The Distinct Binding Specificities Exhibited by Enterobacterial Type 1 Fimbriae Are Determined by Their Fimbrial Shafts*

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Type 1 fimbriae of enterobacteria are heteropolymorphic organelles of adhesion composed of FimH, a mannose-binding lectin, and a shaft composed primarily of FimA. We compared the binding activities of recombinant clones expressing type 1 fimbriae from Escherichia coli, Klebsiella pneumoniae, and Salmonella typhimurium for gut and uroepithelial cells and for various soluble mannosylated proteins. Each fimbria was characterized by its capacity to bind particular epithelial cells and to aggregate mannoproteins. However, when each respective FimH subunit was cloned and expressed in the absence of its shaft as a fusion protein with MalE, each FimH bound a wide range of mannose-containing compounds. In addition, we found that expression of FimH on a heterologous fimbrial shaft, e.g. K. pneumoniae FimH on the E. coli fimbrial shaft or vice versa, altered the binding specificity of FimH such that it closely resembled that of the native heterologous type 1 fimbriae. Furthermore, attachment to and invasion of bladder epithelial cells, which were mediated much better by native E. coli type 1 fimbriae compared with native K. pneumoniae type 1 fimbriae, were found to be dependent on the background of the fimbrial shaft (E. coli versus K. pneumoniae) rather than the background of the FimH expressed. Thus, the distinct binding specificities of different enterobacterial type 1 fimbriae cannot be ascribed solely to the primary structure of their respective FimH subunits, but are also modulated by the fimbrial shaft on which each FimH subunit is presented, possibly through conformational constraints imposed on FimH by the fimbrial shaft. The capacity of type 1 fimbrial shafts to modulate the tissue tropism of different enterobacterial species represents a novel function for these highly organized structures.

Fimbrial lectins, which are filamentous organelles of bacterial adhesion, are generally classified according to the structure of the carbohydrates they recognize (1). Their specificity is usually defined as the simplest carbohydrate structure, typically a monosaccharide, that best inhibits lectin-mediated adhesion. This is referred to as the primary sugar specificity of the lectin (2). Within lectins possessing the same primary sugar specificity, differences in the binding of different oligosaccharides is often observed. This is referred to as the fine sugar specificity (2).

Type 1 fimbriae, the primary sugar specificity of which is for D-mannose, are expressed in Escherichia coli as well as almost in all enterobacteria. Based primarily on studies of E. coli type 1 fimbriae, it is known that these organelles are heteropolymers composed of a major subunit and at least three minor subunits (3–7). The minor subunit FimH is responsible for the sugar specificity of type 1 fimbriae because inactivation of the fimH gene abolishes the binding activity of the bacteria without any apparent effect on fimbrial expression (3, 8). Moreover, isolated FimH mimics many of the mannose-specific binding reactions of type 1 fimbriae (9, 10). On the other hand, FimA is the major subunit that makes up >95% of the fimbrial shaft and is structurally and antigenically heterogeneous among different species (11–22). FimH has been crystallized, and the sugar-binding region was mapped to the N-terminal half (residues 1–156) of the molecule, whereas the region that associates with the fimbrial shaft was mapped to the C-terminal half (residues 160–277) of the FimH molecule (23). Because fimbrial proteins are not typically soluble in solution, FimH was crystallizable only when bound to its periplasmic chaperone, FimC (23).

One of the earliest indications of heterogeneity in the fine sugar specificity among type 1 fimbriae from different strains was the finding that the binding reactions mediated by various enteric type 1 fimbriae exhibit differing sensitivities to competition with defined mannos-containing compounds (24, 25). These reports proposed that the receptor-binding pocket on Salmonella type 1 fimbriae is different from that found on the fimbriae of E. coli or other enteric bacteria. Subsequently, heterogeneity in fine sugar specificity was discovered even within the same species (26). Significantly, it was found that different E. coli type 1 fimbriae can be classified into those that bind either trimannose or monomannose and those that recognize trimannose only (26). Two mechanisms have been suggested in the literature to explain these interspecies and intraspecies differences in the fine sugar specificity of the type 1 fimbrial lectin. Madison et al. (27) reported that the interspecies (E. coli and K. pneumoniae) differences in fine sugar specificity is influenced by the fimbrial shaft, which may induce distinct conformational changes in its FimH subunit. Sokurenko et al. (26) found that the intraspecies heterogeneity in the fine sugar specificity of type 1 fimbriae from different E. coli isolates can be attributed to the allelic variation in the primary amino acid structure of FimH. The heterogeneity between different enterobacterial species and within the same species has been implicated in contributing to different infectious processes. For example, it has been argued that uropathogenic E. coli clones emerge in a process called pathoadaptation to express type 1 fimbriae with specificity for monomannose and trimannose, whereas fecal isolates are specific for trimannose only (28). However, the biological significance of the
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interspecies differences among enterobacteria discussed above has not been studied.

