Probing the receptor recognition site of the FimH adhesin by fimbriae-displayed FimH–FocH hybrids

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Type 1 fimbriae are surface organelles of *Escherichia coli* which mediate D-mannose-sensitive binding to different host surfaces. This binding is conferred by the minor fimbrial component FimH. The binding domain of the FimH adhesin has been studied by constructing hybrids of FimH and a homologous protein, FocH, originating from F1C fimbriae. F1C fimbriae do not bind to D-mannosides or confer agglutination of any known types of erythrocytes or yeast. It was previously shown that the FocH protein can be readily substituted by the FimH adhesin, resulting in hybrid fimbriae with the same binding characteristics as type 1 fimbriae. The receptor binding of fimbriae-presented chimeric FimH–FocH hybrids was studied. FimH–FocH fusions encompassing 72 % of the N-terminus of FimH fused to the complementary sector of FocH conferred agglutination of erythrocytes and yeast cells at a comparable level to FimH. Surprisingly, it was also found that similar fusions containing between 56 and 66 % FimH still conferred binding to yeast cells, D-mannose–BSA and D-mannose–beads but did not give rise to agglutination. The receptor binding capacity of fusions containing 50 % or less of the FimH N-terminal region was virtually abolished. The results point to the presence of a D-mannose-receptor-binding core domain in FimH, the affinity of which is modulated by other sectors of the protein to enable binding to extended mannose-containing targets.

Keywords: adhesin chimeras, bacterial adhesion, FimH receptor site, type 1 fimbriae

INTRODUCTION

Bacterial adherence is normally a prerequisite for successful colonization of a specific host tissue. The best characterized group of bacterial adhesins is constituted by fimbriae (for reviews see Klemm, 1994). The most common of the enterobacterial adhesive surface organelles, and one of the best characterized, is type 1 fimbriae. Type 1 fimbriae are found on the majority of *Escherichia coli* strains and are widespread among other members of the *Enterobacteriaceae* (Klemm & Krogfelt, 1994). Interaction between type 1 fimbriae and receptor structures has, in a number of studies, been shown to play a key role in the colonization by *E. coli* of various host tissues (Bloch *et al.*, 1992; Yamamoto *et al.*, 1990). Recently, the adhesion conferred by type 1 fimbriae in an *E. coli* strain was shown to be linked to urinary tract pathogenesis (Connell *et al.*, 1996). In mouse models, immunization with FimH was shown to prevent urogenital mucosal infection by *E. coli* (Langermann *et al.*,

1997). A detailed dissection of the adhesive properties of type 1 fimbriae and their role in bacterial receptor recognition would undoubtedly help to reach a clearer understanding of important aspects of bacterial pathogenesis.

A type 1 fimbria is a thin, 7 nm wide and approximately 1 µm long, rod-shaped surface organelle. It is a heteropolymer consisting of four different subunits. Approximately 1000 copies of the major building element, FimA, are polymerized into a right-handed helical structure also containing a small percentage of the minor components FimF, FimG and FimH (Brinton, 1965; Klemm & Christiansen, 1987; Krogfelt & Klemm, 1988). It has been shown that the receptor-recognizing element of type 1 fimbriae is the 30 kDa FimH protein (Krogfelt *et al.*, 1990). The FimH protein is located at the tip and is also interspersed along the fimbrial shaft (Jones *et al.*, 1995; Krogfelt *et al.*, 1990). The FimF and FimG components are probably required for integration

Fig. 1. (a) Overview of the wild-type and engineered versions of the *fim* and *foc* gene clusters. Black boxes indicate signal-peptide-encoding sectors. The extent of other recombinant plasmids is indicated by bars. Only DNA inserts are shown. Relevant restriction sites are indicated: B, *Bam*HI; E, *Eco*RI; E5, *Eco*RV; H, *Hind*III; K, *Kpn*I; P, *Pvu*II. The triangle indicates the position of the translational stop linker insertion. (b) Construction of chimeric *fimH-focH* genes. Numbers above the *fimH* gene box indicate the percentage of *fimH* in the fusions. Positions of primers are indicated by boxed numbers. Restriction sites indicated by flags occur naturally in the *focH* gene; sites introduced by PCR are written alongside boxed primer numbers.

of the FimH adhesin into the fimbriae (Jones *et al.*, 1995; Klemm & Christiansen, 1987).

