Recognition of the N-terminal lectin domain of FimH adhesin by the usher FimD is required for type 1 pilus biogenesis

Diana Munera,1 Scott Hultgren2 and Luis Ángel Fernández1*

1Department of Microbial Biotechnology, Centro Nacional de Biotecnología, Consejo Superior de Investigaciones Científicas (CSIC), Campus de Cantoblanco, 28049 Madrid, Spain.
2Department of Molecular Microbiology, Washington University School of Medicine, St Louis, MO 63110, USA.

Summary

In this work we discover that a specific recognition of the N-terminal lectin domain of FimH adhesin by the usher FimD is essential for the biogenesis of type 1 pili in Escherichia coli. These filamentous organelles are assembled by the chaperone–usher pathway, in which binary complexes between fimbrial subunits and the periplasmic chaperone FimC are recognized by the outer membrane protein FimD (the usher). FimH adhesin initiates fimbriae polymerization and is the first subunit incorporated in the filament. Accordingly, FimD shows higher affinity for the FimC/FimH complex although the structural basis of this specificity is unknown. We have analysed the assembly into fimbria, and the interaction with FimD in vivo, of FimH variants in which the N-terminal lectin domain of FimH was deleted or substituted by different immunoglobulin (Ig) domains, or in which these Ig domains were fused to the N-terminus of full-length FimH. From these data, along with the analysis of a FimH mutant with a single amino acid change (G16D) in the N-terminal lectin domain, we conclude that the lectin domain of FimH is recognized by FimD usher as an essential step for type 1 pilus biogenesis.

Introduction

Most Escherichia coli strains express type 1 fimbriae, highly polymeric protein filaments located at the surface of cells and involved in bacterial adhesion to host epithelia of oropharyngeal, gastrointestinal and urinary tracts (Ofek et al., 1977; Bloch and Orndorff, 1990; Bloch et al., 1992). Type 1 fimbriae are composite fibres with a rigid rod (c. 1–2 μm long and 6.9 nm wide) made up of about a thousand copies of the major structural subunit FimA (Hahn et al., 2002), and a short tip fibrillum containing a single copy of two minor structural subunits (FimF and FimG) and the adhesin FimH (Krogfelt and Klemm, 1988; Krogfelt et al., 1990; Jones et al., 1995). FimH adhesin specifically binds to D-mannosyl residues in glycoproteins and, as a consequence, bacterial adhesion mediated by type 1 fimbriae can be inhibited by D-mannose (Old, 1972). Binding of FimH from uropathogenic E. coli strains to mono-mannose-rich receptors on the lumen of the bladder (uroplakins) has a crucial role for the ability of this pathogen to colonize and invade the bladder epithelium during infection (Connell et al., 1996; Mulvey et al., 1998; Martinez et al., 2000; Zhou et al., 2001; Martinez and Hultgren, 2002).

In addition of its function in pathogenesis, FimH plays an essential role for the biogenesis of type 1 fimbriae because E. coli fimH mutants have an impaired capacity to assemble these surface organelles (Klemm and Christiansen, 1987; Schembri et al., 1996). Assembly of type 1 fimbriae is mediated by the chaperone/usher pathway (Thanassi et al., 1998a; Thanassi and Hultgren, 2000a; Sauer et al., 2004), in which the fimbrial subunits are bound in the periplasm by a dedicated fimbrial chaperone (FimC). These binary protein complexes are recognized in an ordered fashion by an outer membrane (OM) protein (FimD), termed ‘ usher’, which promotes dissociation of FimC chaperone, the polymerization of fimbrial subunits, and their secretion across the OM (Saulino et al., 1998; 2000). FimH is the first subunit incorporated into a growing fimbrial filament, which is assembled from tip to bottom. The usher FimD appears to be responsible for this selectivity, since it binds to FimC/FimH complex with higher affinity than to other complexes of FimC with structural subunits (FimC/FimA, FimC/FimG, FimC/FimF) (Saulino et al., 1998). In addition, binding of FimC/FimH appears to activate FimD for polymerization of fimbriae (Saulino et al., 1998; Shu Kin So and Thanassi, 2006). However, the basis of
distinct recognition of FimC/FimH by FimD is not understood.

The 3D structure of FimC/FimH complex (Choudhury et al., 1999) revealed that FimH has a unique structure among type 1 fimbrial subunits having two distinct protein domains assembled by antiparallel β-strands and connected by a short linker (Fig. 1A). The C-terminal domain of FimH (C-FimH) shares high sequence similarity to structural subunits of fimbriae (e.g. FimA, FimG, FimF) and like them folds into an incomplete immunoglobulin (Ig)-like domain lacking the last β-strand. The missing β-strand of all pilin domains leaves a hydrophobic groove exposed to the solvent and makes all fimbrial subunits unstable unless they interact with the chaperone FimC in the periplasm, or a complementary β-strand is ‘donated’ by an N-terminal extension of the following subunit once assembled in the fimbria (Sauer et al., 2002; 2004; Vetsch et al., 2002).

In contrast to the pilin C-domain, the N-domain of FimH (N-FimH) is stable and can be expressed independently of FimC (Schembri et al., 2000). The crystallographic data showed that N-FimH does not interact with FimC, whereas extensive contacts occur along C-FimH (Choudhury et al., 1999) (Fig. 1A). N-FimH consists of 156 amino acids folded in 11 β-strands organized in a barrel-like structure with overall dimensions similar to Ig domains (~2 nm diameter) and stabilized by a disulphide bond (Choudhury et al., 1999). N-FimH is also referred to as receptor-binding domain or lectin domain because it contains a cavity that represents the binding pocket for D-mannosyl residues (Choudhury et al., 1999; Hung et al., 2002). More recently, studies on the function of two type 1 fimbrial ushers (FimD and PapC) have shown that chaperone-subunits complexes are initially recognized by a periplasmic domain of FimD comprising the first 125 residues of the mature protein (Thanassi et al., 2002; Ng et al., 2004). This domain of FimD (FimDν) binds FimC-subunit complexes, including a complex formed exclusively by the C-domain of FimH and FimC (FimC/C-FimH) (Nishiyama et al., 2003; 2005).