Here, we investigate whether the fimbrial shaft-driven modulation of the fine sugar specificity of various enterobacterial species influences tissue tropism. We show that the apparent heterogeneity in binding among type 1 fimbriae is modified by the fimbrial shaft and is responsible for variations in tissue tropism.

EXPERIMENTAL PROCEDURES

Bacterial Strains, Culture, and Plasmids—The bacterial strains and plasmids used in this work are described in TABLE ONE. All strains were cultured statically in Luria broth (Difco) with the appropriate antibiotics (80 μg/ml chloramphenicol for strains containing pACYC184-based plasmids, 100 μg/ml ampicillin for strains containing pUC18-based plasmids, and both antibiotics for strains containing both plasmids).

Culture of Mouse Bladder Epithelial Cells (BECs) and Gut Epithelial Cells (GECs)—The immortalized mouse BEC2 line MM45T.BL was obtained from American Type Culture Collection (Manassas, VA) and cultured in Dulbecco’s modified Eagle’s medium with 10% heat-inactivated fetal bovine serum at 37 °C with 5% CO2. The mouse GEC line SI-H10 was established from LFA1+/+ tsA58 transgenic mice (29) and cultured in Dulbecco’s modified Eagle’s medium containing 20% fetal cell serum (Hyclone Laboratories, Logan, UT) and 50 units/ml penicillin and 50 mg/ml streptomycin (Invitrogen) at 39 °C with 5% CO2. The human BEC line 5637 (ATCC HTB-9) was grown in RPMI 1640 medium (Invitrogen) supplemented with 10% fetal bovine serum (HyClone Laboratories), 2.0 g/liter sodium bicarbonate, 0.3 g/liter L-glutamine, 2.5 g/liter glucose, 10 mmol/L HEPES, and 1 mmol/L sodium pyruvate. Cells were cultured at 37 °C with 5% CO2.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium Bromide (MTT) Bacterial Adherence Assay—The adherence of various fimbriated bacteria to the BECs and GECs was examined as described previously (30). Briefly, cultures of bacterial cells were washed twice with sterile PBS before being resuspended in PBS. 980 μl of each bacterial suspension (A600 nm ~ 1.75) was transferred to a cuvette. 20 μl of the glycoprotein being tested (10 mg/ml in PBS) was then added to each cuvette. The cuvettes were covered with Parafilm and shaken gently for 1 min, at which point they were placed into a spectrophotometer, and a reading at 600 nm was taken. As a control, 980 μl of bacterial suspension (A600 nm ~ 1.75) was mixed with 20 μl of PBS. Subsequent readings were taken at 600 nm after a total elapsed time of 5 and 15 min and every 15 min thereafter for 1 h. The A600 nm reading dropped as the bacteria aggregated and settled at the bottom of the cuvettes. Aggregation was expressed as absorbance as a function of time. To measure agglutination of Salmonella cerevisiae, 950 μl of each bacterial suspension (A600 nm ~ 1.75) was transferred to a cuvette. 50 μl of a 0.5% (w/v) suspension of S. cerevisiae was added to each cuvette, and the absorbance was measured as described above. The type 1 fimbriae-deficient E. coli strain ORN103 was used as a control. All glycoproteins employed in this assay were purchased from Sigma and are listed in TABLE TWO.

DNA Manipulations—Restriction enzymes and DNA-modifying enzymes (New England Biolabs Inc., Beverly, MA) were used as recommended by the supplier. Oligonucleotides were synthesized and purified commercially (Integrated DNA Technologies, Inc., Coralville, IA). A complete list of the plasmids that were generated as well as their gene products are described in TABLE ONE.

Determination of the DNA Sequences of the fimF, fimG, and fimH Genes of E. coli and K. pneumoniae—The sequences of the E. coli strain J96 and K. pneumoniae strain IA551 fimF and fimG genes carried on the pSH2 and pBP7 plasmids, respectively, were determined using primers FimF1 (5’-TGG TCG GTA AAT GCC TGG TCA TTC-3’), FimF2 (5’-CCT GTA TCG CAC TTG C-3’), and FimF3 (5’-GAAT CAC AGG CAT TTA CCC AC-3’) for fimF and primers Gin1 (5’-AAC CCA TGA CAC AGG CAT TTA CCC AC-3’) and Gin2 (5’-ATGG ACC AGG CAT TTA CCC AC-3’) for fimG. The sequence of the E. coli strain J96 fimH gene on the pSH2 plasmid was determined using primers Kp1 (5’-TGG TCG GTA AAT GCC TGG TCA TTG C-3’) and Kp2 (5’-CAT TAG CAA TGT CCT GTG ATT TCT-3’) from the published fimH sequence of E. coli K12 strain strain PC31 (5). To obtain the sequence of the K. pneumoniae strain IA551 fimH gene, a 3.5-kb PvuII fragment containing fimHK was released from the pBP7 plasmid, gel-puriﬁed, blunt-ended, and cloned into the Smal site of the pUC19 cloning vehicle. This construct was named pKT201, and the insert was sequenced completely using an automated fluorescence sequencer (PerkinElmer Life Sciences).