The components of the fimbrial organelle are encoded by the chromosomally located *fim* gene cluster (Klemm *et al.*, 1985). In addition to the structural components, this 9.5 kb DNA segment also encodes the fimbrial biosynthesis machinery as well as regulatory elements. Expression is phase-variable with individual cells switching between a fimbriate and a non-fimbriate state. This is due to inversion of a 314 bp DNA segment located immediately upstream of the *fimA* gene, encoding the major subunit protein (Fig. 1). A promoter residing in this phase switch drives the expression of the *fim* genes (Olsen & Klemm, 1994). The elements of the fimbrial organelle are produced as precursors having an N-terminal signal sequence. This feature is subsequently removed during export across the inner membrane. Thus the FimH protein is produced as a precursor of 300 amino acids that is processed into a mature form of 279 amino acids (Hanson *et al.*, 1988; Klemm & Christiansen, 1987). Further export, from the periplasm and across the outer membrane, is dependent on a fimbriae-

specific export and assembly system constituted by the FimC and FimD proteins (Jones *et al.*, 1993; Klemm, 1992; Klemm & Christiansen, 1990).

By virtue of the FimH adhesin, type 1 fimbriated bacteria confer α -D-mannose-sensitive agglutination of a number of eukaryotic cell types displaying this molecular motif, such as certain erythrocytes as well as yeast cells. To investigate more precisely which regions of the FimH adhesin contribute to receptor recognition we have chosen to exploit the compatibility of type 1 fimbriae with another fimbrial system, namely F1C fimbriae. Unlike type 1 fimbriae, F1C fimbriae do not target to D-mannosides; in fact, they do not agglutinate any known red blood cells or yeasts. However, they are known to confer adhesion to an unknown receptor in the collecting ducts and the distal tubules of the human kidney (Virkola *et al.*, 1988), as well as to cultured renal tubulus cells and various cell lines (Kreft *et al.*, 1992; Marre *et al.*, 1990). The structure of F1C fimbriae closely resembles that of type 1 fimbriae (Riegman *et al.*, 1990; van Die *et al.*, 1984, 1991). They also contain minor components, i.e. the FocF, FocG and FocH proteins. The genetic organization of the two gene clusters encoding the type 1 and F1C fimbriae, viz. the *fim* and *foc* genes, is almost identical (Fig. 1). We have previously shown, by reciprocal exchange of minor structural proteins between the type 1 and F1C systems, that the minor components originating from one of these fimbrial systems can be accommodated by the other. Notably, the FimH and FocH proteins, which show 36% sequence identity, can be readily exchanged between the two systems, resulting in hybrid organelles with receptor specificities defined by the adhesin. The adhesion conferred by FimH integrated in F1C fimbriae precisely mimics that of type 1 fimbriae (Klemm *et al.*, 1994, 1995). The fact that FocH is unable to recognize any of the FimH receptor targets makes it an ideal scaffold into which various parts of the FimH adhesin can be introduced to probe the extent of the receptor-binding domain and associated binding characteristics.

METHODS

Bacterial strains and growth conditions. In this study, we used a variant of the *E. coli* K-12 strain HB101 (F' *lacI* Tn5) (Boyer & Roulland-Dussoix, 1969) as an intermediate host during plasmid construction work and K-12 strain VL751 [*ara* Δ (*lac-pro*) *rpsL* *thi* Δ *fim*; Freitag *et al.*, 1985] for phenotype expression. Cells were grown in either glucose minimal medium (Clark & Maaløe, 1967) containing 0.5% Casamino acids (Difco) or Luria-Bertani (LB) broth (Sambrook *et al.*, 1989) supplemented with the appropriate antibiotics.

Plasmids. Plasmids used in this study are listed in Table 1. Plasmid pMAS1 contained the *fimH* gene from plasmid pMAS4 inserted into pUC19. Plasmid pTBK12 was made by introducing an *Age*I site at position 536 in the *fimH* gene by PCR amplification of pMAS1 using primers MS1 and TB3. In parallel, a *Bsp*EI site was introduced at position 531 in the *focH* gene by PCR amplification of pPKL150 (primers TB1 and TB2). The two segments were ligated and the hybrid gene was inserted in pUC19. The gene was subsequently excised

Table 1. Plasmids

Plasmid	Relevant genotype	Reference/construction
pPKL4	Wild-type <i>fim</i> cluster	Klemm <i>et al.</i> (1985)
pPKL115	<i>fimB</i> ⁺ -G ⁺	Pallesen <i>et al.</i> (1995)
pPKL143	Wild-type <i>foc</i> cluster	Klemm <i>et al.</i> (1994)
pPIL110-512	<i>focB</i> ⁺ -G ⁺	van Die <i>et al.</i> (1984)
pPIL110-518	<i>focB</i> ⁺ -G ⁺	Riegman <i>et al.</i> (1990)
pPKL150	<i>focG</i> ⁺ <i>focH</i> ⁺	Klemm <i>et al.</i> (1994)
pMAS1	<i>fimH</i> ⁺	This work
pMAS4	<i>fimH</i> ⁺	Schembri <i>et al.</i> (1996)
pTBK16	<i>fimfocH</i> ₇₈	First 78 % of <i>fimH</i> fused to last 22 % of <i>focH</i>
pTBK15	<i>fimfocH</i> ₇₂	First 72 % of <i>fimH</i> fused to last 28 % of <i>focH</i>
pTBK14	<i>fimfocH</i> ₆₆	First 66 % of <i>fimH</i> fused to last 34 % of <i>focH</i>
pTBK13	<i>fimfocH</i> ₆₁	First 61 % of <i>fimH</i> fused to last 39 % of <i>focH</i>
pTBK12	<i>fimfocH</i> ₅₆	First 56 % of <i>fimH</i> fused to last 44 % of <i>focH</i>
pTBK19	<i>fimfocH</i> ₅₀	First 50 % of <i>fimH</i> fused to last 50 % of <i>focH</i>
pTBK18	<i>fimfocH</i> ₄₅	First 45 % of <i>fimH</i> fused to last 55 % of <i>focH</i>