Therefore, the current knowledge strongly indicates quite distinct biological functions for the two domains of FimH, being C-FimH required for fimbria assembly through interactions with FimC, FimDν and other fimbrial subunits, whereas N-FimH is responsible for D-mannose binding during bacterial adhesion. Despite this, native FimD was shown to bind N-FimH after its overexpression in the periplasm (Barnhart et al., 2003). However, the significance of this interaction for the assembly of type 1 fimbriae is unknown. In this work we have investigated whether the N-domain of FimH has a specific role during fimbriae assembly by studying the incorporation into pili of FimH chimeras in which this domain has been deleted or substituted by heterologous Ig domains, or in which these Ig domains were fused to the full-length FimH. Results obtained with these chimeras indicated that N-FimH plays an essential role during fimbriae assembly by its interaction with FimD. This conclusion was reinforced by the analysis of a FimH mutant having a single amino acid substitution (G16D) in the N-terminal domain, which showed a dramatic decrease both in its interaction with FimD and in its level of assembly into fimbriae.
Results

A system for expression and detection of FimH variants

Unless indicated otherwise, the E. coli strains used as host for the expression of FimH variants in this work carry mutations or deletions in the chromosomal fim operon (Experimental procedures; Table 1). To study the assembly into fimbriae of FimH chimeras with altered N-domains, we first developed a plasmid-based vector system that allowed their controlled expression, immunological detection, and exchange of N-domains as gene cassettes (Supplementary material; Fig. S1A). To this end, a fimH allele containing a unique BglII site at a permissive position of the C-domain of FimH (Pallesen et al., 1995) was cloned under the control of an isopropyl-1-thio-β-D-galactoside (IPTG)-inducible promoter (Ptac) in the low-copy number plasmid pVLT35 carrying the lacIq repressor (de Lorenzo et al., 1993). This construct, termed pH35, was modified at the permissive site of the C-domain of FimH by inserting a 12-amino-acid epitope (E-tag) for detection with a monoclonal antibody (mAb) anti-E-tag. To facilitate the exchange of N-domains, a unique NotI site was also engineered at the 4-amino-acid linker region between N- and C-domains of FimH, which changed its amino acid sequence from PTGG to PAAA, giving rise to plasmid pFHN10E. Finally, a derivative of pFHN10E with the N-terminal signal peptide (SP) of gene 3 from M13 was constructed (named pFHSN6E). This SP was introduced because Ig domains intended to replace N-FimH (see later) are cloned in M13 phage display vector.

### Table 1. Plasmids and E. coli strains employed in this study.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant genotype</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>HB101</td>
<td>F- (gpt-proA)62 leuB6 glnV44 ara-14galK2 lacY1 Δ(mcrC-mrr) rpsL20 (Strr) xyl-5 mtl-1 recA13</td>
<td>Boyer and Roulland-Dussoux (1969)</td>
</tr>
<tr>
<td>AAEC185</td>
<td>Δ (supE44 hisD17 mcrA mcrB endA1 thi-1 Δ(fimB-fimH) ΔrecA)</td>
<td>Blomfield et al. (1991)</td>
</tr>
<tr>
<td>UT5600</td>
<td>F- ara-14 leuB6 secA6 lacY1 proC14 tsx-67 Δ(ompT-fepC)266 entA403 trpE38 rfbD1 rpsL109(Strr) xyl-5 mtl-1 thi-1</td>
<td>Grodberg and Dunn (1988)</td>
</tr>
<tr>
<td>UTdfim</td>
<td>UT5600 fimD::mini-Tn10Km</td>
<td>Lab collection</td>
</tr>
<tr>
<td>MG1655</td>
<td>F- Δ(lac)</td>
<td>Guter et al. (1981)</td>
</tr>
<tr>
<td>MG1655Δfim</td>
<td>MG1655 ΔfimA-H</td>
<td>Blomfield et al. (1991)</td>
</tr>
<tr>
<td>pLPA30</td>
<td>Ap', pUC18 derivative, Plac promoter</td>
<td>Pallesen et al. (1995)</td>
</tr>
<tr>
<td>pVLT35</td>
<td>Sm'/Sp', Plac expression vector, RSF1010 ori</td>
<td>de Lorenzo et al. (1993)</td>
</tr>
<tr>
<td>pPKL115</td>
<td>Cm', pACYC184 derivative</td>
<td>Pallesen et al. (1995)</td>
</tr>
<tr>
<td>pFH35</td>
<td>pVLT35 derivative</td>
<td>This work</td>
</tr>
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<td>pBAD33 (araC PBAD promoter, pACYC ori, Cm')</td>
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<td>pBAD18 (araC PBAD promoter, pBR322 ori, Ap')</td>
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<td>pCD18his</td>
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<tr>
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<td>Ap', pMMB66 (RSF1010) derivative</td>
<td>Hultgren lab</td>
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<td>Expression of C-FimH with pIII SP</td>
<td>This work</td>
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<tr>
<td>pVLFH</td>
<td>Expression of wild-type C-FimH with FimH SP</td>
<td>This work</td>
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<tr>
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<td>Expression of FimC and FimD (fused to an histidine tail in 3')</td>
<td>Hultgren lab</td>
</tr>
<tr>
<td>pCFH</td>
<td>Expression of FimC and FimD (fused to a histidine tail in 3')</td>
<td>Hultgren lab</td>
</tr>
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vectors. In pFHSN6E, the phenylalanine +1 residue of mature FimH sequence is maintained after cleavage of the SP. Validation of these vectors for expression of functional FimH was demonstrated using yeast agglutination assays (Table S1) and quantitative adhesion assays of E. coli cells to the mannosylated glycoprotein mannan (Supplementary material; Fig. S1B).

