Effective assembly of bacterial fimbriae depends on the TAM nanomachine

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Fimbriae/pili are appendages that extend up to 2 μm beyond the cell surface to function in adhesion for bacterial pathogens[1,2] and which are critical for virulence[3]. The best-studied examples of fimbriae are the type 1 and P fimbriae of uropathogenic *Escherichia coli*[4], the major causative agent of urinary tract infections in humans. These and others, such as the type 3 fimbriae of *Klebsiella pneumoniae*[5], share a common mode of biogenesis orchestrated by a periplasmic chaperone and a molecular assembly platform called "the usher" located in the outer membrane[6,7]. Although the mechanism of chaperone-usher-mediated pilus biogenesis is well characterized, how the usher itself is inserted and assembled at the outer membrane is unclear. Current knowledge would implicate the β-barrel assembly machine (BAM) complex[8,9]. Here we report that a rapid response in usher assembly is crucially dependent on another nanomachine, the translocation assembly module (TAM). We assayed the assembly reaction for a range of ushers and provide mechanistic insight into the TAM-mediated membrane insertion pathway that enables the rapid deployment of bacterial fimbriae.

The crystal structure of the fimbrial usher protein FimD revealed an intricate, five-domain architecture: a β-barrel domain, incorporated into which is a β-sandwich plug domain, an N-terminal periplasmic domain (NTD) and two C-terminal periplasmic domains (CTD1 and CTD2)[6,7] (Supplementary Fig. S1). The mechanism for folding and assembling such a large, multi-domains, protein into the outer membrane is unclear, and currently no assay system is available to study the process *in vivo*. A candidate to catalyze this assembly reaction was the β-barrel assembly machinery (BAM complex): composed of the essential proteins BamA and BamD, assisted by the proteins BamB, BamC and BamE; Fig. 1a. In addition, bacteria have TamA and TamB (Fig. 1a) that constitute a translocation and assembly module (TAM)[10-12]. The BAM complex has been suggested to scaffold the strand-wise insertion of β-barrel proteins into

the outer membrane[8,9]. However, the structural complexity of FimD are seemingly incompatible with the simple assembly mechanism that we understand the BAM complex capable of. In order for bacteria to efficiently respond to a new host environment, FimD assembly has to be rapid and efficient. But how is this achieved?

We developed an assay to monitor the assembly of FimD, optimizing the use of a rifampicin-blocking technique[13] to selectively label proteins of interest with ^{35}S-labelled amino acids *in vivo* (Supplementary Fig. S2a). Two BAM-dependent proteins, the β-barrel proteins PhoE and TolC, were used as controls. PhoE is a 12-stranded β-barrel protein (Fig. 1b): an oligomer of three barrels are stable on SDS-PAGE if the samples are not boiled prior to electrophoresis[14]. A time course of PhoE expression showed the assembly of the folded oligomers (Fig. 1c), with a defect in the rate of oligomer formation in a Δ*bamE* mutant background (Supplementary Fig. S2b, S2c). Thus, this new assay reflects the activity of the BAM complex in assembling β-barrel proteins[15-17]. TolC requires the BAM complex for its assembly[18] and, while diminished in the Δ*bamB* and Δ*bamE* mutants, the assay revealed that the rate of TolC assembly is unaffected in the Δ*tamA* or Δ*tamB* mutants (Supplementary Fig. S2d,e,f,g). These important controls demonstrated the mutants were capable of membrane protein translation, as well as translocation and assembly by the BAM complex.

Having validated the assay, we examined the requirements for assembly of the FimD usher. The FimD β-barrel has a surface-exposed, protease-sensitive loop between β-strands 13 and 14[6,19] (Fig. 2a). In correctly assembled FimD, the protease-sensitive loop can be cleaved[6]: when Proteinase K was added to wild-type *E. coli*, fragmentation of ^{35}S-labelled FimD into ~50 kDa and ~40 kDa fragments was observed (Fig. 2b). Mass spectrometry (Supplementary Fig. S3) revealed that the larger fragment (Fig. 2b, "N") corresponds to the N-terminal region including