and inserted into the unique *EcoRV* site of pBR322. Plasmid pTBK13 was made by introducing a *HincII* site at position 573 in the *fimH* gene by PCR (primers MS1 and TB4), and joining this with the pre-existing *HincII* site in the *focH* gene; this fragment was inserted into *EcoRV*- and *BsmI*-digested pBR322. Plasmids pTBK14, pTBK15, pTBK16, TBK18 and TBK19 were made following a similar strategy of PCR amplification of selected segments of the *fimH* and *focH* genes, assembly of these in pUC19, and subsequent transfer of the chimeric genes into pBR322 downstream of the *tet* promoter (Fig. 1). It should be noted that introduction of restriction sites did not alter the amino acid sequence.

DNA techniques. Isolation of plasmid DNA was carried out by the mini-lysate method of Del Sal *et al.* (1989). Restriction endonucleases were used according to the manufacturer's specifications (New England Biolabs or Pharmacia). PCRs were made as previously described by Stentebjerg-Olesen *et al.* (1997). The following primers were used: MS1, 5'-GTG-ATAAGCTTACCTATACCTACCTACAGC; TB1, 5'-TGTACGGATCCGCCACAACCTGCATTCTAG; TB2, 5'-TCCAGTCCGGAACATGCAGGGTCTCATC; TB3, 5'-CAGCCACCGGTAGGCACCACCACATC; TB4, 5'-GGC-AGGTTAACGGTGACATCACG; TB6, 5'-ACGCCGCCG-GCAGGTGAAAACGACG; TB7, 5'-GTTCACTGCCAAT-TCCCTCTTACTGTACGTTGCGACC; TB8, 5'-GTATTA-CCTCTCCGGCACACACAGGAAGTGGTAATACT-GTATTC; TB9, 5'-ACAACTATAACAGCGATG; TB10, 5'-GCGATTAAAGCTGGCTCATTAGTGGCCACAGTTTA-TG; TB11, 5'-CATAACTGTGGCCACTAATGAGCCA-GCTTTAATCGC; TB12, 5'-GACCAACAACATAAC-AGCGAGAGAAATTTACCTGGCG; TB13, 5'-CGCCA-GGTGGAATTTCTCTCGCTGTTATAGTTGTTGGTC; TB14, 5'-ACTCTTTCCCAACAGTTAATGTGGGGCA-AAACCTGGTCG; TB15, 5'-CTGGTTTGGCCACATT-AACTGTTGGGAAAGAGTGATATTG; TB16, 5'-GTG-TTATGTCAGAATGAATATCCGGAACCATTTACAG-AC; TB17, 5'-TGTAATGGTTTCCGGATATTCATTCTG-ACATAACACCAG; TB18, 5'-GCAAGCTTACTGTACCT-TATACCTATGCG. The nucleotide sequences of constructs were determined by the dideoxynucleotide chain-termination method (Sanger *et al.*, 1977). Oligonucleotide primers were purchased from Gibco-BRL.

Immunofluorescence microscopy. Surface presentation of FimH-FocH chimeras was assessed by immunofluorescence microscopy employing a monoclonal antibody that recognizes a similar conformational epitope in FimH and FocH (Chanteloup *et al.*, 1991). Cell fixation, immunolabelling and microscopy were carried out as previously described by Pallesen *et al.* (1995).

Electron microscopy. Electron microscopy was carried out essentially as described previously (Klemm *et al.*, 1994; Pallesen *et al.*, 1995). In short, a 10 µl aliquot of bacterial suspension was placed on a carbon-coated, glow discharged grid for 30 s. Grids were washed in two drops of PBS (0.145 M NaCl; 0.15 M sodium phosphate), dehydrated in increasing concentrations of ethanol, blotted dry, and shadowed with tungsten.

Receptor blot. Receptor blots of FimH and FimH-FocH to α-D-mannosylated BSA (Sigma) were carried out essentially as previously described by Krogfelt *et al.* (1990). In short, filter blots were first incubated with α-D-mannose-BSA (0.5 mg l⁻¹; Sigma) followed by incubation with rabbit anti-BSA serum and finally with peroxidase-conjugated anti-rabbit serum.