Finally, purification of fimbriae from E. coli AAEC185/pPKL115 cells confirmed that the E-tagged FimH protein encoded by pFHSN6E was incorporated into type 1 fimbriae (Fig. 1B, lane 1). As expected, no protein band was detected with anti-E-tag mAb in mock purifications from E. coli AAEC185 cells transformed with pPKL115 and pVLT35 (empty vector; lane 2) or with pFHSN6E and pFC33 (encoding FimC chaperone alone; lane 3). It is interesting to note that the amount of FimA is lower in fimbria purifications from E. coli cells that do not express FimH (Fig. 1B). This is because FimH is required for efficient polymerization of type 1 fimbria (Saulino et al., 1998). When the fim operon is in a single (chromosomal) copy, expression of FimH seems to be essential for type 1 fimbria polymerization (Connell et al., 1996; Langermann et al., 1997; Saulino et al., 1998). When the fim operon is provided in a multicopy plasmid (e.g. pPKL115) fimbria polymerization occurs in the absence of FimH, albeit at reduced levels. Therefore, vector pFHSN6E reproduces the wild-type behaviour of full-length FimH in mannose-binding and triggering of type 1 fimbria assembly.

Expression of a truncated FimH lacking its N-terminal domain

To assess whether N-FimH domain has a role during the incorporation of this adhesin into type 1 fimbria, we generated a derivative of pFHSN6E, termed pCFH, encoding a truncated FimH variant lacking its N-terminal domain (Fig. 2A). After cleavage of its SP, the mature C-FimH protein encoded by pCFH maintains two alanine residues from the linker region followed by cysteine 161 as the first residue from C-FimH. Fimbria purifications of E. coli AAEC185/pPKL115 cells harbouring pCFH revealed that C-FimH was not assembled into fimbriae (Fig. 2B). Albeit detectable in the periplasm, the level of C-FimH was much lower than that of full-length FimH (Fig. 2B). Similarly, FimA was also recovered at reduced levels from fimbriae in E. coli cells expressing C-FimH (Fig. 2B, lane 3). These data suggested that expression of C-FimH may induce some periplasmic stress that lowers the levels of pilin subunits and, subsequently, of type 1 fimbriae. It is well documented that accumulation of pilin subunits in the periplasm induces a specific cell envelope stress leading to an increase in periplasmic proteases (Raivio and Silhavy, 1999; Ruiz and Silhavy, 2005; Rowley et al., 2006).

To rule out the possibility that the lack of incorporation of C-FimH could be due to the specific modifications introduced by our vector (i.e. a heterologous SP and the E epitope tag), we made a new construct in pVLT35 in which the wild-type SP and C-domain of FimH were fused in frame, thus creating plasmid pCFHwt. Upon SP cleavage, the wild-type C-FimH domain encoded by this plasmid maintains at its N-terminus two glycine residues from the wild-type FimH linker. Fimbriae purifications of E. coli AAEC185/pPKL115 cells harbouring pCFHwt, or pFHSN6E or pFVLT35 confirmed that C-FimH was not assembled into fimbriae (Fig. S2). Western blot analysis with a polyclonal antibody raised against C-FimH clearly detected a ~29 kDa band corresponding to wild-type FimH in type 1 fimbriae purified from cells harbouring pFHSN6E. On the contrary, C-FimH was not detected in fimbriae purified from cultures carrying pCFHwt. The periplasmic levels of C-FimHwt were even lower than those coming from pCFH and much lower than those corresponding to wild-type FimH from pFHSN6E (Fig. S2), being barely detectable by Western blot after film overexposure. The lower accu-
mulation of C-FimHwt in the periplasm may indicate a higher susceptibility of this protein to degradation by periplasmic proteases, compared with C-FimH-Etag. Similar low levels of FimA were recovered from type 1 fimbriae purified from cells carrying pCFHwt or the empty vector pVLT35 (Fig. S2). Therefore, the results obtained with both deletion constructs confirm that C-FimH domain is not incorporated into fimbriae in vivo in the absence of the N-FimH domain.

**Chimeras replacing the N-domain of FimH by stable Ig domains**

Deletion of N-FimH strongly suggested a function of this domain during fimbriae assembly. To obtain additional evidence of this function, we set up an alternative approach in which stable Ig domains derived from antibodies replaced N-FimH. It has been previously shown that the lectin N-domain of FimH folds as an Ig-like domain (Buts et al., 2003; Bouckaert et al., 2005), being a globular domain of ~2 nm composed by antiparallel β-sheets and stabilized by an intradomain disulphide bond. Thus, contrary to C-FimH construct, chimeras having an Ig domain replacing N-FimH maintain not only the size of full-length FimH (c. 29 kDa) but also its overall structural characteristics (i.e. two Ig-like domains). This structural similarity should also facilitate the translocation of the chimeras through FimD channel (Saulino et al., 2000; Thanassi, 2002; Li et al., 2004).

In order to minimize any aggregation or instability associated with Ig domains derived from standard antibodies (i.e. those having heavy and light chains) (Wörm and Plückthun, 1999; 2001), we chose single domain recombinant antibodies that have proven to be soluble, monomeric and stable in the periplasm of E. coli (Muyldermans, 2001; Muyldermans et al., 2001; Dumoulin et al., 2002; Holliger and Hudson, 2005). Two single domain antibodies were chosen: (i) a VHH domain from a natural heavy chain-only camel antibody (i.e. lacking light chains in Igs) (Muyldermans et al., 2001; Veiga et al., 2004); (ii) a VL domain from a human antibody engineered to be stable in the absence of a heavy chain (van den Beucken et al., 2001). A comparison of the 3D structures of N-FimH, a camel VHH and human VL domains, is shown in Fig. 3A. Topological models of these domains are shown in Fig. S3 according to conventions for Ig domains (Bork et al., 1994) and as previously reported for N-FimH (Buts et al., 2003; Bouckaert et al., 2005). Two FimH hybrids were constructed replacing N-FimH by stable VHH or VL domains, encoded by plasmids pVHCFFH and pVLCFH respectively (Fig. 3B). Upon transformation of E. coli/pPKL115 cells with these plasmids, type 1 fimbriae were purified and the presence of the corresponding E-tagged FimH chimera detected by Western blot. Neither VHH-CFimH nor VL-CFimH hybrid was assembled into type 1 fimbriae (Fig. 3C, lanes 3 and 4). VHH-CFimH and VL-CFimH substitution hybrids were detected in the periplasmic fraction and remained soluble upon high-speed centrifugation (100 000 g) (Fig. 3C, lanes 3 and 4, lower panel). Therefore, replacing the N-domain of FimH by a heterologous Ig domain does not allow the assembly of the hybrid chimera in fimbriae.
Chimeras containing stable Ig domains fused to full-length FimH

