacterial surface occurs only after contact with the host cell. The proposed mechanism is not unlike the aggregation of K. pneumoniae on human intestinal cells where the interaction of a newly synthesized capsule-like material and an, as yet, unidentified fimbriae have been suggested (33). Aggregative adherence could be beneficial to the bacteria because it enables them to increase their load on an epithelial cell even when the specific fimbrial receptor density is limiting.

Several lines of evidence allow us to conclude that the Salmonella fimbrial adhesin is the product of the fimH gene as follows: (i) a protein in Salmonella fimbriae corresponding to the size of the fimH gene product cross-reacted with anti-FimH antibodies, (ii) inactivation of the fimH gene resulted in abolishment of binding activity in the mutant ORN103-(pKT303) without concomitant loss of fimbriae as determined by electron microscopy and by biochemical analyses of mutant fimbriae, (iii) recombinant FimHs, when expressed in the absence of other fimbrial proteins bound mouse enterocytes in a D-mannose-inhibitable fashion. Our findings confirm and extend the earlier report of an association between the presence of 33–36-kDa proteins and functional Salmonella type 1 fimbriae (34) and the prediction of Clegg and Swenson (25) that the product of the fimH gene was the adhesin. Previous attempts to inactivate fimHs have resulted in loss of fimbrial expression which has made it difficult to define precisely the functional role of the gene product (35). This is probably attributable to the polar effects of the fimHs mutation on the downstream gene, fimF, which is critical to fimbrial expression. Our studies have delinked fimbrial expression from FimHs expression and in doing so revealed that the adhesin is not required for the assembly of fimbriae.

Clegg and colleagues (25, 35–37) have reported on distinct aspects in the genetic organization, expression, and regulation of fim gene cluster of the S. typhimurium in relation to other enterobacterial fim gene clusters. Here, we have noted several differences between FimHs and its counterpart on other enterobacteria. First, its size is appreciably higher than the size of the FimH molecule of other enterobacteria which typically approximate 29 kDa (15). Second, fimHs maps at the center of the fim cluster and not at the distal end as is the case in other enterobacteria (25). Third, the amount of FimHs relative to FimAs in the fimbriae appears to be higher (1:10) rather than the 1:60 ratio found in E. coli type 1 fimbriae (15). Fourth, FimHs exhibits very limited amino acid sequence similarity with other enterobacterial FimH. Fig. 7 depicts the extent of homology in covalent structure even after progressive multiple sequence alignments of FimHs and FimH proteins. Although the significance of these distinct traits of FimHs is currently unknown, they may reflect evolutionary advances over other...
enterobacterial type 1 fimbrial adhesins.

A very surprising finding was that FimH S, when isolated from the fimbriae, failed to exhibit the selective binding specificity of native Salmonella fimbriae. Thus, in addition to binding enterocytes and to PAPM-BSA, MalE/FimH S exhibited strong binding to bladder cells and to HRP. This indicated that FimHS is intrinsically capable of recognizing the same mannose motifs bound by *E. coli* type 1 fimbriae. This is remarkable when one considers that there is little homology in primary structure between FimHS and FimHE. These findings led us to conclude that the binding pattern exhibited by the Salmonella fimbriae is not exclusively dependent on the primary structure of the adhesin. Since the adhesin is intimately associated with the fimbrial shaft, it is likely that the latter influences the binding traits of the former. We sought to assess the relative contribution of the shaft in modulating binding pattern of the fimbriae. Since it was reported that the receptor binding domain on FimHE is comprised of epitopes formed by amino acid residues in the first half of the molecule (19), we replaced the amino-terminal half of FimHS with the corresponding region on FimHE that contains the receptor binding domain on the *E. coli* adhesin. The resulting hybrid fimbriae contained a chimeric FimHES moiety with the receptor binding domain of FimHE but was presented on a Salmonella fimbrial shaft. The level of binding to enterocytes mediated by the hybrid fimbriae was less than that of native *Salmonella* type 1 fimbriae, and this is probably a reflection of reduced levels (by about 40%) of the adhesin in the fimbriae. Nevertheless, its binding pattern did not differ markedly from that of native *Salmonella* fimbriae as indicated by their binding to enterocytes and PAPM-BSA and their inability to bind bladder cells and HRP. Evidently, the loss of the amino-terminal half of FimHS was not accompanied by a concomitant loss of the distinct binding traits of *Salmo-

Valuable clues on the structure of *Salmonella* type 1 fimbriae-mediated tropism for enterocytes may be gleaned from the morphological and biochemical characterization of the hybrid fimbriae containing FimHES.
Fig. 8. Model to explain the differential binding traits of FimHs in its isolated state and when expressed on the fimbrial filament. It is hypothesized that in its isolated state, FimHs displays the ability to recognize various mannosylated motifs (as depicted in the figure) which allows the molecule to bind different mannosylated molecules on various cells or in soluble proteins. However, when incorporated at the tips of the Salmonella fimbrial shaft, FimHs is only able to bind a narrow range of mannosylated motifs because of the structural constraints imposed by the shaft, hence the narrow range of binding exhibited by S. typhimurium type 1 fimbriae. We have also depicted FimHs that are intercalated periodically along the fimbrial length. As indicated, the binding pocket in these intercalated FimHs are inaccessible in the quaternary conformation of the fimbriae, and thus these molecules are essentially nonfunctional until they are exposed by breakage. It is suspected that the sites of intercalation of FimHs are more susceptible to breakage from shear forces than other regions of the fimbriae. Thus even after the loss of a fimbrial fragment, the residual filament can still mediate attachment. It should be noted that the FimHs are likely to be located much further apart on the filament than is depicted in the figure.

That the hybrid fimbriae appeared wavy throughout its entire length and was unstable suggests that FimHs was intercalated longitudinally at multiple sites in the structure. Two models have been proposed for the location of FimHs in native E. coli fimbriae. In the first model, FimHs is located exclusively at the tips (38), which is analogous to the location of the PapG adhesin of the well characterized E. coli P fimbriae. In the second model, FimHs is located not only at the tips of the filament, but also intercalated longitudinally (15). The second model was supported by the subsequent finding that native E. coli type 1 fimbriae were significantly more prone to fragmentation than the adenin-deficient variant fimbriae when subjected to repeated cycles of freeze-thaw (39). Apparently, sites of FimHE incorporation were the preferred sites of fimbrial breakage during freeze-thawing since adhesion properties of fimbrial fragments had increased (39). In view of the extraordinary instability of the hybrid fimbriae bearing the heterologous FimHs, it is likely that the adhesin is incorporated more frequently into the structure than exclusively at the tips. More definitive studies are currently underway in this laboratory to localize FimHs more precisely in the fimbriae.

In summary, our studies indicate that the type 1 fimbriae on S. typhimurium is potentially critical to the infectious process because it determines the initial targeting of the bacteria to the gut. The type 1 fimbriae of S. typhimurium mediates high level of bacterial binding to enterocytes but not to bladder cells. The specific determinant of adhesion on the fimbriae is its structurally distinct 35-kDa subunit, FimHs. Surprisingly, in the absence of other fimbrial components, FimHs exhibited a broad range of mannosose-sensitive binding reactions and did not display the selective binding associated with Salmonella type 1 fimbriae. We hypothesize that the tissue tropism exhibited by Salmonella type 1 fimbriae is determined at least in part by the fimbrial shaft which bears FimHs. It has previously been assumed that the fimbrial shaft is an innocuous structure on which the adhesin is strategically poised. Our studies indicate that these structures play a more active role in the pathogenic process by defining the specific receptor molecules and host cells that interact with the bacteria.

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