Generation of male/fimHE, male/fimHK, and male/fimHS Gene Fusion Constructs—Plasmids pSH2, pBP7, and pISF101, which contain the entire type 1 fim gene clusters of E. coli, K. pneumoniae, and S. typhimurium, respectively, were used as the PCR templates to generate fimHE, fimHK, and fimHS (E. coli, K. pneumoniae, and S. typhimurium fimH genes, respectively) DNA sequences. The PCR products were cloned into plasmid vector pMAL-p2 (New England Biolabs Inc.) using standard techniques as described previously (30) to produce plasmids pKT100, pKT213, and pKT304. Primers KP7 (5’-GCC GGA ATG CTG TAA AAC CGG CAA TGG TAC C-3’) and KP8 (5’-GGG CAA ACT TAT GGT CGT CTA TCC C-3’).
TABLE ONE
Strains and plasmids employed in this study

| Strains and plasmids | Properties | Ref./source |
|----------------------|------------|-------------|
| E. coli ORN103       | thr-1 leu-6 thi-1 Δ(argF-lac) U169 xyl-7 ara-13 mtl-2 gal-6 rpsL tonA2 fhuA2 minA minB recA13 Δ(fimABCDEFGH) | Ref. 4 |
| E. coli ES1301 mutS  | lacZ53 thyA36 rha-5 metB1 deoC1 in(rrnD-rrnE) (mutS201::Tn5) | Promega Corp. |
| E. coli TB1           | F' ara Δ(lac-proAB) rpsL (StaI) (8081 lacΔ(lacZ-M15Δ) hsdR (rK- mX-)) | New England Biolabs Inc. |
| E. coli XLI-Blue      | recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac (F' proAB lacP'ZΔGM15 Tn10 (Ter)) | Stratagene |
| **Plasmids**         |            |             |
| pSH2                 | E. coli pVHE type 1 fimbriae encoding operon in pACYC184 vector | Ref. 4 |
| pUT2002              | E. coli FimHE type 1 fimbriae encoding operon in pACYC184 vector | Ref. 8 |
| pBP7                 | K. pneumoniae IA551 type 1 fimbriae encoding operon in pACYC184 vector | Ref. 57 |
| pBP7/99              | K. pneumoniae FimHK type 1 fimbriae encoding operon in pACYC184 vector | Ref. 27 |
| pMD7                 | K. pneumoniae FimHE type 1 fimbriae encoding operon in pACYC184 vector | This study |
| pISF101              | S. typhimurium type 1 fimbriae encoding operon in pACYC184 vector | Ref. 58 |
| pKT303               | S. typhimurium FimHS type 1 fimbriae | Ref. 30 |
| pKT201               | K. pneumoniae fim operon 3.5-kb PvuII-Sall fragment in pUC19 vector | This study |
| pBM10                | K. pneumoniae fim operon 3.5-kb BamHI-Sall fragment (fimFGH) in pUC18 vector | Ref. 27 |
| pBM20                | E. coli fim operon 5.0-kb BamHI fragment (fimFGH) in pUC18 vector | Ref. 27 |
| pMD108               | K. pneumoniae fimHE in pUC18 vector | This study |
| pMD109               | E. coli fimHE in pUC18 vector | This study |
| pMAL-p2              | MalE affinity tag expression vector | New England Biolabs Inc. |
| pKT100               | MalE/FimHE expression plasmid | This study |
| pKT213               | MalE/FimHK expression plasmid | This study |
| pKT304               | MalE/FimHS expression plasmid | This study |

TCT ATT ATT GAT AAA CAA AAG TCA C-3') were used to generate the fimHE and fimHK DNA fragments employed in this study. Primers KP9 (5'-GCC GGA ATT CTG CCG TAA TTC AAA CGG GAC G-3') and KP10 (5'-GGG CAA GCT TCT ATT AAT CAT AAT CGA CTC GTA G-3') were used to generate the fimHS DNA fragment.

Construction of Bacterial Strains Expressing Hybrid Type 1 Fimbriae—These strains were produced as described previously (27). Briefly, a 5.0-kb BamHI fragment from plasmid pSH2 or a 5.5-kb BamHI-Sall fragment from plasmid pBP7 was inserted into a similarly cut site in a pUC18 cloning vector to make plasmids pBM20 and pBM10, respectively. These fragments contained fimH and fimF and FimG, two additional fimbrial subunit genes (5'). DNA-derived protein sequence data have been described (30).