the NTD, the first 13 strands of the β-barrel and the plug domain. The smaller fragment (Fig. 2b, "C") corresponds to the remaining 11 β-strands and the CTD1 and CTD2 domains. To verify the site of cleavage biochemically, a TEV cleavage site was engineered into the loop between β-strands 13 and 14 (Fig. 2a, asterisk), and the products generated by TEV cleavage were observed to be of the predicted size (Fig. 2c), and consistent with the mass spectrometry data (Fig. 2d, Supplementary Fig. S3). More detailed analysis of the early minutes of the assembly time-course (Fig. 2e) showed that the C-terminal fragment forms earlier than the N-terminal fragment (Fig. 2f). This first experimental demonstration of the polarity of the mechanism for strand-wise β-barrel assembly reveals that the process is initiated from the C-terminal end of the substrate.

We assayed Δ*bamB*, Δ*bamC* and Δ*bamE* mutants for effects on the assembly of the FimD usher protein. Under the conditions of this assay, all mutants expressed similar amounts of the usher protein (Fig. 3a). FimD assembly proceeded in the Δ*bamC* mutant strain at a similar rate to wild-type *E. coli* (Fig. 3b, Supplementary Fig. S4), while defects were observed in the Δ*bamB* and Δ*bamE* mutants. This is in accordance with a previous assessment of the steady-state level of FimD in null mutations in Δ*bamC* (no defect), Δ*bamE* (a small reduction) and Δ*bamB* (a substantial reduction)[20]. Notably, the Δ*tamA* and Δ*tamB* mutants show a more significant defect in FimD assembly (Fig. 3b, Supplementary Fig. S4). Curiously, a single protease-protected fragment of 45 kDa predominates in the Proteinase digests (Fig. 3b, "*") and epitope-tagging experiments (Supplementary Fig. S5) and mass spectrometry (Supplementary Fig. S3) revealed that this represents a central portion of FimD excised by Proteinase K (Supplementary Fig. S5). An extended assay for FimD assembly in the Δ*tamA* or Δ*tamB* mutants showed that after four hours of incubation, some correctly assembled FimD was observed as judged by the substantial appearance of the N-terminal and C-terminal fragments (Fig. 3d). These data suggest the BAM

complex can eventually assemble FimD into the outer membrane, but the presence of the TAM is required for its efficient assembly from the C-terminus.

Bacterial pathogens produce fimbriae to adhere to host tissues[21,22]. In the case of uropathogenic *E. coli* (UPEC), the rapid deployment of fimbriae enables the pathogen to adhere to bladder epithelial cells and resist the hydrodynamic flow of urine that would otherwise lead to expulsion from the urinary tract. The FimH adhesin located at the tip of the type I fimbriae (Supplementary Fig. S1b) allows the pilus to bind α-D-mannosylated glycoproteins such as the uroplakins on the luminal surface of the bladder epithelium[23]; this mannose-binding activity, essential for bladder recognition and colonisation, can be assayed to measure fimbrial-dependent cell agglutination activity[24]. Consistent with the defects observed for FimD assembly, the *E. coli* mutants lacking TAM function were significantly diminished in fimbrial-dependent mannose binding activity (Fig. 3e). To mimic a scenario in which the rapid response of fimbriae could be measured, we engineered a tightly controlled inducible promoter upstream of the genes encoding type 1 fimbriae on the chromosome of the UPEC reference strains CFT073 and UTI89 (CFT073-P_{tet}-*fim* and UTI89-P_{tet}-*fim*; Supplementary Fig. S6). The addition of the inducer anhydrotetracycline (AHT) is used as a proxy for the urinary tract stimulus that leads to the activation of *fim* transcription. In CFT073-P_{tet}-*fim*, the addition of AHT led to the rapid production of functional type I fimbriae as measured by cellular agglutination (Fig. 3f). In contrast, deletion of the Δ*tam*A and Δ*tamB* in CFT073-P_{tet}-*fim* significantly delayed the onset of type 1 fimbrial biosynthesis; at 20 minutes post induction with AHT the time to agglutination for the CFT073-P_{tet}-*fim* Δ*tam* mutant was approximately 3-fold slower than for CFT073-P_{tet}-*fim*. Eventually, the agglutination time for both strains was equivalent. This functional data confirms that while the BAM complex can mediate translocation of the FimD usher, it does so inefficiency in the absence of the TAM (Fig. 3f). Comparable results were obtained in the UPEC strain UTI89 (Supplementary Fig. S6).