At this point we hypothesized that, if N-FimH was required for assembly, a fusion of an Ig domain to the N-terminus of the mature full-length FimH sequence may be compatible with its incorporation into type 1 fimbriae. To test this possibility, two additional constructs were made by fusing the V_{HH} or V_{L} domain to full-length FimH sequence (Fig. 3B). The plasmids encoding these fusion hybrids, pVHFH and pVLFH, were introduced into E. coli/pPKL115 cells and type 1 fimbriae were purified and analysed in SDS-PAGE and Western blot. As shown in Fig. 3C (lanes 5 and 6), both fusion hybrids (V_{HH}-FimH and V_{L}-FimH) were detected in type 1 fimbria purifications, albeit with reduced efficiency than FimH (~20-fold). These data strongly indicate that N-domain of FimH is needed for assembly of the adhesin into type 1 fimbria. Regardless of their higher structural complexity (having three protein domains), V_{HH}-FimH and V_{L}-FimH were found in the periplasm at higher levels than the substitution hybrids (Fig. 3C, lower panel).

Surface display of FimH chimeras

The incorporation in fimbriae of the FimH chimeras was investigated by enzyme-linked immunosorbent assays (ELISA) to detect their display on the surface of E. coli cells. As shown in Fig. 4, anti-E-tag mAb detects the display of E-tagged FimH on the surface of intact E. coli (pPKL115, pFHSN6E) cells but not on E. coli cells transformed with pFC33 (encoding FimC) and pFHSN6E. Interestingly, permeabilization of the OM of these cells with EDTA indicated that the pool of periplasmic E-tagged FimH was not being recognized by the mAb (although it was easily detectable by Western blot, see Fig. 1B). The absence of reactivity of FimH in the periplasm, but not once is assembled in fimbriae, suggests that FimC may hinder the E epitope from mAb recognition. As a control of the permeabilization procedure, a recombinant antibody (scFvH ~30 kDa) with an E-tag at its C-terminus was expressed in the periplasm of these cells from plasmid pFvHp3 (Veiga et al., 1999). This protein was clearly detected in the ELISA upon OM permeabilization (Fig. 4).

Taking advantage of the specific recognition in ELISA of the FimH protein assembled into type 1 fimbriae, we analysed the surface display of the FimH chimeras. As shown in Fig. 4, the fusion hybrids V_{HH}-FimH and V_{L}-FimH were detected with anti-E-tag mAb on the surface of E. coli/pPKL115 cells carrying either pVHFH or pVLFH. The level of surface display of these chimeras was c. 5–10% of wild-type FimH, being the V_{HH}-FimH chimera displayed at higher levels than the substitution hybrids (Fig. 4). No reactivity (<0.5% of wild-type FimH) was detected in cells expressing the substitution hybrids, V_{HH}-CfimH or V_{L}-CfimH (Fig. 4). These results are in accordance with fimbriae purification data and indicate that FimH chimeras containing N-FimH can be assembled into type 1 fimbriae, albeit with reduced efficiency.

FimH chimeras do not inhibit the function of FimD usher

To gain an insight into the molecular basis underlying the need of the N-FimH, we investigated whether expression of the FimH chimeras could block the activity of FimD usher. To this end, we transformed a wild-type E. coli strain (MG1655), expressing type 1 fimbria and wild-type FimH, with the plasmids encoding the FimH chimeras. Upon induction of the chimeras, the level of wild-type FimH on the surface of E. coli cells was quantified in a standard haemagglutination assay with guinea pig erythrocytes (Hultgren et al., 1985; 1986) using serial twofold dilutions.
of the cultures. As shown in Table 2, identical haemagglutination indexes ($2^n = 256$) were obtained for *E. coli* MG1655 cells transformed with the empty vector (pVLT35) or with any of the plasmids encoding the FimH chimeras (regardless of being deletion, substitution or fusion chimeras). A moderate twofold increase in the agglutination index ($2^n = 512$) was observed for *E. coli* MG1655 cells transformed with pFHSN6E (full-length FimH), which can be explained by the increased dose of FimH expressed in these cells. Therefore, FimH chimeras were not inhibiting FimD (even when expressed from a single chromosomal copy), which was capable of assembling normal levels of type 1 fimbriae containing wild-type FimH.

**Table 2.** Coexpression of FimH chimeras and FimH wt.

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>−O-mannose</th>
<th>+O-mannose</th>
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<tbody>
<tr>
<td>pVLT35</td>
<td>256</td>
<td>2</td>
</tr>
<tr>
<td>pFHSN6E</td>
<td>512</td>
<td>2</td>
</tr>
<tr>
<td>pCFH</td>
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<td>2</td>
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<td>pVHCFFH</td>
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</tr>
<tr>
<td>pVLFH</td>
<td>256</td>
<td>2</td>
</tr>
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</table>

a. Plasmids in *E. coli* MG1655 (*fim+ fimH*).

b. HA index = $2^n$, where $n$ is the greatest twofold dilution of the culture inducing agglutination of guinea pig erythrocytes.

Three independent experiments were performed with identical results.