**Assays for Fimbriation**—Bacterial cell expression of type 1 fimbriae was confirmed by mannose-sensitive yeast agglutination.

**Purification of MalE/FimH Fusion Proteins, SDS-PAGE, and Western Blotting**—The fusion proteins were expressed and purified as described previously (10). SDS-PAGE analysis and immunostaining of specific proteins were performed as described previously (10) using antisera raised against FimHE-(1–100).

**Verification of FimA and FimH Expression**—To examine the relative amounts of the FimA protein subunit and the FimH adhesin subunit expressed by each bacterial strain, each strain was grown statically for 48 h and washed with PBS by centrifugation. Bacteria (~1 × 10^9 cfu) of each strain were boiled in acid for 5 min to dissociate the fimbrial subunits, neutralized by the addition of NaOH, brought to 1 X SDS-PAGE sample buffer, and boiled for an additional 2 min (33). The protein concentration of each sample was determined using the Bio-Rad protein assay, and 100 μg of total protein from each sample was subjected to SDS-PAGE and transferred to a polyvinylidene difluoride membrane. Western blotting was performed using rabbit antisera against E. coli FimA or FimH-(1–100).

**Mouse BEC and GEC Overlay Assay**—This assay was performed as described previously (10). Briefly, purified fusion proteins (MalE/FimHE, MalE/FimHK, and MalE/FimHS) and MalE were subjected to SDS-PAGE and transferred to nitrocellulose membranes. After block-
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ing in 3% BSA in PBS for 1 h, the nitrocellulose blot was overlaid with 5 × 10⁵ biotinylated mouse BECs or GECs in the presence and absence of 100 mM methyl α-L-mannopyranoside. BECs and GECs were biotinylated as described previously (12). After 1 h of incubation at room temperature, the blot was rinsed several times with PBS, and the bound cells were probed with alkaline phosphatase-conjugated avidin, followed by the substrate 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium.

Glycoprotein Overlay Assay—This assay was performed as described previously (30). Briefly, purified fusion proteins (MaE/FimHE, MaE/FimHK, and MaE/FimHS) and MaE were subjected to SDS-PAGE and transferred to nitrocellulose membranes. After blocking the nitrocellulose blot in 3% BSA in PBS for 1 h, the membrane was overlaid with a glycoprotein-containing solution at a concentration of 100 μg/ml in PBS in the presence and absence of 100 mM methyl α-L-mannopyranoside. After 1 h of incubation at room temperature, the blot was rinsed several times with PBS. Bound glycoproteins were probed with alkaline phosphatase-conjugated concanavalin A (Sigma), followed by 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium.

Peptide Synthesis and Generation of Antiserum—FimHE-(1–100) was multiple antigen peptide-synthesized as five overlapping peptides using standard Fmoc (N-(9-fluorenyl)methoxycarbonyl) synthesis on an Advanced ChemTech 396 peptide synthesizer (34). These peptides were employed to immunize rabbits.

RESULTS

Bacteria Expressing E. coli, K. pneumoniae, or S. typhimurium Type 1 Fimbriae Exhibit Different Binding Specificities for Mouse BECs and GECs—We found that ORN103(pSH2) bacteria expressing E. coli strain J96 type 1 fimbriae bound well to BECs and moderately to GECs, whereas ORN103(pBP7) bacteria expressing K. pneumoniae strain IA551 type 1 fimbriae bound moderately to BECs but exhibited minimal binding to GECs. In contrast, ORN103(pISF101) bacteria expressing S. typhimurium strain 6704 type 1 fimbriae exhibited minimal binding to BECs and bound very well to GECs (Fig. 1, A and B). Thus, there exists remarkable variation among enterobacterial type 1 fimbriae in their ability to bind mucosal cells of gut and urinary tract origin. The binding of the fimbriated bacteria belonging to the three species was minimal in strains containing plasmids pUT2002, pBP799, and pKT303, which encode E. coli, K. pneumoniae, and S. typhimurium fimbriae, respectively, which are deficient in FimH. In addition, binding was inhibited by methyl α-L-mannopyranoside (data not shown). Taken together, the data suggest that FimH is the decisive determinant on the fimbriae responsible for epithelial cell binding.

