Structural basis of tropism of *Escherichia coli* to the bladder during urinary tract infection

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Summary

The first step in the colonization of the human urinary tract by pathogenic *Escherichia coli* is the mannose-sensitive binding of FimH, the adhesin present at the tip of type 1 pili, to the bladder epithelium. We elucidated crystallographically the interactions of FimH with D-mannose. The unique site binding pocket occupied by D-mannose was probed using site-directed mutagenesis. All but one of the mutants examined had greatly diminished mannose-binding activity and had also lost the ability to bind human bladder cells. The binding activity of the monosaccharide D-mannose was delineated from this of mannotriose (Man(a1–3)(Man(a1–6))Man) by generating mutants that abolished D-mannose binding but retained mannotriose binding activity. Our structure/function analysis demonstrated that the binding of the monosaccharide α-D-mannose is the primary bladder cell receptor for uropathogenic *E. coli* and that this event requires a highly conserved FimH binding pocket. The residues in the FimH mannose-binding pocket were sequenced and found to be invariant in over 200 uropathogenic strains of *E. coli*. Only enterohaemorrhagic *E. coli* (EHEC) possess a sequence variation within the mannose-binding pocket of FimH, suggesting a naturally occurring mechanism of attenuation in EHEC bacteria that would prevent them from being targeted to the urinary tract.

Introduction

Bacterial attachment to mammalian tissue constitutes the first step in the colonization of their hosts. *Escherichia coli*, the aetiological agent in greater than 80% of all urinary tract infections (UTIs) (Hooton and Stamm, 1997), mediates mannose-sensitive attachment to receptors found lining the urinary tract, an event that has been shown to be critical in bladder disease (Martinez et al., 2000). The adhesive fibre required for these host–pathogen interactions is the type 1 pilus. Type 1 pili are 1–2-μm-long composite fibres consisting of a cylindrical rod comprising repeating immunoglobulin-like (Ig) FimA pilin subunits, and a short and stubby tip fibrillum (Brinton, 1965). They are assembled by the chaperone/usher pathway (Soto and Hultgren, 1999) and in their mature form the Ig fold of every subunit is completed by an amino-terminal extension from a neighbouring subunit in a process termed ‘donor strand exchange’ (Choudhury et al., 1999; Sauer et al., 1999; Barnhart et al., 2000). FimH is the mannose recognizing adhesin. It is a two-domain protein consisting of an amino-terminal receptor binding domain linked to a carboxy-terminal pilin domain (Jones et al., 1995; Choudhury et al., 1999). At the tip of the FimH receptor-binding domain, distal to the pilin domain, there is a pocket capable of accommodating a mannose unit (Knight et al., 2000). In the crystal structure of the FimH–FimC complex (Choudhury et al., 1999), this pocket was shown to accommodate cyclohexylbutanoyl-N-hydroxyethyl-D-glucamide (C-HEGA), an additive for the crystallization, which rather distantly resembles the physiologically relevant receptor, D-mannose. The interactions of FimH with D-mannose have yet to be characterized.

FimH is a critical factor in the ability of *E. coli* to colonize the bladder and persist in the urinary tract. A consequence of bacterial adherence via FimH is the activation of a cascade of innate defences that leads to the exfoliation of bladder epithelial cells and an inflammatory response (Mulvey et al., 1998). *Escherichia coli* are able to evade these host responses, in part, by invading into the bladder epithelium in a process that is facilitated by
FimH (Martinez et al., 2000). Mice and primates vaccinated with the FimH adhesin are protected from infection (Langermann et al., 1997). FimH binds to D-mannose and mannotriose receptors, with the former being more highly associated with cystitis isolates (Sokurenko et al., 1995; 1997; 1998; 2001). Attachment of uropathogenic E. coli (UPEC) to the bladder epithelium is proposed to be mediated by FimH binding to the uroplakins 1a and 1b (Wu et al., 1996). These cellular receptors contain a single N-linked carbohydrate of the high-mannose type, common to most glycoproteins recognized by FimH. Several other reported FimH receptors include Tamm–Horsfall protein (Pak et al., 2001), CD48 (Baorto et al., 1997), laminin (Kukkonen et al., 1993), collagen (Pouttu et al., 1999), fibronectin (Schembri et al., 2000) and abiotic surfaces (Pratt and Kolter, 1999). Based on inhibition studies, the carbohydrate binding site of FimH has been suggested to be an extended pocket with optimal fit for a trisaccharide.