Agglutination of red blood cells and yeast cells. The capacity of bacteria to express a D-mannose-binding phenotype was assayed by their ability to agglutinate guinea pig erythrocytes or yeast cells on glass slides. Aliquots of washed bacterial suspensions at OD₅₅₀ 0.5 and 5 % erythrocytes or yeast cells were mixed and the time until agglutination occurred was measured. Furthermore, clones which did not cause any agglutination under these conditions were also tested at OD₅₅₀ 20 and/or low temperature, but still did not react.

Binding to mannose beads and yeast cells. Binding of bacteria to mannose beads and yeast cells was carried out essentially as described previously (Klemm *et al.*, 1990). In short, bacteria from exponentially growing cultures were harvested, washed three times, and resuspended in PBS. Aliquots of bacteria, with identical OD₅₅₀ values in all cases, and beads (methyl-α-D-mannopyranoside-coated beaded agarose; Sigma) or yeast cells (*Saccharomyces cerevisiae*) were mixed and incubated under gentle agitation for 20 min. Beads or yeast cells were collected by low-spin centrifugation, gently washed and examined microscopically. Binding to beads and yeast cells

was additionally assayed by incubating aliquots of bacteria with either beads or yeast cells for 2 min, after which bacteria adhering to beads or yeast cells were separated from unbound bacteria by low-speed centrifugation. Unbound bacteria were removed by aspiration, the procedure was repeated twice, and finally attached bacteria were released by addition of methyl α -D-mannose. Microscopy revealed that no bacteria remained attached to the beads or yeast cells at this point. The bacteria were then quantified by plating out, the experiments were repeated four times.

Adhesion inhibition assay. Inhibitor titration, with methyl α -D-mannose, of bacterial binding to mannan bound to 96-well plates was carried out essentially as previously described by Sokurenko *et al.* (1997). In short, wells were coated with mannan (Sigma) at a concentration of $10 \mu\text{g ml}^{-1}$, washed three times with PBS and quenched with 0.2% BSA in PBS. Bacterial suspensions containing identical cell numbers were mixed with increasing concentrations of methyl α -D-mannose, added to the wells and incubated for 40 min at 37 °C. The wells were then washed with PBS, and 160 μl LB containing 100 mM methyl α -D-mannose was added to each well and incubated for 5 h at 37 °C. The number of bound bacteria was determined by measuring OD₆₀₀ values with a microtitre plate reader.

RESULTS

Construction of genes encoding hybrid FimH–FocH proteins

A series of chimeric genes was created which harboured sectors of the *fimH* gene encoding 45, 50, 56, 61, 66, 72 or 78 % of the N-terminal part of the FimH protein. These sectors were fused to segments of the *focH* gene encoding the complementary C-terminal sectors of the FocH protein. The result was a nested series of genes encoding FimH–FocH fusions containing increasingly larger segments of the N-terminal part of the FimH protein, i.e. between 125 and 220 amino acid residues, fused to correspondingly smaller segments of the C-terminal part of the FocH protein (Table 1; Fig. 1). DNA sequence analysis established that the chimeras authentically mimicked the expected sectors of the parent genes and that the reading frame was conserved in the fusion points in all cases.

Agglutination conferred by FimH–FocH hybrids

To investigate the binding potential of the FimH–FocH hybrids, we initially performed ‘classic’ agglutination assays. Auxiliary plasmids, encoding either all the *fim* genes except *fimH* or all the *foc* genes except *focH*, were used to complement plasmids encoding the FimH–FocH chimeras. The resulting hosts therefore display the chimeras as either constituents of type 1 or F1C fimbriae, respectively. Hybrid FimH–FocH containing 72 or 78 % of the N-terminal part of FimH conferred agglutination times that were only slightly slower than the wild-type FimH displayed by either of the parent fimbrial systems (Table 2). Furthermore, the titres were basically unaffected by the display system, i.e. either type 1 or F1C fimbriae. However, substitution of larger sectors between FimH and FocH, i.e. the 45–66 % fusions, was

unable to induce agglutination of erythrocytes and yeast cells, which provide a rich source of mannan (Table 2).

Surface presentation of the adhesins

Surface presentation of the FimH–FocH hybrids was assessed by immunofluorescence microscopy employing a monoclonal antibody which recognizes an identical conformational epitope in both FimH and FocH. In all cases, similar signals that differed significantly from the negative controls were observed. In addition, fimbrial morphology and number were assessed by electron microscopy. Two representative recombinant strains were investigated, i.e. cells containing plasmids pPKL115 plus pTBK14 expressing the FimH–FocH₆₆ hybrid adhesin and cells containing plasmids pPKL115 plus pTBK15 expressing the FimH–FocH₇₂ hybrid adhesin, and compared to the wild-type control (Fig. 2). In both cases, fimbriated cells were seen where the morphology and number of the fimbriae were similar to the wild-type.