Enteroxerobacterial Type 1 Fimbriae Exhibit Considerable Diversity in Their Ability to Aggregate Soluble Mannose-containing Glycoproteins—We examined the ability of each of the strains to bind and aggregate 10 soluble glycoproteins, most of which are known to be mannosylated. The capacity of type 1 fimbriated bacteria to aggregate mannosylated proteins is dependent not only on the availability of mannose residues in these molecules, but also on their number and correct spatial arrangement within the molecule. Only mannan and p-aminophenyl α-L-mannopyranoside/BSA were aggregated by all three type 1 fimbriated bacteria, albeit at different rates (Fig. 2, A and B; and TABLE TWO). The two glycoproteins were aggregated the fastest by bacteria expressing Salmonella fimbriae, followed by bacteria expressing E. coli fimbriae, with bacteria expressing Klebsiella fimbriae exhibiting the slowest and the lowest level of aggregation (Fig. 2, A and B). In addition, horseradish peroxidase (HRP) and porcine thyroglobulin were readily aggregated by bacteria expressing E. coli fimbriae, but not by either of the other type 1 fimbriated bacteria (Fig. 2C and TABLE TWO), whereas RNase B was not aggregated by any of the strains (Fig. 2D). Thus, E. coli, S. typhimurium, and K. pneumoniae type 1 fimbriae were highly selective in the nature of the mannoproteins that they aggregated. Moreover, there appeared to be distinct differences between fimbriae as to the level of aggregation in each case. Because none of the bacteria expressing FimH− fimbriae exhibited any aggregating ability, FimH is a critical determinant of glycoprotein aggregation by type 1 fimbriated bacteria.

The Distinct Binding Traits of Enterobacterial Fimbriae Cannot be Readily Ascribed to Variation in the Primary Structures of Their Respective FimH Adhesins—The DNA sequences of the fimH genes from plasmid pSH2 containing the E. coli strain J96 fim cluster and plasmid pBP7 containing the K. pneumoniae strain IA551 fim cluster (GenBankTM accession numbers AY914173 and AY914172, respectively) were determined, and the amino acid sequences were deduced (supplemental Fig. 1). Earlier work had determined the fimH sequences of E. coli K12 strain PC31 (5) and K. pneumoniae strain IA565 (15); however, our use of the fim operon from two different strains (E. coli strain J96 and K. pneumoniae IA551) required us to determine their respective fimH sequences. The primary structure of S. typhimurium strain 6704 FimH (FimHS) encoded by pISF101 has already been reported (GenBankTM accession number L19338). A comparison of the predicted amino acid sequences of E. coli strain J96 FimH (FimHE) and K. pneumoniae strain IA551 FimH (FimHK) revealed a high level of identity (98.6%). Both
**TABLE TWO**

| Glycoproteins                          | Glycoprotein aggregation by bacteria expressing fimbriae from different species | Glycoprotein binding by various MalE/FimH molecules |
|----------------------------------------|---------------------------------------------------------------------------------|----------------------------------------------------|
|                                        | **Ec** | **Kp** | **St** | **FimHE** | **FimHK** | **FimHS** |
| *S. cerevisiae* mannan                  | +      | +      | +      | +          | +          | +          |
| *p*-Aminophenyl α-d-mannopyranoside/BSA | +      | +      | +      | +          | +          | +          |
| Horseradish peroxidase                 | +      | -      | -      | +          | +          | +          |
| Porcine thyroglobulin                  | +      | -      | -      | +          | +          | +          |
| Bovine pancreas RNase B                | -      | -      | -      | +          | +          | +          |
| Soybean agglutinin                     | -      | -      | -      | +          | +          | +          |
| Human colostrum IgA                    | -      | -      | -      | +          | +          | +          |
| Chicken ovalbumin                      | -      | -      | -      | +          | +          | +          |
| Galactose/BSA                          | -      | -      | -      | -          | -          | -          |
| Fetuin                                 | -      | -      | -      | -          | -          | -          |

*Ec, *E. coli*; Kp, *K. pneumoniae*; St, *S. typhimurium.*

*a* Glycoproteins with little or no mannose.
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located in the C-terminal pilin domain (amino acids 160–277), which mediates the interactions of FimH with the fimbrial shaft (23). Mature Salmonella FimH is composed of 313 amino acids and is therefore considerably larger than either FimHE or FimHK. Moreover, even after multiple sequence alignments of mature FimHS and FimHE or FimHK, very limited homology can be seen (supplemental Fig. 1). Therefore, few clues to the molecular basis for the diversity in binding among enteric type 1 fimbriae are apparent from determining the primary sequences of their respective FimH lectins.