Taken together, the data shows that the presence of the TAM provides a selective advantage to a bacterial pathogen that needs to deploy fimbriae urgently.

FimD is but one of many diverse usher proteins that work to drive fimbrial production[22,25]. We sought to address whether suppressing the function of the TAM would impact generally on the production of fimbriae, and four additional usher proteins (Supplementary Fig. S7a) were subjected to biochemical assembly assays. PapC is the usher required for the production of P fimbriae that mediate UPEC adherence to α-Gal(1-4)β-Gal receptor epitopes in the upper urinary tract[26], while HtrE is the usher for the assembly of Yad fimbriae[27]. YbgQ and YfcU are distinct "π group" ushers encoded by genes which are prevalent and highly conserved in a range of *E. coli* pathotypes[27,28,29].

These usher proteins are readily assembled into the outer membrane of *E. coli* (Fig. 4a). The extracellular loops of FimD and PapC differ in structure (Supplementary Fig. S7b), and assembly measurements of all other usher proteins suggest them to be structurally more similar to PapC than they are to FimD: the predominant form being ~95 kDa protease-resistant proteins (Fig. 4a), suggesting that no single loop in these ushers is protease-sensitive. All of these usher proteins were observed to have defective assembly into the outer membrane in Δ*tamA* mutants. In each case, the ~95 kDa usher remains relatively sensitive to Proteinase K, gradually generating more protease-resistant fragments through the timeframe of these assays (Fig. 4a).

We conclude that the role of the TAM in catalysing the assembly of FimD will be general to the diverse usher proteins found in bacteria[22,25]. In BAM+ TAM+ cells, FimD assembly proceeds from the C-terminal end of the barrel domain and is completed rapidly (Fig. 4b). In bacteria lacking the TAM (i.e. BAM+ TAM$^-$ cells), the BAM complex initiates assembly from strands

amidst the protein and assembly proceeds very inefficiently, with the exposure of non-native, protease-sensitive regions of FimD not found in the crystal structure of the correctly folded usher. Ultimately, the usher will be assembled into a functional form with or without the TAM, but time matters in the harsh environments that pathogens must colonize, and success in colonization requires a rapid and efficient deployment of fimbriae to dock onto host cell surfaces.

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AUTHOR CONTRIBUTIONS

CS, MJB, IDH, KMP, MDP, AWL designed and carried out analysis.

CS, MJB, IDH, JL, MAS, HHS, KT and MJS provided expertise to analyses,

MAS, MJB, IDH, JL, RAS, GW and TL supervised experimental work and evaluated data.

MAS, RAS, GW and TL evaluated results and wrote manuscript.

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FIGURE LEGENDS

Figure 1. Mutation of the *tam* genes in *E. coli* does not affect the assembly of PhoE β-barrels. a, Representation of the subunits constituting the BAM complex and the TAM, including what is known of their membrane topography. OM, outer membrane; IM, inner membrane. **b,** Crystal structure of PhoE oligomer of β-barrels showing the arrangement of 12 anti-parallel β-strands from a single polypeptide in each of the three β-barrels. (PDB: 1PHO). Graphic prepared using PyMOL. **c,** *E. coli* strains harbouring pKS07(PhoE) starved of methionine and cysteine were pulse-labelled with ^{35}S-methionine and ^{35}S-cysteine. The triangles above the graphs represent the time increment where aliquots were taken at 10 seconds, 2, 4, 8, 16 and 32 minutes. Duplicate aliquots were removed to tubes containing Semi-Native Sample Buffer (0.2 % w/v SDS, 10% v/v glycerol, 0.01% w/v bromophenol blue, 40 mM Tris, pH 6.8) followed by 5 minute incubation at either 37 °C or 100 °C prior to electrophoresis. o – oligomeric forms of PhoE; m – PhoE monomers.