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and with a hydrophobic region in or close to the combining site (Firon et al., 1983; 1987).

Here, we present the crystal structure of the FimCH chaperone–adhesin complex bound to the D-mannose receptor, together with a mutational analysis of the FimH residues defining the mannoside-binding pocket. Bacteria expressing these mutants were analysed for the ability to mediate haemagglutination of guinea pig red blood cells, the adherence and invasion into a human bladder cell line, as well as to human bladder sections in situ. Purified mutant FimH proteins were analysed for their ability to bind D-mannose and mannotriose in vitro. Our interdisciplinary studies revealed the molecular basis for the association of UTI with the binding of FimH to mannose and provide a molecular snapshot of the critical interactions necessary for persistent bladder infections.

Results

Crystal structure of FimCH in complex with α-D-mannopyranoside

The structure of the FimCH chaperone–adhesin complex bound to D-mannopyranoside was determined to 2.8 Å resolution. The mannose is buried in a unique site at the tip of the receptor-binding domain, an elongated 11-stranded β-barrel comprising residues 1–157 (Fig. 1A), in a deep and negatively charged pocket (Fig. 1D). Choudhury and co-workers (Choudhury et al., 1999) found the glucamide moiety of C-HEGA bound in this same pocket, in a conformation closely resembling a pyranoside. The position, configuration and orientation of the D-mannose are unambiguously defined by the electron density (Fig. 1C). FimH selects out the alpha configuration around the free reducing anomeric oxygen O1 of the D-mannose. All of the D-mannose hydroxyl groups other than O1 interact extensively with the receptor-binding pocket of FimH. Residues Phe1, Asn46, Asp47, Asp54, Gln133, Asn135, Asp140 and Phe142 of FimH interact with D-mannose by hydrogen bonding and hydrophobic interactions. All of the residues involved in carbohydrate binding are situated at the ends of β-strands or in the loops extending from them, with the exception of Gln133, which is located within strand 10 (Fig. 1B). Asp54 makes co-operative hydrogen bonds with O4 and O6 of the D-mannose (Fig. 1C). O6 further directly interacts with the backbone of Asp47, the side-chain oxygen of Asn46 and the NH2 terminal group of the FimH polypeptide. The NH2-terminal group makes additional interactions with O2 and O5. O2 also interacts with the only water molecule inside the pocket (W1 in Fig. 1C). Gln133 and Asp140 bind to O3. Asn135 makes a bifurcated hydrogen-bonding interaction with O3 and O4. Phe142 makes a hydrophobic interaction with the C2-C3 bond. Phe1 stabilizes the pocket by stacking its aromatic ring with the side-chains of Gln133 and Phe144. Interestingly, the mannoside-binding pocket is surrounded by a hydrophobic ridge comprising Ile13, Tyr48, Ile52 and Phe142 (Fig. 1A–D).

Functional analysis of mannoside-binding pocket

A mutational analysis of Asn46, Asp54, Gln133, Asn135 and Asp140 of FimH was carried out to probe the importance of the size and charge of these residues in interacting with the mannoside receptor (Table 1). In addition to these residues, we investigated the role of Ser62. Position 62 is located outside the mannoside-binding pocket but has been implicated in the binding of FimH to collagen type I and type IV (Pouttu et al., 1999) when it exists as an alanine. Thus, as a control, Ser62 was changed to alanine. The effect of each mutation on mannoside recognition was analysed using a number of functional assays including measuring haemagglutination (HA) titre as well as analysing adherence and invasion into bladder epithelial cells. The ability of each mutant FimH protein to be assembled into the pilus was confirmed to rule out any possible assembly defects caused by a mutation. Co-expression of each of the FimH mutants with the chaperone FimC led to stable FimCH complexes, an event that is necessary for FimH assembly into a pilus (Sauer et al., 2000), and immunofluorescence staining demonstrated that all of the FimH mutant proteins were assembled into pilus (Table 1). To enhance our insight into structure–function, correlates of the mannoside-binding pocket, one of the mutant FimCH complexes (Q133N) was crystallized (see below). All of the mutations abolished FimH function as determined by the HA (Table 1) and bladder adherence assays (Fig. 2A and B), with the exception of N46D (threefold reduction) and the S62A control. Accordingly, all N46D and S62A FimH mutants were able to facilitate invas...
Fig. 2. Adherence to and invasion of 5637 bladder cells.