Enterobacterial FimH Subunits in the Absence of Their Respective Fimbrial Shafts Exhibit Similar Binding Traits—We investigated whether the differential binding among enteric type 1 fimbriae could be attributed to the intrinsic properties of their respective FimH lectins. We subcloned each of the fimH genes and expressed them as fusion proteins with MalE. MalE stabilizes FimH and also allows the fusion protein to be readily isolated from the recombinant clones (10). The periplasmic fraction from each of the isopropyl-β-D-thiogalactopyranoside-induced clones was isolated, and the MalE/FimH fusion proteins were affinity-purified on an amylose column and analyzed by SDS-PAGE. As shown in Fig. 3A, pure preparations of each of the fusion proteins were obtained. Most of the lower molecular mass bands in lanes 2–4 correspond to partially degraded forms of the full-length fusion proteins as validated by Western blotting using anti-FimH antibody (lanes 6–8). The difference in migration among the MalE (lane 1), MalE/FimHE (lane 2), MalE/FimHK (lane 3), and MalE/FimHS (lane 4) fusion proteins corresponds to the size differences of the respective FimH molecules. Western blotting using antibodies to MalE (data not shown) and to FimHE (lanes 5–8) confirmed that the bands in lanes 2–4 were indeed fusions of FimH and MalE. It is noteworthy that FimHE antisera are broadly cross-reactive with other enteric FimH molecules (10, 35).

Each of the fusion proteins was subjected to SDS-PAGE and then electrophoretically transferred to nitrocellulose strips. The immobilized proteins were then exposed to biotinylated BECs or GECs. In contrast to the selective binding traits that are characteristic of their respective fimH genes, all three FimH lectins bound readily to both BECs (Fig. 3B) and GECs (Fig. 3C). No epithelial cell binding was seen with MalE alone, indicating that MalE was functionally inert. All of the epithelial cell binding reactions exhibited by the MalE/FimH proteins were inhabitable by methyl α-D-mannopyranoside (data not shown), confirming the mannose specificity of the binding reaction.

We next examined the ability of the three MalE/FimH fusion proteins to bind the battery of glycoproteins listed in TABLE TWO. MalE/FimH fusion proteins were electrophoretically transferred to nitrocellulose and incubated with each glycoprotein. To detect any bound mannoproteins, we probed the nitrocellulose blots with alkaline phosphatase-labeled concanavalin A (a well known probe for the detection of mannosylated compounds), followed by an appropriate substrate. Interestingly, not only did the three fusion proteins exhibit comparable binding activity, but the binding range of each of the FimH proteins included most of the mannose-containing glycoproteins tested. The binding of each of the fusion proteins to HRP and RNase B is shown in Fig. 3 (D and E), and this is typical of other mannoproteins tested. Moreover, each of these binding reactions was inhabitable by 100 mM methyl α-D-mannopyranoside (data not shown). Thus, all three isolated FimH subunits bound comparably to a wide range of mannosylated substrates.

Bacteria Expressing Hybrid Fimbriae Composed of FimH on a Heterologous Fimbrial Shaft Have an Altered Binding Specificity—In previous studies, Madison et al. (27) found that the fimbrial shaft can modify the fine sugar specificity of FimH. In these studies, the FimH subunits of E. coli and K. pneumoniae were swapped on the fimbrial shafts of the two species. To see whether such a presentation also affects tissue tropism, we expressed FimHE on the K. pneumoniae fimbrial shaft (FimHE/ShaftK) and vice versa (FimHK/ShaftE). Because the hybrid fimbriae are derived from genes encoded on two separate replicons (diagrammed in supplemental Fig. 2), the stoichiometry of the fimbrial proteins produced may differ from that of proteins encoded by fimbrial genes present on a single replicon, such as pSH2 and pBP7. Therefore, as a control, we created reconstituted wild-type fimbriae with the plasmids used to create hybrid fimbriae by complementing fimH-deficient fimbrial gene clusters with homologous fimH. The strain designations and the type of fimbriae produced are shown in Fig. 4A. Attempts to con-
of the glycoproteins (Fig. 5, B and C). Thus, FimH expressed on a heterologous shaft acquired the glycoprotein specificity of its heterologous FimH (e.g. FimHK on the E. coli type 1 fimbrial shaft acts like FimHE on the E. coli type 1 fimbrial shaft). Neither of the hybrid fimbriae aggregated RNase B, indicating no dramatic increase in the breadth of mannosides recognized by FimH expressed on a heterologous type 1 fimbrial shaft (Fig. 5D). To rule out the possible effects of heterologous FimF and FimG, we performed the previous aggregation experiments using strains expressing heterologous FimH in the absence of heterologous FimF and FimG and found the same results (supplemental Fig. 3).

We next tested the ability of wild-type E. coli, wild-type K. pneumoniae, and hybrid type 1 fimbriae to mediate the previously described tropism for the bladder epithelium (Fig. 1A). Both wild-type fimbriae mediated adherence to BECs, but E. coli type 1 fimbriae (FimHE-ShaftE and FimHE/ShaftE) demonstrated a much greater capacity to mediate both adherence and the previously described bacterial invasion of BECs compared with wild-type K. pneumoniae type 1 fimbriae (FimHK-ShaftK and FimHK/ShaftK) (Fig. 6, A and B) (31, 36). FimHK/ShaftK hybrid fimbriae mediated adherence to and invasion of BECs at levels equal to those of wild-type E. coli type 1 fimbriae, whereas FimHE/ShaftK hybrid fimbriae mediated BEC interactions that were much more similar to those of wild-type K. pneumoniae type 1 fimbriae, thus further demonstrating the potential importance of the role the type 1 fimbrial shaft plays in determining FimH binding specificity and tissue tropism.