The N-terminal domain of FimH is required for efficient binding to FimD usher

As inhibition of FimD was ruled out, we tested whether FimH chimeras were impaired in their capacity to bind FimD in vivo. To investigate this issue, we compared their level of association to a FimD usher tagged with six histidines (6xhis) at its C-terminus. It has been reported that FimD$_{6xhis}$ can be purified from the OM in complex with FimH and FimC using immobilized metal affinity chromatography (IMAC) (Saulino et al., 1998; Barnhart et al., 2003). To this end, *E. coli* MG1655 Δ*fim* cells were co-transformed with pCD18his (encoding *fimC* and *fimD*$_{6xhis}$ under the control of the arabinose BAD promoter) and one of the plasmids encoding the FimH variants (pFHSN6E, pVHCFFH, pVLCFH, pVHFH or pVLFH). After induction of FimC, FimD$_{6xhis}$ and the corresponding FimH chimeras in these cells, the proteins from the OM fractions were solubilized with detergent, and FimD$_{6xhis}$ complexes were purified by IMAC. After imidazole elution of the resin, the presence of the FimH chimeras associated to eluted FimD$_{6xhis}$ was revealed by Western blot with anti-E-tag mAb (Fig. 5). Full-length FimH was efficiently recovered associated to FimD$_{6xhis}$ (Fig. 5, middle panel), whereas V$_{Cer}$-CFimH and V$_{C}$-CFimH chimeras were hardly detectable in FimD$_{6xhis}$ eluates (Fig. 5, middle panel, lanes 4 and 5) indicating that the substitution chimeras do not bind FimD$_{6xhis}$ at significant levels. On the contrary, fusion chimeras V$_{Nter}$-FimH and V$_{C}$-FimH were both clearly associated to FimD$_{6xhis}$ (Fig. 5, middle panel, lanes 6 and 7). In accordance to surface ELISA data (Fig. 4), the V$_{Nter}$-FimH fusion bound to FimD$_{6xhis}$ more efficiently. Identical purification efficiency of FimD$_{6xhis}$ in all samples was monitored by Western blot with an anti-His mAb (Fig. 5, upper panel). Homogeneous expression of all the chimeras was controlled by Western blot of whole cell protein extracts (Fig. 5, lower panel). Specificity for FimD$_{6xhis}$ during purification was assessed using OM fractions from control cells expressing FimH and FimC alone (pFHSN6E and pFC18) (Fig. 5, lane 3). Therefore, these data show that binding to FimD usher in vivo only occurs when FimH carries its N-terminal domain.

**Mutation G16D in N-FimH diminishes FimH assembly and FimD recognition**

From an independent project of mutagenesis of N-FimH in pFHSN6E (D. Munera and L.Á. Fernández, unpublished), lane 2), whereas V$_{Cer}$-CFimH and V$_{C}$-CFimH chimeras were hardly detectable in FimD$_{6xhis}$ eluates (Fig. 5, middle panel, lanes 4 and 5) indicating that the substitution chimeras do not bind FimD$_{6xhis}$ at significant levels. On the contrary, fusion chimeras V$_{Nter}$-FimH and V$_{C}$-FimH were both clearly associated to FimD$_{6xhis}$ (Fig. 5, middle panel, lanes 6 and 7). In accordance to surface ELISA data (Fig. 4), the V$_{Nter}$-FimH fusion bound to FimD$_{6xhis}$ more efficiently. Identical purification efficiency of FimD$_{6xhis}$ in all samples was monitored by Western blot with an anti-His mAb (Fig. 5, upper panel). Homogeneous expression of all the chimeras was controlled by Western blot of whole cell protein extracts (Fig. 5, lower panel). Specificity for FimD$_{6xhis}$ during purification was assessed using OM fractions from control cells expressing FimH and FimC alone (pFHSN6E and pFC18) (Fig. 5, lane 3). Therefore, these data show that binding to FimD usher in vivo only occurs when FimH carries its N-terminal domain.

**Function of N-domain of FimH in pilus biogenesis**

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we identified a mutant clone (G16D) with a dramatic reduction in the level of FimH assembled in type 1 fimbriae (13.4% ± 8.3% of the level obtained with wild-type vector pFHSN6E) as determined both by surface ELISA (Fig. 6B) and Western blot analysis of purified type 1 fimbriae (Fig. 6C, upper and middle panels). Mutant G16D carries a single amino acid substitution in glycine 16 of N-FimH domain (referred to the mature protein) substituted to aspartic acid. This mutation is located in a surface-exposed loop of N-FimH, near the D-mannose-binding pocket (Fig. 6A). Importantly, mutant G16D accumulates in the periplasm of E. coli cells at identical levels than its wild-type counterpart, and in a fully soluble form (as judge by ultracentrifugation at 100 000 g) (Fig. 6B, bottom panel).

Next, we investigated whether mutant G16D had an impaired capacity to bind FimD in vivo, which could explain its reduced assembly into fimbriae. As described for the FimH chimeras, mutant G16D was induced in E. coli MG1655Δfim cells co-transformed with pCD18his and the protein complexes containing FimD_{his} were purified by IMAC. The level of G16D mutant protein associated to the eluted FimD_{his} was detected by Western blot with anti-E-tag mAb. Parallel inductions and IMAC purifications were performed for E. coli MG1655Δfim pCD18his cells co-transformed with wild-type pFHSN6E, or the empty vector pVLT35. Three independent induction and purification experiments for mutant and wild-type proteins were performed, which showed identical results. In Fig. 7 a representative Western blot of these experiments is shown, which clearly indicates a reduced level of association of mutant G16D to FimD_{his}. Quantification of band intensities in these experiments (normalized by the purification efficiencies of FimD_{his} with anti-His mAb; Fig. 7, upper panel) revealed that mutant G16D binds to FimD_{his} at a level of 25.9% ± 10% that found for the wild-type FimH, which was in fair agreement with their different level in fimbriae.