A and B. AAEC185/pUT2002 bacteria complemented with FimH did not exhibit any significant binding with the exception of S62A and N46D mutants. The x-axis represents the percentage of total input bacteria (surface bound and/or invaded).

C. Invasion of bound bacteria expressing mutant FimH proteins into 5637 cells.

D–G. Binding of type 1-piliated bacteria to human bladder sections. AAEC185/pUT2002 bacteria complemented with wild-type (D), N46D (F) and N46A (G) FimH (Q133A, Q133K, and D54A FimH behaved as in G). Wild-type FimH binding inhibited by methyl-α-D-mannose (E). Wild-type FimH binding inhibited by methyl-α-D-mannose (E).

Mannose- and mannotriose-binding activities of FimH

Different allelic variants of FimH exist among clinical isolates of UPEC and have been shown to affect the mannos-binding properties of FimH (Sokurenko et al., 1998). These allelic variants can be broadly divided into two functional groups: those that bind mannotriose...
only and those that also are capable of binding D-mannose. D-Mannose binding activity has been correlated to an increased virulence phenotype amongst UPEC (Sokurenko et al., 1997). Therefore, we investigated the effect of each mutation on D-mannose and mannotriose binding using purified FimCH complexes in enzyme-linked immunoabsorbance assay (ELISA), POROS (which is a measure of mannotriose binding) and D-mannose chromatography assays.

We first examined D-mannose binding. All mutant FimHs except for N46D and the S62A control were defective in D-mannose binding when tested by ELISA (Fig. 3A). The N46D mutant, not S62A, has a reduced binding to D-mannose as detected by ELISA. Although the mutated N46A, N46D, D140A, D140N and D140E FimH proteins bound mannose-coated beads to approximately the same extent as wild-type FimH, they were eluted by methyl-α-D-mannose in significantly greater relative amounts than the wild-type or S62A FimH (Fig. 3C), indicating that these FimH mutants have decreased mannose binding affinity.

The binding to mannotriose was next examined and compared with both mannose binding and binding to the bladder. Wild-type FimH was able to bind mannotriose approximately 10 times better than mannose (Fig. 3A and B). The binding of N46D FimH to mannotriose was unaffected by the mutation (Fig. 3B and D). In contrast, the N46A mutation drastically reduced binding to mannotriose (Fig. 3D). Although mutations at Asp140 greatly reduced (D140N) or abolished (D140A, D140E) mannose binding in the ELISA and mannotriose bead-binding assays (Fig. 3A and C), these mutant FimHs retained their ability to bind mannotriose (Fig. 3B). In fact, D140N bound mannotriose (Fig. 3B) with the same relative affinity as wild-type FimH bound mannotriose (Fig. 3A). N46D and D140N FimH were retained in the POROS assay (Fig. 3D) similarly to wild-type FimH, confirming their ability to bind mannotriose. The N46D mutation reduced binding to bladder epithelial cells by only threefold, whereas the N46A, D140A, D140N, and D140E mutations abolished binding (Fig. 2A and B). Hence, mannose and not mannotriose, appears to be the critical receptor in the colonization of uropathogenic E. coli to the bladder. Consistent with this conclusion, Q133A, N135A and N135D FimH proteins are impaired in their ability to bind bladder epithelium (Fig. 2A and B) and mannose (Fig. 3A and C), yet, their ability to...

Fig. 3. A and B. Mannose-binding studies of FimCH complexes. Binding of FimCH complexes to mannose-BSA-coated wells (A) and mannotriose-BSA wells (B) in ELISA studies (N46A is not included, but consistently did not bind in both A and B). C. Coomassie-stained SDS–PAGE gels show the binding of purified FimCH complexes to mannose-coated beads and their elution by methyl-α-D-mannose. Top: Most FimH mutants retained the ability to bind mannose-coated beads. Bottom: Elution with methyl-α-D-mannose. D. POROS chromatography of FimH mutants on mannotriose-BSA. A decreasing peak retention time ratio indicates a faster OFF-rate relative to wild-type FimH.