**DISCUSSION**

Because type 1 fimbriae are expressed by virtually all enterobacterial species, including nonpathogenic strains, there is controversy regarding their contribution to the infectious process. This controversy was fueled, at least in part, by the fact that, for many years, the only information regarding the binding properties of type 1 fimbriae was their uniform binding specificity for D-mannose (37, 38). However, once it was realized that type 1 fimbriae from various strains exhibit differences in their fine sugar specificity, studies were initiated examining the relevance of such differences to bacterial virulence. For example, uropathogenic E. coli strains that avidly bind uroepithelial cells and efficiently colonize the mouse bladder express type 1 fimbriae that possess a fine sugar specificity for monomannose and trimannose residues (26, 28). In contrast, fecal E. coli isolates that bind weakly to uroepithelial cells and poorly colonize the mouse bladder expressed type 1 fimbriae with specificity for only trimannose residues (26, 28). These studies revealed that the intraspecies variation in E. coli type 1 fimbrial fine binding specificity correlates with the capacity of distinct E. coli strains to colonize a particular tissue.

In this study, we have shown that interspecies variation in tissue tropism can be ascribed, in part, to differences in the fine sugar specificity driven by the association of FimH with the fimbrial shaft rather than the primary structure of FimH. This notion is based on the following findings. (i) Different enterobacterial species differentially adhere to BECs and GECs. S. typhimurium bound avidly to GECs; E. coli bound well to BECs and moderately to GECs; and K. pneumoniae bound moderately to BECs only. (ii) When K. pneumoniae FimH was expressed on the shaft of E. coli, the tissue tropism of the strain expressing the hybrid fimbriae was similar to that of the strain expressing the native E. coli fimbriae and vice versa. (iii) Once FimH was disengaged from the fimbrial shaft and expressed as a fusion protein with MalE, the differences in binding to epithelial cells were abolished irrespective of the origin of FimH. MalE/FimHE, MalE/FimHK, and MalE/FimHS also exhibited comparable binding to various soluble mannose-containing proteins as
well. Therefore, the distinct binding property exhibited by each enterobacterial fimbria is not solely an intrinsic property of their respective FimH adhesins. We determined that, although the primary structures of FimH\textsubscript{E} and FimH\textsubscript{K} are 98.6% identical, the FimH\textsubscript{S} structure exhibits very limited homology to either of the other two FimH proteins. The \textit{E. coli} FimH mannose-binding pocket is created by discontinuous regions of the molecule and seems to be conformation-dependent, so the similar basic mannose specificity of both \textit{E. coli} and \textit{S. typhimurium} FimH could arise from similar tertiary conformations, even though their primary structures are divergent (23, 39, 40). Because the MalE/FimH fusion proteins mediated mannose-binding properties on the overlay assays following SDS-PAGE, these proteins must have renatured and presumably regained their functionally competent conformation. Many proteins are known to renature and regain their conformation during electrophoretic transfer from SDS-polyacrylamide gels to nitrocellulose membrane (41–43).

Presumably, the mannose-binding pocket in each FimH is sufficiently flexible to interact with many mannose configurations. The role of the fimbrial shaft in modulating the binding spectrum of FimH could be by restricting the flexibility of the mannose-binding pocket or by limiting its accessibility to particular mannose configurations. The heterogeneity in the binding traits of FimH\textsubscript{E}, FimH\textsubscript{K}, and FimH\textsubscript{S} after they are incorporated into their native fimbriae suggests that the restrictive impact of each shaft is distinct. Because FimA, which constitutes >95% of the fimbrial shaft, is structurally and antigenically heterogeneous among various enterobacteria with sizes ranging from 14 to 22 kDa (11–22), the conformational change undergone by FimH is likely to be different in each fimbrial structure.