D-Mannose recognition by FimH–FocH hybrids

The binding capacity of the chimeras was also assessed by a different parameter, namely the ability to bind to D-mannosylated BSA. In this assay, it was found that FimH–FocH hybrids consisting of 56 % or more of the N-terminal region of FimH fused to the complementary C-terminal sectors of FocH, readily bound to mannan–BSA. By fivefold overloading of the gel, a weak signal was observed with the 50 % fusion, whereas the 45 % fusion did not react under these conditions (Fig. 3). Addition of methyl α -D-mannose to the assay completely abolished binding to mannan–BSA.

D-Mannose recognition is distinct from the ability to cause agglutination

At this point it was apparent that the ability of a FimH–FocH hybrid to recognize D-mannose was not concomitant with the ability to cause agglutination of erythrocytes or yeast cells. All hybrids containing 56 % or more of the FimH N-terminus clearly recognized mannan–BSA but only hybrids containing 72 % or more were able to induce agglutination (Table 3). To investigate this aspect further, we looked at the ability of the clones to bind to methyl- α -D-mannopyranoside-coated beaded agarose. It was found that exactly the same hybrids, i.e. the 56, 61, 66, 72 and 78 % fusions, that readily bound to mannan–BSA in the receptor blot assay also conferred binding of bacteria to the beads (Fig. 4). The 45 and 50 % fusions did not differ from the negative control. Quantification of the percentage of bacteria adhering to the monosaccharide mannan target on the beads revealed only minor differences among the recombinant strains. The exceptions were the negative control, expressing FocH, and to a degree the wild-type control (Fig. 4).

Additionally, we investigated the ability of the hybrids to bind to yeast cells, since the receptor target, mannan,

Table 2. Adhesive phenotypes conferred by FimH-FocH hybrids expressed in both type 1 and F1C backgrounds assayed by the ability to agglutinate guinea pig erythrocytes of *E. coli* VL751 (Δ *fim*) derivatives

Agglutination of guinea pig erythrocytes or yeast cells is indicated in seconds before reaction occurred. The mean values of three measurements are given.

Plasmid(s)	Relevant genotype	Haemagglutination	Yeast agglutination
pMAS4	<i>fimH</i> ⁺		
+ pPKL115	<i>fimB</i> ⁺ -G ⁺	12	9
pMAS4	<i>fimH</i> ⁺		
+ pPIL110-512	<i>focB</i> ⁺ -G ⁺	10	8
pTBK16	<i>fimfocH</i> ₇₈		
+ pPKL115	<i>fimB</i> ⁺ -G ⁺	19	11
pTBK16	<i>fimfocH</i> ₇₈		
+ pPIL110-512	<i>focB</i> ⁺ -G ⁺	24	13
pTBK15	<i>fimfocH</i> ₇₂		
+ pPKL115	<i>fimB</i> ⁺ -G ⁺	20	12
pTBK15	<i>fimfocH</i> ₇₂		
+ pPIL110-512	<i>focB</i> ⁺ -G ⁺	26	13
pTBK14	<i>fimfocH</i> ₆₆		
+ pPKL115	<i>fimB</i> ⁺ -G ⁺	> 600	> 600
pTBK14	<i>fimfocH</i> ₆₆		
+ pPIL110-512	<i>focB</i> ⁺ -G ⁺	> 600	> 600
pTBK13	<i>fimfocH</i> ₆₁		
+ pPKL115	<i>fimB</i> ⁺ -G ⁺	> 600	> 600
pTBK13	<i>fimfocH</i> ₆₁		
+ pPIL110-512	<i>focB</i> ⁺ -G ⁺	> 600	> 600
pTBK12	<i>fimfocH</i> ₅₆		
+ pPKL115	<i>fimB</i> ⁺ -G ⁺	> 600	> 600
pTBK12	<i>fimfocH</i> ₅₆		
+ pPIL110-512	<i>focB</i> ⁺ -G ⁺	> 600	> 600
pTBK19	<i>fimfocH</i> ₅₀		
+ pPKL115	<i>fimB</i> ⁺ -G ⁺	> 600	> 600
pTBK19	<i>fimfocH</i> ₅₀		
+ pPIL110-512	<i>focB</i> ⁺ -G ⁺	> 600	> 600
pTBK18	<i>fimfocH</i> ₄₅		
+ pPKL115	<i>fimB</i> ⁺ -G ⁺	> 600	> 600
pTBK18	<i>fimfocH</i> ₄₅		
+ pPIL110-512	<i>focB</i> ⁺ -G ⁺	> 600	> 600
pPKL143	Wild-type <i>foc</i> cluster	> 600	> 600
pPKL115	<i>fimB</i> ⁺ -G ⁺	> 600	> 600
pPIL110-512	<i>focB</i> ⁺ -G ⁺	> 600	> 600