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bind mannotriose is in part retained (Fig. 3D). The D54A, D54N, Q133N and Q133K FimH mutants were dramatically impaired in D-mannose binding, as revealed by the fact that they exhibited virtually no detectable binding to mannose (Fig. 3A and C), mannotriose (Fig. 3B and D) or bladder epithelial cells (Fig. 2A and B). Thus, D-mannose binding seems to be the most specific and stringent reaction that is strictly correlated with pathogenicity in the urinary tract.

Conservation of mannose-binding pocket

All of the mutations in the receptor binding pocket reduced or abolished binding to mannose and bladder epithelial cells, suggesting that the binding pocket may be highly conserved in nature. To investigate this, we sequenced the fimH gene in over 200 uropathogenic strains of E. coli and one EHEC. The alignment results can be divided into 17 groups as shown in Fig. 4. Overall, there was very little heterogeneity in the FimH sequences among different isolates. There were no sites that varied by more than two residues suggesting systematic variation or structural restrictions. All of the residues involved in D-mannose binding and the regions around them were invariant, giving strong evidence that the mannose-binding pocket is an invariant feature of FimH in all uropathogenic strains of E. coli. EC189, the serotype O157 enterohaemorrhagic strain, shares an identical FimH sequence as O157:H7 and O157:H7 EDL933 (GenBank accession nos BAB38702 and AAG59502 respectively). All three EHEC isolates contain a mutation in the mannose-binding pocket at position 135. In UPEC, position 135 is an asparagine, whereas in the three EHEC strains it is a lysine. Mutations in UPEC FimH at position 135 (N135A and N135D) abolished the ability of FimH to bind bladder cells.

Crystal structure of the Gln133Asn mutant

The structure of the FimCH complex containing the Q133N mutation in FimH was derived from crystals grown in the presence of methyl-α-D-mannopyranoside and refined to 3 Å resolution. The mannose was bound in the mannose-binding pocket (Fig. 5C), consistent with the residual binding affinity that Q133N FimH had to mannosyl-coated beads (Fig. 3C). Asn133 did not link to O3 of the mannose. Interestingly, the mutation not only affected the interactions originally made by Gln133, but the mannose also lost all interaction with Asn135 and Asp140 (Fig. 5C). A shift of 0.7 Å in the protein backbone at Asp140 and the mannose sugar ring from its position in the wild type, together with changes in the side-chain conformations of Asn133, Asn135, Asn138 and Asp140 (Fig. 5A), enables these residues to take part in a very different hydrogen bonding network (Fig. 5C) to that which was present in the wild-type FimCH–mannose structure (Fig. 5B) (the latter being identical to in the C-HEGA-bound FimCH structure; Choudhury et al., 1999). The new hydrogen bonding network stabilized the modified binding pocket and thereby included a new water molecule, W2.

![Fig. 4. Alignment of the amino acid sequences of the FimH receptor-binding domain from over 200 representative clinical isolates. The grey-coloured boxes show the residues building up the mannose-binding pocket. Residues identical to the amino acid sequence of the FimH crystal structure (J96) were indicated by ‘.’ The other positions shown were found to be heterogeneous. Those sequences not shown were found to be conserved among all isolates.](image-url)
W2 interacted directly with O3 of mannose. W1 was conserved and interacted both with O2 of mannose and the amide group of amino acid 133, as in the wild-type complex. The α-linked methyl group on the anomic O1 of D-mannose pointed out of the pocket and made a hydrophobic interaction with Tyr48 (3.7 Å). It is unlikely that the methyl group α-linked to the D-mannose could be responsible for any of the structural rearrangements that we observe within the pocket of the Q133N mutant, as it is physically located outside the pocket. The hydrophobic part of the Gln133 side-chain makes close van der Waals contacts with the Phe1 aromatic ring (Fig. 5B). The shorter Asn133 side-chain compensated for the lack of the penultimate carbon Cg of Gln133 by establishing an amino–aromatic stacking interaction: Asn133 pointed its amide nitrogen atom towards the Phe1 ring (Fig. 5C). These results explain how mutating a single side-chain can dramatically affect the geometry of the mannos-binding pocket.