FimH is not only located at the tips of the fimbriae, but is also intercalated at intervals along the fimbrial shaft (35, 44). One explanation for our results is that the location (tip versus shaft) of FimH influences its fine sugar specificity; and therefore, if FimH is intercalated into the shaft of one fimbria but is present only at the tip of another, they would differ in their fine sugar specificity. However, for this to be true, FimH presented at the fimbrial tip as well as FimH intercalated along the shaft would both have to be able to mediate adhesion. Much previous work has indicated that only FimH present at the tips of fimbriae is functional in mediating adhesion (35, 45–48). For example, type 1 fimbriae have been observed to bind to erythrocyte membranes as well as the uropelithelium by the tips of the fimbriae (46, 49). In addition, non-aggregated cell-free type 1 fimbriae do not agglutinate erythrocytes, whereas aggregated type 1 fimbriae and whole bacteria both cause hemagglutination (45, 46, 48), strongly indicating that, with respect to adhesion, type 1 fimbriae are monovalent. The work of Ponniah et al. (47) supports this idea by showing that fragmentation of type 1 fimbriae greatly increases their mannose-binding activity by exposing FimH that was previously inaccessible.
intercalated into the fimbrial shaft, indicating that shaft-located FimH is not functional in adhesion. In addition, the expression of FimH on a heterologous fimbrial shaft leads to the acquisition of a specificity that appears to be dependent on the background of the fimbrial shaft, not the background of FimH, indicating that heterologous FimH and homologous FimH are incorporated into the shaft in a very similar manner. For example, the specificity of FimHK expressed on the E. coli type 1 fimbrial shaft closely mimics the specificity of FimHE expressed on the E. coli type 1 fimbrial shaft, indicating that FimHK and FimHE are incorporated into the E. coli type 1 fimbrial shaft in a similar manner, viz. at the fimbrial tip and intercalated along the length of the shaft. We therefore believe that the differences in fine sugar specificity are influenced by the manner in which FimH present at the fimbrial tip interacts with the fimbrial shaft.

Although FimA is the main component of the fimbrial shaft, the fimbrial tip structure is made up not only of FimH but also the minor fimbrial subunits FimF and FimG (50, 51). The potential for these subunits to modulate FimH specificity has not been ruled out by these studies; however, we demonstrated that strains expressing heterologous FimH in the absence or presence of heterologous FimF and FimG did not differ in their ability to aggregate yeast, HRP, or porcine thyroglobulin (supplemental Fig. 3). In addition, sequencing fimF and fimG of E. coli strain J96 (GenBank™ accession number DQ090770) and K. pneumoniae strain IA551 (GenBank™ accession number DQ090769) revealed 99.4% (one dissimilarity at amino acid residue 41) and 100% homology between mature E. coli and K. pneumoniae FimF and mature E. coli and K. pneumoniae FimG, respectively (supplemental Fig. 3), further minimizing the likelihood of their impact on our studies of type 1 fimbrial specificity.

Most work to date has directly related differential binding among E. coli type 1 fimbriae to alterations in the primary sequence of FimH (52–55). Sequence variations that diminish the mannose-binding ability of FimH were found to be located within or close to the sequences that make up the FimH binding pocket as defined by the FimH crystal struc-
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ture (23, 40, 54), although the location of the FimH binding pocket in intact fimbriae may vary somewhat from that determined by the crystal structure of FimH complexed with only its chaperone, FimC (23). However, sequence alterations that increase the monomannose-binding ability of FimH, which is thought to play a role in the tropism of uropathogenic E. coli for the bladder epithelium, are not located near the FimH binding pocket, but are instead located in the lower part of the FimH lectin domain defined by the tertiary structure of FimH (26, 28, 39, 40, 54). Therefore, rather than altering the FimH binding pocket directly, these sequence variations may instead alter the conformational stability of the binding pocket, such as we hypothesize would occur by altering the FimH interaction with the fimbrial shaft. In fact, replacement of the pilin domain of FimH (amino acids 160–279), which directs incorporation of FimH into the type 1 fimbrial shaft, with the corresponding pilin segment of the E. coli type 1C fimbrial adhesin FocH significantly alters the binding phenotype of the FimH/FocH hybrid type 1 fimbriae without altering the primary sequence of the FimH lectin domain (amino acids 1–156) (23, 39). Presumably, this is because of an altered conformation of the FimH binding site when incorporated into an altered fimbrial shaft via the FocH pilin domain (39). The importance of quaternary association in polymeric adhesins in determining binding specificity was recently revealed in studies of the plant snowdrop and garlic lectins (56). Although subunits of both lectins are structurally similar in many respects, their carbohydrate specificity varies considerably, and this is directly linked to differences in their oligomerization (56).

In summary, we have determined that enterobacterial FimH intrinsically has the ability to exhibit a broad range of mannose-specific binding interactions, but that this trait is modulated by the fimbrial shaft on which it is presented. We believe that the influence of the shaft on FimH forms part of the molecular basis for the tissue tropism exhibited by different type 1 fimbriae. This supplemental binding mechanism (e.g., modulation of the basal specificity of FimH for d-mannose by the fimbrial shaft) may have evolved as different enterobacteria adapted to colonize a particular niche. This capacity of each fimbrial shaft to modulate the binding activities of its FimH subunit could be critical to microbial pathogenesis because it enables the pathogen to selectively colonize sites in the host that are presumably supportive to its growth. Our findings reveal a novel and physiologically relevant function for these highly organized cell-surface organelles.

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