Discussion

Polymerization of type 1 fimbriae in E. coli is a highly ordered process in which a single FimH adhesin is incorporated first at the tip of a growing pilus filament, followed by the structural subunits FimG and FimF (assembling the tip fibrillum) and over a thousand copies of FimA, which makes up the main rod of the filament (Jones et al., 1995; Hahn et al., 2002). The specific recognition of FimH by the usher FimD in the OM is essential for the ordered assembly of type 1 fimbriae. First, FimD displays different affinities for the periplasmic binary complexes formed by the pilin subunits and FimC chaperone, having FimG/FimC complex the highest affinity (Dodson et al., 1993; Saulino et al., 1998; 2000). Second, FimD becomes activated and initiates polymerization upon recognition of FimH/FimC complex, which triggers a conformational change in FimD (Saulino et al., 1998; Shu Kin So and Thanassi, 2006).
A previous work had shown a physical interaction between FimD and N-FimH upon overexpression of this domain in the periplasm (Barnhart et al., 2003). However, the biological significance of this interaction for the assembly of type 1 fimbriae was not investigated. The results presented in this paper highlight the biological significance of this interaction showing that recognition of N-FimH by FimD is required for the assembly of FimH in type 1 fimbria. FimH variants lacking the lectin N-domain (or having its N-domain substituted by a heterologous Ig domain) did not associate to FimD in vivo (Fig. 5) and were not assembled in fimbriae (Figs 2–4). On the contrary, chimeras in which an Ig domain was fused to the mature sequence of full-length FimH bound to FimD in vivo (Fig. 5) and were assembled into fimbriae (Figs 3 and 4). The levels of the fusion chimeras in fimbriae were lower than those obtained with wild-type FimH (Figs 3 and 4) but very significant considering their higher structural complexity with three protein domains being one of them a heterologous Ig domain. In addition, we have shown that a single amino acid substitution in N-FimH (G16D) is capable of dramatically diminishing the association of FimH to FimD (to just c. 25% of the wild-type level) and, consequently, the amount of FimH assembled in type 1 fimbriae (c. 14%) (Figs 6 and 7). This mutation does not affect FimH solubility, processing of its SP, or its periplasmic location (Fig. 6). Mutant G16D suggests a high degree of specificity in the recognition of N-FimH by FimD. The exact specificity of FimD (or other fimbrial ushers) for FimH, or altered versions of the adhesin, has not been investigated in detail. However, some reports have suggested a certain degree of flexibility in the recognition. For instance, an exchange of adhesins between F1C and type 1 fimbriae of *E. coli* has been reported (these systems share 68% identity in their chaperones, 58% in their ushers, and 40% in their adhesins) (Klemm et al., 1994; 1995). Although hybrid fimbriae filaments were not purified in these reports, the data strongly indicated some promiscuity in FimD for recognition of FocH adhesin. Clearly, further work is needed to clarify the structural motifs that are essential for N-FimH recognition by FimD in order to determine their conservation in other adhesins or in natural variants of FimH (Bouckaert et al., 2006). Interestingly, residue G16 is conserved in all FimH sequences in current data bases.

Another interesting question is the specific site(s) of FimD involved in the interaction with N-FimH. FimD is a large OM protein (833 residues) that recognizes binary pilin/FimC complexes in the periplasm and mediates the translocation of folded pilin subunits across the OM (Klemm and Christiansen, 1990; Saulino et al., 2000). FimD also catalyses fibre formation by facilitating interactions between the donor and acceptor pilin subunits during donor β-strand exchange reaction (Vetsch et al., 2006). By analogy to the homologous usher PapC, FimD

In this work we have disclosed a previously unrecognized function of the N-terminal lectin domain of FimH for fimbria assembly. This domain had been involved in bacterial attachment and invasion of bladder epithelial cells through its specific binding to mannosylated residues in glycoproteins (Martinez et al., 2000; Zhou et al., 2001; Mulvey, 2002). However, a role of the N-domain of FimH in fimbria assembly had not been defined. Earlier investigations had well established the importance of the C-terminal pilin domain of FimH for its assembly in fimbria. The crystal structure of FimH/FimC complex revealed extensive interactions between the chaperone FimC and the C-domain of FimH (Choudhury et al., 1999). A binary complex formed by C-FimH/FimC was able to bind the N-terminal periplasmic domain of FimD (FimD\(_\text{N}\)) in vitro (Nishiyama et al., 2003). More recently, resolution of a cocrystal of the ternary complex FimD\(_\text{N}/C\)-FimH/FimC has shown specific interactions of C-FimH/FimC and FimD\(_\text{N}\) that allow the usher to discriminate between loaded and unloaded FimC (Nishiyama et al., 2005).
is supposed to contain a hydrophilic channel, 2–3 nm wide, through which folded pilins are transported (Thanassi et al., 1998b; Li et al., 2004). The soluble N-terminal periplasmic domain FimD_N has been shown to be the initial targeting site for recognition of binary pilin/ FimC complexes (Nishiyama et al., 2003; 2005; Ng et al., 2004). Biochemical and structural data indicate that FimD_N does not bind N-FimH (Nishiyama et al., 2003; 2005). Thus, N-FimH should probably bind to a different site in FimD, from which much less information is available. The large central domain of FimD is predicted to have 20 amphipathic β-strands (http://bioinformatics.biol.uoa.gr/PRED-TMBB/) and is inserted in the OM likely forming a β-barrel with a central hydrophilic channel (Koebnik et al., 2000; Henderson et al., 2004). Some evidences indicate that the last ~150 amino acids of FimD may form a second soluble periplasmic domain that seems to participate as a ‘second site’ in pilin/FimC complex recognition and in subsequent steps of fimbria assembly (Thanassi et al., 2002; Shu Kin So and Thanassi, 2006). Given the L-shape of FimH, it was suggested that the N-terminal domain may insert into the hydrophilic channel of FimD (Barnhart et al., 2003) whereas the pilin C-domain bound to FimC contacts the periplasmic domains of FimD. Further work is needed to prove this hypothesis and define the interaction between FimD and N-FimH.