is different from the α -D-mannose or the methyl α -D-mannopyranoside targets on mannose-BSA and mannose-beads, respectively. Microscopy of yeast cells incubated with the individual clones revealed that the 72 and 78 % fusions, which were able to induce agglutination, also attached to the yeast cells (Fig. 4). Surprisingly, the non-agglutinating clones, expressing FimH-FocH₅₆ through FimH-FocH₆₆, were also seen to attach to yeast cells (Fig. 4). Enumeration of attached bacteria, however, revealed two distinct groups. In one group conferring agglutination, between 65 and 85 % of the added bacteria bound to yeast, whereas in the non-agglutinating group only between 30 and 45 % of the

bacteria adhered to the yeast cells under the assay conditions (Fig. 4). Judging from the above, a D-mannose-recognizing phenotype seemed to be distinct from and superimposed on the ability to confer agglutination. It should also be noted that the binding phenotype of the hybrids was independent of whether type 1 or F1C fimbriae were used as display organelles.

Inhibition tests

To probe the binding of the hybrids from a different angle, we also looked at the inhibition profiles, i.e. the ability of recombinant cells to bind to mannan in the

Fig. 2. Electron micrographs showing fimbriation of *E. coli* strain VL751 cells containing recombinant plasmids which in addition to a *fim* gene cluster with a stop-linker in the *fimH* gene encode the following adhesins: (a) FimH-FocH₇₂ (pTBK15+pPKL115); (b) wild-type FimH (pMAS4+pPKL115); (c) FimH-FocH₆₆ (pTBK14+pPKL115). Bar, 0.2 μ m.

presence of increasing concentrations of methyl α -D-mannoside (Fig. 5). The binding of all strains to mannan could be inhibited by methyl α -D-mannoside; however, the concentrations of methyl α -D-mannoside required to inhibit adhesion by 50%, viz. the IC₅₀, differed significantly among the strains. Two groups were apparent, with values differing by about one order of magnitude. One group which conferred agglutination and D-mannose binding, i.e. cells expressing FimH, FimH-FocH₇₈ and FimH-FocH₇₂, had IC₅₀ values of 1.60, 4.7 and

0.80 mM, respectively, whereas hybrids unable to cause agglutination but which still bound to mannose, i.e. FimH-FocH₆₆, FimH-FocH₆₁ and FimH-FocH₅₆, had IC₅₀ values of 0.08 (extrapolated), 0.10 and 0.20 mM, respectively.

DISCUSSION

One of the decisive factors determining the range of host organisms, tissues and cell types that bacteria are able to colonize is their ability to recognize and bind to specific receptor molecules on host cell surfaces. In the case of fimbriae-mediated adhesion, this target tropism is determined by the molecular architecture of the binding domain of the fimbrial adhesin. The archetypical fimbrial adhesin is undoubtedly the FimH adhesin, a constituent of the (among enterobacteria) ubiquitously present type 1 fimbriae. The type 1 fimbriae with the associated adhesive phenotype was one of the first bacterial adhesins to be described and defined by virtue of its haemagglutinating activity which could be inhibited by the simple monosaccharide α -D-mannose (Duguid *et al.*, 1955; Duguid & Gillies, 1957). Since then, a number of powerful tools from the domains of biochemistry, genetics and molecular biology have disclosed many interesting features of this adhesin. In the present study, we have used the compatibility of FimH and FocH with either of the two parental organelle systems to display chimeric versions of the two proteins on the bacterial surface to study the receptor-binding domain of FimH. The inability of FocH to recognize any of the receptor targets of FimH makes it an ideal scaffold onto which sectors of FimH can be grafted. Proteins or protein domains with identity scores above 20% are generally considered to have similar three-dimensional structures (Chotia & Lesk, 1986; Sander & Schneider, 1991). The primary structures of the FimH and FocH proteins exhibit an overall 36% identity indicative of similar folding. However, it is noteworthy that the homology between the proteins is not evenly distributed, and the homologies of the N-terminal and C-terminal halves of the proteins are below or above this mean, respectively.

The adhesive capacity of the FimH-FocH fusions was tested against two classes of receptor molecules containing either a single α -D-mannose or methyl α -D-mannose residue coupled to BSA or agarose beads, or complex α -D-mannose-containing polysaccharides as present on yeast cells (a rich source of mannan), mannan and guinea pig red blood cells. Hybrids which contained 72% or more of the N-terminal part of FimH fused to the corresponding C-terminal parts of FocH were shown to confer binding characteristics resembling those of the wild-type FimH adhesin, i.e. agglutination of red blood cells and yeast cells (Table 2). Cells expressing such hybrids also bound in high numbers to yeast cells and methyl- α -D-mannopyranoside-conjugated beaded agarose (Fig. 4); the hybrid proteins were shown to bind to mannose-BSA (Fig. 3). Surprisingly, chimeras harbouring less of the N-terminal part of FimH, viz. from