Discussion

The three-dimensional structure of the FimH adhesin bound to its mannos-binding pocket was determined, and the binding pocket was characterized by site-directed mutagenesis and an extensive functional analysis. Our structure–function studies have revealed the molecular basis of a highly conserved host–pathogen interaction that is necessary for the establishment of persistent urinary tract infections (Mulvey et al., 1998). The FimH pocket is perfectly designed to engage in tight interactions with a monosaccharide receptor. It establishes an unusually large interface involving multiple specific interactions with its monosaccharide receptor: the solvent-accessible surface area buried at the interface between the D-mannose and FimH is 368 Å² compared with 312 Å² for methyl-α-D-mannopyranoside bound to concanavalin A (Naismith et al., 1994), or 716 Å² between the P pilus adhesin, PapGII, and its tetrasaccharide receptor (Dodson et al., 2001). A function of the hydrophobic ridge around the pocket may be to direct the sugar into the pocket in a manner that facilitates polar interactions within the FimH pocket. The reducing anomic O1 of the D-mannose bound to wild-type FimH in our crystal structure does not interact with any FimH residue and projects into the solvent. The FimH protein imposes the α-configuration on the C1-O1 bond of the D-mannose so that the free O1 is in a position axial to the sugar ring. Both features confirm functional binding of the D-mannose receptor in our crystal structure. In its axial position, the free O1 can make a α-glycosidic linkage, which is a prerequisite for a terminal mannos binding to high-mannose or hybrid type asparagine-linked oligosaccharides. A terminal mannos residue on glycoproteins was found to be a basic requirement for binding of FimH (Sokurenko et al., 1997). On the bladder epithelium, the uroplakins 1a and 1b each expose a single N-linked carbohydrate of the high-mannose type (Wu et al., 1996) and they are therefore presumably physiological FimH receptors.

The increase in affinity of FimH for mannotriose and mannopentaose compared with D-mannose is probably due in part to the stacking of the hydrophobic face of the saccharides onto the hydrophobic ridge around the pocket, formed by Ile13, Tyr48, Ile52 and Phe142. The methyl group on O1 of methyl-α-D-mannose in the Q133N FimH structure is involved in such a hydrophobic stacking interaction with Tyr48. The increase in affinity for the specific binding of oligomannosides is approximately to the same extent (30 times) as for the binding of mannosides carrying aromatic substituents like p-
From nitrophenyl-mannopyranoside (Firon et al., 1987). Similar increases in affinity due to the addition of either sugar rings or hydrophobic substituents is a feature also observed in plant lectins that have a hydrophobic patch next to the primary binding site, e.g. concanavalin A (Bouckaert et al., 1999). The hydrophobic character of the FimH pocket ridge distinguishes E. coli type 1 adhesins from Salmonella sp. type 1 adhesins. The latter do not seem to have increased affinity for mannose with hydrophobic substituents or for larger mannosides (Firon et al., 1984).

Our structure–function study led us to define the structural basis for the tropism of UPEC FimH variants for terminal α-D-mannose. From all mutations in the mannose-binding pocket of FimH, N46D is the only mutation that does not completely abolish binding to bladder epithelium. The N46D mutation reduced binding to bladder cells by about threefold (Fig. 2B), similar to its threefold reduced affinity for mannose (Fig. 3A). On the other hand, it retained the ability to bind mannnotriose with the same relative affinity as wild-type FimH (Fig. 3B). The retained, albeit reduced, ability of N46D FimH to bind bladder cells is probably related to the fact that Asp46 can engage, at least in part, in the same interactions stabilizing the pocket as Asn46. The D140N FimH mutant retained an ability to bind mannnotriose (Fig. 3B), similar to wild-type FimH binding of mannose (Fig. 3A), but was unable to bind bladder tissue (Fig. 2G). Thus, mannose but not mannnotriose binding appears to be strictly correlated with the physiologically relevant function of FimH in binding bladder epithelium. The co-operative hydrogen bonding of Asp54 to O4 and O6 of the mannose, and the role of this residue in the construction of the binding pocket is predominant because mutation of Asp54 (D54A and D54N) leads to complete loss of both D-mannose and mannnotriose binding. This suggests that the functional mannose-binding pocket is part of the mannnotriose binding site. In conclusion, even the slightest change in the mannose-binding pocket, in an atom (Asn46 Nδ2) that does not directly bind to mannose (Fig. 1C), significantly reduces binding. These data and the invariant nature of the pocket in over 200 uropathogenic isolates emphasize the finely tuned nature of this interaction. The structure of the Q133N FimH mutant (in complex with FimC) gives a novel insight into the effect of such a single residue mutation. The mutation leads not only to the loss of multiple FimH–mannose interactions, four instead of the one normally established by the mutated residue alone, but also results in a different hydrogen bonding network and a different geometry of the pocket, which render mannose binding less favourable.