Figure 2. Assembly of the usher FimD. a, Crystal structure of FimD showing the position of the extracellular loop (yellow). (PDB: 3RFZ). The N-terminal domain is shaded cyan, the plug domain shaded green, and the C-terminal domains shaded blue. An asterisk indicates the proposed site of cleavage by Proteinase K, and the position at which a TEV site was engineered. The cartoon below depicts the membrane topology of FimD. β-strands 1, 6, 12 and 24 are labelled. **b,** *E. coli* harbouring pKS02(FimD) were starved of methionine and cysteine, pulse-labelled with ^{35}S-methionine/^{35}S-cysteine for 40 seconds, followed by a 60-minute chase with unlabelled methionine and cysteine. The triangles above the graphs represent the time increment where aliquots were taken at 2, 5, 10, 15, 30 and 60 minutes. At each timepoint, aliquots were treated with Proteinase K for 10 minutes, followed by TCA precipitation. Analysis was by SDS-

PAGE and densitometry, the 50 kDa N-terminal fragment and 40 kDa C-terminal fragment are indicated. **c,** *E. coli* cells harbouring pKS02 (FimD) or pCJS29 (FimD with an engineered TEV site in extracellular loop 7) were incubated for 30 minutes to allow assembly of FimD, and then aliquots were treated with TEV protease or Proteinase K, followed by TCA precipitation, SDS-PAGE and densitometry. **d,** MS1 chromatographic peak area-based quantitation where peptide coverage is plotted as a normalised heat-map: red shows high abundance in peptide recovery, pale pink shows minimal detection of peptides, white shows complete absence of peptides (details Supplementary Fig. S3). **e,** *E. coli* cells harbouring pKS02 were used to measure the assembly of FimD over 4 minutes. At increments of 20 seconds, aliquots were treated with Proteinase K, followed by TCA precipitation for analysis by SDS-PAGE and densitometry. **f,** A plot of normalized band density of each fragment versus time (N-terminal in red circles and C-terminal as cyan boxes), from the primary data shown in Fig. 2e. Error bars represent the standard deviation of 4 biological replicates.

Figure 3. *E. coli* Δ*tamA* or Δ*tamB* mutants have defects in the assembly of FimD. a, The indicated strains of *E. coli* harbouring pKS02(FimD) were used to measure the synthesis of the 95 kDa protein FimD. Analysis was by SDS-PAGE and densitometry. **b**, *E. coli* strains containing pKS02(FimD) were pulse-labelled with ^{35}S-methionine/^{35}S-cysteine for 40 seconds, followed by a chase with unlabelled methionine and cysteine. The triangle above the graphs represents the time increment where aliquots were taken at 10 seconds, 2, 5, 10, 15, 30 and 60 minutes. At each timepoint, aliquots were treated with Proteinase K for 10 minutes, followed by TCA precipitation. Analysis was by SDS-PAGE and densitometry. **c,** Histogram of the fitted first order observed rate constants corresponding to the appearance of the N-terminal FimD fragment (N) or the single FimD fragment found in the Δ*tamA* and Δ*tamB* mutants (*). Error bars represent the 95% confidence level of fit. ** $p < 0.001$. **d,** The indicated *E. coli* strains

harbouring pKS02(FimD) were used to measure the formation of PK-sensitive FimD fragments was assessed at 10 seconds, 2, 4, 8, 16, 32, 64, 128 and 256 minutes. **e,** The change in optical density (600 nm) corresponding to the level of cell agglutination of cultures of wild-type *E. coli* BW25113, or the corresponding isogenic Δ*fimD* mutant or Δ*tamA* mutant strains were measured in the absence or presence of the polyvalent mannose polymer (Methods). ** $p < 0.001$. **f.** The UPEC strain CFT073-P_{tet}-*fim*, engineered to contain a tightly controlled inducible promoter upstream of the genes encoding for type 1 fimbriae (Supplementary Fig. S6), was subject to induction with Anhydrotetracycline (AHT). The time taken for agglutination of type 1 fimbriae expressing bacterial cells was measured at 20, 30, 40, 50 and 60 minutes post induction. Data was collected for CFT073-P_{tet}-*fim* (TAM+) (■) and for CFT073-P_{tet}-*fim* (Δ*tamAB*) (•). All experiments were performed in triplicate; error bars indicate standard deviation. Complementation experiments, as well as equivalent experiments on a second UPEC reference strain (UTI89) are presented in Supplementary Fig. S6