Fig. 3. Analysis of binding to α -D-mannose-BSA by FimH–FocH hybrids. Equivalent samples were subjected to SDS-PAGE and transferred to a PVDF filter. Bound α -D-mannose-BSA was detected by reaction with anti-BSA rabbit serum followed by peroxidase-conjugated anti-rabbit serum. Lanes are shown in doublets with the adhesin expressed by type 1 or F1C fimbriae, respectively. The position of the FimH–FocH hybrids is indicated by an arrow. Lanes: 1, wild-type FimH; 2, wild-type FocH; 3, FimH–FocH₄₅; 4, FimH–FocH₅₀; 5, FimH–FocH₅₆; 6, FimH–FocH₆₁; 7, FimH–FocH₆₆; 8, FimH–FocH₇₂; 9, FimH–FocH₇₈. The insert on the right-hand side represents a fivefold overloading of the gel with equivalent samples of wild-type FimH (lane 10), wild-type FocH (lane 11) and FimH–FocH₅₀ (lane 12).

Table 3. Summary of phenotypes conferred by FimH–FocH hybrids

The extent of FimH in the hybrids is indicated. In all cases, a helper plasmid encoding all *fim* genes except *fimH* was also present in the host strain (*E. coli* VL751).

Hybrid	Residues of mature FimH	Mannose-BSA binding	Mannose-bead binding	Yeast cell binding	Agglutination*
FimH	1–279	+	+	+	+
FimH–FocH ₇₈	1–217	+	+	+	+
FimH–FocH ₇₂	1–201	+	+	+	+
FimH–FocH ₆₆	1–184	+	+	+	–
FimH–FocH ₆₁	1–170	+	+	+	–
FimH–FocH ₅₆	1–158	+	+	+	–
FimH–FocH ₅₀	1–139	(+)	–	–	–
FimH–FocH ₄₅	1–125	–	–	–	–
FocH	0	–	–	–	–

* Agglutination of guinea pig erythrocytes or yeast cells.

56 through 66%, were unable to confer agglutination but were nevertheless able to bind to D-mannose-BSA, methyl- α -D-mannopyranoside-conjugated beaded agarose and yeast cells (Table 2; Figs 3 and 4). It should be mentioned that such clones were agglutination-negative, even when the assays were carried out with concentrated bacterial suspensions and/or at low temperature. This suggests that D-mannose binding and agglutination actually can be distinct phenotypes and that the two characteristics can be related to physically distinct regions in FimH. Finally, the 50% fusion did bind weakly to D-mannose-BSA, but was negative in all other binding assays. The 45% fusion tested negatively by all criteria we used to monitor binding with, indicative of the extent of the D-mannose-binding domain of FimH. Taken together, our results from the chimeric FimH–FocH approach suggest that the minimal information for D-mannose recognition in FimH is located within the first 50% of FimH. Furthermore, this ‘core domain’ can be complemented with steric information residing in the remaining part of FimH. Notably, the 185–201 segment apparently contains information that

upgrades the D-mannose-binding core domain to additionally confer agglutination, reflected in the different binding phenotypes endowed by FimH–FocH₆₆ and FimH–FocH₇₂.

Two other sets of observations indicate that the agglutinating- and mannose-binding-only phenotypes are probably a reflection of higher avidity for the receptor target in the first phenotype. Mannan is a branching polymer consisting of several hundred mannose residues (Ballou, 1976). When presented with such a target, fusions having 72% or more of the N-terminal sector of FimH bound in significantly higher numbers to yeast cells and had higher IC₅₀ values than hybrids that only conferred binding to monosaccharide mannose targets (Figs 4 and 5). Utilizing a large spectrum of saccharides, Sharon and coworkers found that the binding domain of FimH is probably an elongated pocket that recognizes a trisaccharide motif (Firon *et al.*, 1987; Sharon, 1987). Increased affinity for oligosaccharides by many lectins is derived from extended binding sites in which more than just terminal sugar residues of an oligosaccharide make

Fig. 4. Bacterial attachment to monosaccharide (methyl- α -D-mannopyranoside-coated beads) and polysaccharide (yeast cells) targets. (a–c) Micrographs of *E. coli* strain VL751 expressing FimH-FocH₆₁ (pTBK13+pPKL115), FimH-FocH₇₈ (pTBK16+pPKL115) and FimH (pMAS4+pPKL115), respectively, adhering to beads and yeast cells (inserts). The block diagrams in (d) and (e) show binding of recombinant bacteria to mannose-beads and yeast, respectively. The adhesin hybrids expressed by recombinant strains are: A, FimH-FocH₅₆ (pTBK12+pPKL115); B, FimH-FocH₆₁ (pTBK13+pPKL115); C, FimH-FocH₆₆ (pTBK14+pPKL115); D, FimH-FocH₇₂ (pTBK15+pPKL115); E, FimH-FocH₇₈ (pTBK16+pPKL115); F, FimH (pMAS4+pPKL115); G, FocH (pPKL143). The results shown are the means of four experiments $\pm$ SD. For experimental details see Methods.