Interestingly, only EHEC possess a sequence variation within the mannose-binding pocket of FimH. Enterohaemorrhagic strains (EHEC) are the cause of haemolytic uraemic syndrome, which results in acute kidney failure (Noel and Boedeker, 1997). This syndrome is thought to be the effect of the Shiga toxin, which enters the blood stream and subsequently locates to the kidney because of its receptor binding specificity (Cooling et al., 1998; Kiyokawa et al., 1998). An inspection of the FimH gene sequences from three different O157:H7 enterohaemorrhagic strains revealed that the binding pocket residue Asn135 was changed to a lysine. Based on the FimH–mannose crystal structure, a lysine at this position would be predicted to exclude mannose from the binding pocket. A dysfunctional mannose-binding pocket would render EHEC strains unable to colonize the bladder and establish an infection. Indeed, although EHEC strains possess the type 1 pilus gene cluster, there is a lack of an association of EHEC strains with urinary tract infections. This may represent a natural selection for a less virulent phenotype, as colonization of the urinary tract would lead to a direct delivery of the toxin to the kidney, causing drastic and rapid consequences to the host. However, there have been several reported cases of non-diarrhoeal haemolytic–uraemic syndrome in association with urinary tract infections (Starr et al., 1998; Miedouge et al., 2000; Hogan et al., 2001). These patients were infected with Shiga toxin-producing, non-O157:H7 E. coli. In the absence of the sequence information for these strains, we would predict that the FimH genes in these isolates do not contain mutations in the mannose-binding pocket.

The recently determined crystal structure of the PapGII receptor binding domain in complex with its specific receptor, globotetraoside (Dodson et al., 2001), suggests structural convergence with the FimH receptor binding domain. PapGII is the adhesin on E. coli Pap pili involved in pyelonephritis. Superposition of the two receptor binding domains (r.m.s. deviation of 1.9 Å), shows that the overall shape and size of the receptor binding domains of these adhesins are remarkably similar (Fig. 6), despite their dissimilar sequences and folds. They both are elongated molecules, approximately 22 Å wide, but variable in length. This implies a structural constringency to which these molecules should satisfy, most probably due to the passage through the usher during pilus assembly. The location and the shape of the carbohydrate binding sites grafted onto these structural platforms differs strongly and is related to the tropism of these proteins. The deep FimH binding pocket is located at the tip, whereas PapG has a more shallow and extended binding site at the side of the molecule (Fig. 6). FimH carries the small deep pocket with an unusually large interaction surface to allow sufficiently high affinity for a single D-mannose, which is a prerequisite for pathogenicity in UTIs. It could easily interact via its tip side, with the terminal mannose on glycoproteins in the bladder. PapGII has a fourfold larger minimal receptor, and the position of the binding site at the side of...
the molecule may allow additional interactions of the hydrophobic tip of PapG with the lipidic part of its glycolipid receptor in the kidney (Dodson et al., 2001).

This study combined molecular biology and macromolecular crystallography to provide a detailed structure–function analysis of the receptor-binding pocket of FimH. The data obtained in this study provide the foundation for the development of vaccines and therapeutic drugs that target the type 1 pilus adhesin and are effective in the treatment of UTIs. In addition, because mannose binding is known to block FimH binding to human collagens (Poulttu et al., 1999) (although the mechanism is currently unknown), a mannose mimic that inserts into the sugar-binding pocket could also prove useful in preventing septicemia and meningitis caused by E. coli of collagen-binding phenotype in newborns.