Finally, our findings also have biotechnological implications. FimH has been used as a display system of small peptides and protein fragments in the surface of bacteria for bioremediation and the generation of live vaccines (Schembri and Klemm, 1998; Schembri et al., 1999; Klemm and Schembri, 2000). Most of these strategies used a permissive site of the pilin C-domain of FimH for display of these peptides (Pallesen et al., 1995) that is used in this work for E-tag insertion. Our data indicate that peptide and protein fusions to the mature full-length FimH will be displayed at the tip of a type 1 fimbria filament as long as they do not interfere with translocation through FimD channel.

**Experimental procedures**

**Bacterial strains, growth and induction conditions**

The *E. coli* K-12 strains used in this study are listed in Table 1. The fim mutant strain *E. coli* UTdfim contains a mini-Tn10Km (de Lorenzo and Timmis, 1994) inserted in position 1735 of fimD (with Km cassette in the opposite transcriptional orientation). Bacteria harbouring the plasmids indicated in each case were grown at 37°C on Luria–Bertani (LB) agar plates (Miller, 1992) containing 2% (w/v) glucose (for repressing the *Plac* and the *Pbac* promoter) and appropriate antibiotics for plasmid selection. For fimbria expression, the colonies were grown overnight (o/n) in liquid Brain Heart Infusion medium (2–10 ml) containing appropriated anti-biotics at 37°C without agitation. The next day, the o/n cultures were diluted 10 times in the same medium and incubated at 37°C until the OD_600 reached ~0.6. At this point, 0.05 mM IPTG was added to the cultures, which were further incubated without agitation for 18 h. For binding experiments to FimD_{N-lola}, bacteria were grown o/n at 30°C without agitation in 10 ml liquid LB medium containing 0.2% (w/v) glucose and appropriated antibiotics. The next day, the o/n cultures were used for inoculation of a shake flask containing 20 ml liquid LB medium without glucose (initial OD_600 ~0.05). All cultures were incubated at 37°C with agitation (200 r.p.m.) until the OD_600 reached ~0.5. At this point, 0.02% (w/v) L-arabinose (Sigma) was added and after 15 min, 0.05 mM IPTG was added to the cultures, which were further incubated with agitation for 90 min. Antibiotics were used at the following concentrations: ampicillin (Ap) 150 μg ml⁻¹; chloramphenicol (Cm) 30 μg ml⁻¹, spectinomycin (Sp) 50 μg ml⁻¹.

**Plasmids, DNA constructs and oligonucleotides**

Standard cloning methods were used to purify and manipulate DNA (Ausubel et al., 1994). All DNA constructs were sequenced using the Dideoxy method and an automated DNA sequencer (Perkin Elmer). Oligonucleotides were synthesized by Sigma Genosys. All the PCR reactions were done with Vent DNA polymerase (New England Biolabs). Plasmids used in this study are summarized in Table 1. Details of plasmid constructions are described in the Supplementary material.

**Protein extracts preparation and cellular fractionation**

Whole cell protein extracts were obtained from *E. coli* cells harvested from 1 ml induced cultures (4000 g, 5 min) resuspended in 100 μl of Tris-HCl 100 mM, pH 8, and adding 100 μl of 2× SDS-PAGE sample buffer (see below). Protein samples were boiled for 10 min, sonicated briefly (5 s; Labasonic B Braun) to diminish viscosity, and centrifuged (14 000 g, 5 min) to remove insoluble material (i.e. peptidoglycan). Periplasmic protein extracts were prepared as described previously (Jurado et al., 2002). For solubility experiments, either periplasmic extracts or total cellular lysates (as indicated) were centrifuged (100 000 g, 1 h) using a SW55-Ti rotor (Beckman) and the supernatants from this centrifugation were considered the soluble fractions. Total cellular lysates were obtained from the cells harvested from 80 ml of induced cultures, resuspended in 15 ml of 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, and lysed in a French Cell Press (Sim-AminoN, Electronic Instruments) at 14 000 psi.

**SDS-PAGE and Western blots**

SDS-PAGE was performed using the MiniProtein III electrophoresis system (Bio-Rad) and following standard protocols (Ausubel et al., 1994). The sample buffer for SDS-PAGE (1X) was 60 mM Tris-HCl pH 6.8, 1% w/v SDS, 5% v/v glycerol, 0.005% w/v bromophenol blue and 1% (v/v) 2-mercaptoethanol. For immunoblotting, proteins separated by SDS-PAGE were transferred to a polyvinylidene difluoride membrane (Immobilon-P, Millipore) using a semi-dry electro-
phoresis transfer apparatus (Bio-Rad). For immunodetection of the E-tagged proteins, blots were incubated for 1 h at room temperature with an anti-E-tag mAb-peroxidase (POD) conjugate (Amersham Bioscience). FimD<sub>bact</sub> was detected with anti-His mAb-POD conjugate (Sigma). For detection of FimHwt and C-FimHwt, a mouse polyclonal anti-C-FimH serum (D. Munera and L.A. Fernández, unpublished) was employed in combination with an anti-IgG mouse-POD conjugate (Sigma). Membranes were blocked, washed and developed as previously described (Jurado et al., 2002; Fraile et al., 2004).

Enzyme-linked immunosorbent assays

ELISA conditions were based on those described previously both for assaying E. coli adhesion or surface display of E-tagged proteins (Veiga et al., 1999; 2004). See also Supplementary material. For assaying the surface display of the chimeric FimH constructs, E. coli cells expressing the different variants were resuspended at OD<sub>bac</sub> = 3 in PBS (intact cells) or in PBS containing 20 mM EDTA (permeabilized cells) and adsorbed to immunoplates for 2 h. After this incubation, plates were washed with PBS to remove unbound cells and blocked with PBS-milk as above. Chimeric FimH variants were detected with the anti-E-tag mAb-POD conjugate (1:2000). After three additional washes with PBS, the ELISA were developed as described previously (Veiga et al., 1999; 2004).