contact (Weis & Drickamer, 1996). In the light of this, it is tempting to speculate that such an elongated binding site in FimH, capable of accommodating an extended oligosaccharide motif, is gradually affected in our FimH-FocH fusions when we decrease the FimH portion, resulting in different binding characteristics. We also constructed reverse fusions, i.e. *focH-fimH* hybrids encoding N-terminal sectors of FocH fused to complementary regions of FimH. However, all efforts to detect the corresponding gene products or related phenotypes were unsuccessful.

Previous comparisons of FimH variants showed that minor differences in the amino acid sequence resulted in different binding characteristics of the adhesin (Sokurenko *et al.*, 1994, 1995, 1997). Such differences were located between residues 32 and 166 (Fig. 6). Further information on the structure-function architecture of the FimH adhesin was provided by linker insertion analysis of the *fimH* gene. A systematic change of codons in the wild-type sequence in a number of positions, spanning the entire sequence, resulted in a series of FimH variants with a wide range of adhesive phenotypes. Notably, changes in positions 56 and 136/137 abolished receptor recognition, whereas ma-

nipulation of positions 8, 225 and 259 did not (Schembri *et al.*, 1996). In addition, a 16–18 kDa truncate of FimH, having the same N-terminal sequence as the mature FimH protein, was reported to confer binding to D-mannose since it was isolated from D-mannose beads (Jones *et al.*, 1995). Comparable-sized truncates were also observed in this study indicative of similar processing of the chimeric adhesins (Fig. 3). Very recently it was reported (Thankavel *et al.*, 1997) that a Male fusion containing residues 1–100 of FimH (actually residues 3–102, according to the primer sequences) recognized α -D-mannose-containing targets. A weak signal, about 25-fold weaker than the other signals, could indeed be seen with FimH-FocH₅₀ in the mannose-BSA receptor blot (Fig. 3). Overloading of the gel confirmed this observation. This would suggest that some modicum of D-mannose-binding activity can be ascribed to the first 50% of FimH.

Although a peptide segment encompassing the N-terminal 50% of FimH seems to confer binding to D-mannose targets, sectors outside this segment heavily influence the receptor binding. These are perhaps involved in accommodation of more extensive targets than just a single monosaccharide motif. Previous

Fig. 5. Inhibition of binding of strains expressing hybrid adhesins to mannan by methyl α -D-mannopyranoside (α -MM). Data were converted to percentage of bound cells by correlating with the number of cells bound in the absence of inhibitor. The dashed line indicates IC_{50} . ●, FimH; ■, FimH-FocH₇₈; ▲, FimH-FocH₇₂; ○, FimH-FocH₆₆; △, FimH-FocH₆₁. Data from a single experiment are shown, but the experiment was repeated several times and the results were essentially the same.

findings also confirm this (Sokurenko *et al.*, 1994, 1995). The minimal sector of FimH that was observed to confer binding to a D-mannose monosaccharide target with the FimH-FocH technique was contained within the N-terminal 140 residues of FimH. The importance of this region can also be seen from the fact that abolition of receptor recognition was previously reported by site-directed mutagenesis causing alteration, but not deletion, of the amino acids in positions 136 and 137 (Schembri *et al.*, 1996). Also, in this study we find that a segment straddling position 200 seems to be involved in the modulation of receptor binding of FimH since replacement of this segment with the corresponding FocH segment causes abolition of agglutination but not of D-mannose recognition. It therefore seems that although part of the FimH receptor recognition domain appears to be restricted to the first half of the molecule, other sectors of the protein must significantly contribute to the receptor recognition. The modulating effect of such regions on FimH receptor recognition might only be observable in the context of intact fimbrial organelles as reported here. The present approach, using fusion constructs of two homologous adhesins to dissect receptor-binding sites within one of them, is novel and should have a high potential for the study of structure-function relationships of bacterial adhesins.

Fig. 6. Comparison of the amino acid sequences of the mature FimH and FocH proteins. Gaps are included to obtain maximum fit. FimH-FocH hybrids are indicated by arrows and percentage of FimH in the chimeric protein. Identical amino acids are indicated by colons and functionally similar amino acids with stops. Positions in which amino acid changes alter binding phenotype (Sokurenko *et al.*, 1994, 1995) are indicated in bold. Binding characteristics of FimH variants created by linker-insertion or site-directed mutagenesis (Schembri *et al.*, 1996) are indicated by circled '+' and '-'. The minimum segments of FimH which in the context of FimH-FocH hybrids were required to confer efficient D-mannose binding and additionally agglutination are indicated by boxing with thick or thin lines, respectively.

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