**Experimental procedures**

**Plasmid constructions, bacterial strains, eukaryote cell lines and mutagenesis**

Plasmid pHACW18, containing the fimH gene of the urinary tract infection (UTI) strain J96, within the EcoRI–BamHI sites of pUC18, was used as the template for the site-directed mutagenesis. Plasmid pHJ9205 contained the fimC gene driven by the inducible arabinose promoter (S. J. Hultgren and C. Hal Jones, unpublished work) and was used in combination with the pHACW18 derivatives (in pMMB66 as described below) for the expression of FimCH complexes. The plasmid pUT2002, having a fimH-deleted type 1 operon driven by the natural promoter, was described previously (Minion et al., 1989). The isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible expression vector, pMMB66 (Furste et al., 1986), and the E. coli strains MC1061 (Wertman et al., 1986), ORN103 (Orendorf and Falkow, 1984), C600 (Sambrook et al., 1989) and AAEC185 (Blomfield et al., 1991) (type 1 pilus null) were used in this study. Site-directed mutagenesis was performed using a two-step polymerase chain reaction (PCR) as described (Hung et al., 1999), using pHACW18 as the template. The PCR products were cloned into EcoRI and XcmI site of pHACW18. Mutations in FimH were confirmed by sequencing. Each mutant and the wild-type FimH were subcloned as an EcoRI–BamHI I full-length fimH gene into pMMB66. The original pMMB66 expression vector was used as the negative control plasmid. All bacteria used were grown in Luria–Bertani broth (LB) with appropriate antibiotics. The human bladder epithelial cell line 5637 (ATCC HTB-9) was grown in RPMI1640 supplemented with 10% fetal bovine serum (FBS) and cultured according to standard tissue culture techniques.

**Haemagglutination assays**

ORN103/pUT2002 complemented with FimH expression constructs were induced to express FimH and type 1 pili. Briefly, bacteria were first grown overnight in shaking incubators at 37°C. Bacteria were diluted 10-fold and subcultured statically overnight in the presence of 1 μM IPTG. Haemagglutination assays with guinea pig erythrocytes were performed following published protocols (Hultgren et al., 1990; Slonim et al., 1992).

**Bacterial surface staining of FimH proteins**

Bacterial strain AAEC185 transformed with pUT2002 was complemented with either wild-type or each of the mutant FimH expression plasmids. These bacteria were cultured in the same manner as described above for FimH and type 1 pili expression. Overnight cultures were diluted to OD∞00 = 1, and 1 ml of bacteria was used to immunostain for FimH on the bacterial surface. Surface-located FimH proteins were detected with anti-FimH antiserum followed by Oregon Green-conjugated goat anti-mouse IgG (H + L) secondary antibody. Bacteria were then fixed with 2% glutaraldehyde with 1 μg/ml of Hoechst stain (Sigma) for 5 min at room temperature (RT). The staining of FimH on bacterial surfaces was visualized with an Olympus BX60 microscope system.

**Adherence and invasion assays**

AAEC185/pUT2002 transformed with FimH expression plasmids were used to assay FimH-mediated bacterial adherence and invasion into a human bladder cell line, 5637. Bacteria were cultured as described above for type 1 pili expression. Adherence and invasion assays were performed with 1 h infection (Elsinghorst, 1994; Martinez et al., 2000). Results were obtained from at least two different infection experiments with duplicate wells in each experiment. In situ binding to human bladder tissues was performed similarly to the previously described protocol with minor modifications (Falk et al., 1993; Striker et al., 1995). Non-diseased human bladder sections were obtained from the surgical pathology...
and autopsy files of the Department of Pathology at Washington University. Human bladder tissues on microscope slides were deparaffinized and incubated with 100 ml of freshly FITC-labelled bacteria for 2 h at RT in a humidified chamber. Subsequently, slides were washed extensively with phosphate-buffered saline (PBS) (0.12 M NaCl, 2.7 mM KCl, 10 mM phosphate, pH 7.4), fixed for 5 min with 2.5% glutaraldehyde in PBS and counterstained with 1 mg l–1 of Hoechst stain for 5 min. Visualization of bound bacteria was performed on an Olympus BX60 microscope system.

**FimCH purification, crystallization, data collection and refinement**

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**Table 2** Crystallographic data and refinement statistics.