Fimbriae purification and analysis

The protocol used was adapted from reported methods (Thanassi et al., 2002). E. coli cells expressing different plasmids were grown in the conditions described for fimbria expression (see above). One hundred OD<sub>bac</sub> units were harvested (6000 r.p.m., 10 min, 4°C), resuspended in 800 µl of Tris-HCl 5 mM pH 8, 75 mM NaCl and incubated at 60°C, 20 min. The fimbriae were detached from the bacteria at 4°C using a homogenizer (PRO-200; PRO Scientific) in position 2 (5 mM pH 8, 75 mM NaCl and incubated at 60°C, 20 min). The fimbriae were boiled during 5 min and immediately neutralized with 6 µl NaOH 1 M before loaded onto SDS-polyacrylamide gels.

Yeast aggregation and haemagglutination assays

For yeast aggregation assays (Slonim et al., 1992) see Supplementary material. For haemagglutination assays (Hultgren et al., 1985; 1986), E. coli MG1655 cells expressing different constructs were induced in liquid using the conditions previously described for fimbria expression. Two OD<sub>bac</sub> units of these cultures were harvested (6000 r.p.m., 10 min, 4°C), washed with PBS and resuspended in 130 µl PBS. Twenty-five microlitres of serial 2<sup>x</sup> dilutions of these samples were added to microtiter V plates (Costar) containing 25 µl PBS or PBS-mannose 2% (as a control for mannose-dependent haemagglutination). Forty microlitres of guinea pig blood (Charles River laboratories), at OD<sub>bac</sub> = 6 previously washed with PBS, were added to each well. After gently mixing the plates were incubated at 4°C o/n. The haemagglutination index reported represent 2<sup>n</sup>, where n is the greatest dilution of the cultures that prevented a positive haemagglutination reaction.

Co-purification of chaperone–subunit complexes bound to FimD<sub>bact</sub>

Analysis of the interaction between the FimC–FimH complex with the FimD<sub>bact</sub> usher in the OM (Thanassi and Hultgren, 2000b; Ng et al., 2004) was done in E. coli host strain MG1655/Δfim transformed with pCD18his and a pVLT35-based vector encoding the different FimH chimeras or the FimH mutant G16D. The same strain transformed with pFC18, encoding FimC alone, and pFHSHN6E was used as negative control. The cultures were grown and induced as described in growth culture conditions. After induction, 10 ml of each culture was harvested (4000 r.p.m., 15 min, 4°C), washed with PBS and resuspended in 1 ml Tris-HEPES 20 mM, pH 8. The samples were lysed by sonication during 4 min (15 s on, 15 s off) on ice. Unbroken cells were discarded by centrifugation (4000 r.p.m., 10 min, 4°C) and the supernatant was considered the clarified cellular protein extract. To selectively solubilize the inner membrane proteins, sarkosyl (0.5% w/v, Sigma) was added and the samples were incubated at room temperature during 5 min. The OM fraction was recovered by centrifugation (100 000 g, 1 h; SW55-Ti rotor, Beckman) and resuspended in 500 µl Eluent (1% v/v, Calbiochem) in HEPES 20 mM pH 7.5 and incubated at 4°C o/n in an orbital platform. Next day 200 µl of the solubilized OM was incubated with 25 µl cobalt affinity resin (Talon, Clontech) previously washed with HEPES 20 mM pH 7.5. The incubation was done in an orbital platform for 90 min at 4°C and the resin was recovered by centrifugation (14 000 r.p.m., 1 min). After washing the samples twice with 250 µl Wash Buffer (HEPES 20 mM pH 7.5, 1% Eluent, 100 mM NaCl, 10 mM imidazole) the resin was incubated with 50 µl Elution Buffer (HEPES 20 mM pH 7.5, 0.1% Eluent, 100 mM NaCl, 250 mM imidazole) during 15 min at 4°C. The ternary FimC-FimH-FimD<sub>bact</sub> complexes were recovered from the supernatants after centrifugation (14 000 r.p.m., 1 min). SDS-PAGE sample buffer (5×) was added, and the protein samples were boiled for 10 min and analysed by Western blot.

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References


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Supplementary material

The following supplementary material is available for this article online:

Data S1. Validation of FimH expression in vectors pFH35, pFHN10E and pFHSN6E.

Data S2. Assays of E. coli adhesion using ELISA.

Fig. S1. Validation of FimH vectors.

A. Schematic representation of FimH variants in vectors pFH35, pFHN10E and pFHSN6E. The fimH sequence of pFH35 is modified in pFHN10E and pFHSN6E to facilitate construction and immunological detection of FimH variants with altered N-domains. The E-tag epitope is inserted at a permissive site or C-domain. In pFHSN6E the endogenous signal peptide (sp) is replaced by that of gene 3 from M13 phage (sp g3). Relevant restriction sites are also shown.

B. ELISA showing the specific adhesion of mannans of E. coli HB101 (pPKL115) cells expressing FimH variants from pFH35, pFHN10E or pFHSN6E. The empty vector pVLT35 was used as a negative control. Serial dilutions of the cultures were used in the assay as indicated. Bound E. coli cells were detected with anti-E. coli polyclonal serum. No adhesion was observed to a non-mannosylated protein control (BSA) (OD490 = 0.05).

Fig. S2. Expression of C-FimHwt. Coomassie and Western blot analysis of purified fimbriae (left panels) from E. coli AAEC185/pPKL115 cells harbouring pFH35 (lane 1), or pVLT35 (empty vector; lane 2), or pCFHwt (lane 3). The accumulation of C-FimHwt in these cells was analysed by Western blot (right). Western blots were developed with a polyclonal serum. No adhesion was observed to a non-mannosylated protein control (BSA) (*). For detection of C-FimHwt, the film was overexposed.

Fig. S3. Structure of N-FimH and Ig domains. 3D structures (top) and topological models (bottom) of N-FimH domain, a camel VHH, and human VL from PDB entries 1QUN, 1KXV and 1F6L. Numbering is according to conventions established for antibody domains (Bork et al., 1994).

Table S1. Validation of vector system for FimH chimeras.

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