<table>
<thead>
<tr>
<th>PDB entry code</th>
<th>FimH-D-mannose-FimC</th>
<th>Q133N FimH-Me-α-D-Man-FimC</th>
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<tr>
<td>space group</td>
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<td>C2</td>
</tr>
<tr>
<td>unit cell</td>
<td>a (Å) 138.077</td>
<td>a (Å) 138.349</td>
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<td></td>
<td>b (Å) 138.130</td>
<td>b (Å) 138.334</td>
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<td></td>
<td>c (Å) 215.352</td>
<td>c (Å) 213.212</td>
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<tr>
<td>β (°)</td>
<td>90.005</td>
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<tr>
<td>Molecules per asymmetric unit</td>
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<td>8</td>
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<tr>
<td>Resolution (Å)</td>
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<td>45–3.0</td>
</tr>
<tr>
<td>Number of observed reflections</td>
<td>370 427</td>
<td>197 848</td>
</tr>
<tr>
<td>Number of unique reflections</td>
<td>99 138</td>
<td>72 289</td>
</tr>
<tr>
<td>Last resolution shell (Å)</td>
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<td>3.11–3.00</td>
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<tr>
<td>R-merge (%)</td>
<td>6.9 (47.8)</td>
<td>8.7 (51.0)</td>
</tr>
<tr>
<td>completeness (%)a</td>
<td>99.8 (99.9)</td>
<td>87.1 (65.9)</td>
</tr>
<tr>
<td>&lt;(σ(I))/I&gt;</td>
<td>13 (2.7)</td>
<td>10.6 (2.1)</td>
</tr>
<tr>
<td>Reflections with I &gt; 2σ(I) (%)a</td>
<td>83.8 (52.4)</td>
<td>82.3 (60.8)</td>
</tr>
<tr>
<td>Number of protein atoms</td>
<td>29 168</td>
<td>29 160</td>
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<tr>
<td>Number of water molecules</td>
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<tr>
<td>Sigma cut-off used in refinement</td>
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<tr>
<td>Crystallographic R-factora</td>
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<tr>
<td>Rfree –factora</td>
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<td>0.280 (0.39)</td>
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<tr>
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<tr>
<td>r.m.s. bond angles (deg.)</td>
<td>1.4</td>
<td>1.3</td>
</tr>
</tbody>
</table>

*a value between brackets is for the last resolution shell.

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**Binding of FimCH complexes to mannose-coated sepharose beads**

Sepharose 6B were coated with saturating amounts of α-D-mannose (Sigma) and resuspended as 50% (v/v) slurry in 20 mM MES, pH 6.8. FimCH complexes (20 μg) were incu-
bated with 100 μl of the mannoside beads for 2 h in a reaction volume of 200 μl. Unbound proteins were removed and beads were washed extensively with PBS. The beads were divided into two equal portions. To one portion, 50 μl of SDS–PAGE loading buffer was added (bound FimCH); to the other half, 50 μl was incubated for 60 min with 1% methyl-α-D-mannose (eluted FimCH). Proteins in the bound and eluted fractions were resolved on 15% SDS–PAGE gels and stained with Coomassie stain.

ELISA

For ELISA experiments, Immulon 4 plates were coated overnight at 4°C with 0.1 μg per well of mannos- or mannotriose-BSA. FimCH samples were diluted in PBS/0.05% Tween-20/0.1% BSA, and 100 μl of protein samples were added into each well. Bound FimCH complexes were detected with biotin-conjugated anti-FimC monoclonal Ab followed by horseradish peroxidase-conjugated streptavidin (Tropix). The ELISA reaction was developed with 3,3¢,5,5¢-Tetramethylbenzidine (KPL) substrate for 10 min and stopped with 50 μl per well of 2 N H₂SO₄. Reaction plates were read on SOFTmax at 450 nm.

POROS chromatography

To evaluate the mannotriose-binding affinities of FimH mutants, a high-performance liquid chromatography (HPLC)-format assay was developed using methacrylate resins (PE Biosystems). The mannotriose (Man(α1–3)[Man(α1–6)]Man)-BSA conjugate was coupled to the resin via epoxide chemistry. The column, which has a bed volume of 0.2 ml, was equilibrated with modified PBS (33.3 mM phosphate, 150 mM NaCl, pH 7.2) and run at a flow rate of 1 ml min⁻¹. Purified FimCH complexes were diluted to 10 μg ml⁻¹ in modified PBS with 0.5% Tween-20 (mPBST), and 50 μl was injected into the column to allow interactions with the mannotriose moieties. After a 0.5 min wash with modified PBS, bound FimCH is eluted with 0.1 M H₃PO₄ and 0.15 M NaCl for 2 min and detected by intrinsic tryptophan fluorescence, using an excitation wavelength of 280 nm and an emission wavelength of 325 nm. Affinity measurements rely on glycolipid traffic. Nature 389: 636–639.

References


specificity of the surface lectins of *Escherichia coli*, *Klebsiella pneumoniae* and *Salmonella typhimurium*. *Carbohydr Res* **120**: 235–249.


Sokurenko, E.V., Chesnokova, V., Dykhuizen, D.E., Ofek, I., and