Figure 4. The TAM is required for the assembly of multiple different usher proteins. a, Wild-type and Δ*tamA E. coli* were used to measure the formation of Proteinase K-sensitive fragments of PapC, HtrE, YbgQ and YfcU, assessed at 10 seconds, 2, 4, 8, 16 and 32 minutes. At each timepoint, aliquots were treated with Proteinase K for 10 minutes, followed by TCA precipitation, and analysed by SDS-PAGE and densitometry. -PK, samples were incubated for 32 minutes, but Proteinase K was omitted from sample processing. WT, wild-type. **b,** Model for usher assembly by the beta-barrel assembly machinery, with or without the activity of the TAM. At a fixed timepoint where the BAM+TAM+ cells have completed FimD folding, Proteinase K (PK) generates 50 kDa and 40 kDa fragments because of the correct exposure of Loop 7 at the cell surface. At this time, the BAM+TAM- cells have partially folded a ~45 kDa core of FimD

that did not start from the C-terminus. Ultimately, a correctly folded FimD is generated in both conditions.

SUPPLEMENTARY MATERIAL

Supplementary Figure S1. The five domain architecture of usher proteins, exemplified by FimD. a, Structural map of FimD shows the domain boundaries according to the crystal structure (pdb 3RFZ). Trans-membrane β-strands shown as arrows within the three segments of the β-barrel domain. The plug domain, shown in green, is incorporated between β-strands 6 and 7. A protease-sensitive extracellular loop (Loop 7) is displayed between β-strands 13 and 14. The N-terminal domain (NTD) and two C-terminal domains (CTD1 and CTD2) are displayed in the periplasm. **b,** Schematic of the chaperone-usher system for fimbrial manufacture. Multiple copies of the major fimbrial subunit FimA are built into the growing filament by the action of the usher (FimD). The tip subunit (FimH) is a lectin with mannose-binding activity. The domains of FimD have been color-coded as shown in panel a, and the β-barrel pore domain is represented as a wireframe to show the β-strands within the outer membrane. **c,** Crystal structure of FimD (pdb 3RFZ) highlights the position corresponding to the loop between β-strands 13 and 14 (asterisks). The extracellular loop is protease sensitive and was degraded with trypsin in order to crystallize FimD[6]. This represents the basis of the assay employing Proteinase K susceptibility as a measure of correct folding in the FimD assembly assays.

Supplementary Figure S2. A rifampicin/T7-based assay to measure outer membrane protein assembly rates in *E. coli*. a, Cartoon depiction of the rifampicin blocking technique and ^{35}S pulse-labelling, and subsequent detection techniques of correctly folded outer-membrane proteins. In the case of FimD, correct folding and assembly in the outer membrane results in the a protease-sensitive loop between β-strands 13 and 14 being displayed on the extracellular surface of the outer membrane, and Proteinase K treatment converts the ~85 kDa FimD to two fragments that migrate on SDS-PAGE at ~50 kDa and ~40 kDa. **b,** Densitometry analysis of PhoE assembly in the various mutants, corresponding to data shown in Fig. 1c. The normalized

density of the monomer band is plotted versus time, where the solid line represents the fitted exponential decay, and error bars are the SEM calculated from 4 biological replicates. **c,** Quantitation of the assembly of PhoE trimers from monomers at 37 °C. The histogram depicts the fitted first order observed rate constants of PhoE oligomer assembly from the data in Supplementary Fig. S2b. **d,** Crystal structure of TolC (PDB: 1EK9): the oligomer is assembled from three subunits, each of which contributes 4 β-strands to a 12-stranded β-barrel. The long helical extensions delve into the periplasm. **e,** *E. coli* strains harbouring pMB11(TolC) were used to measure the assembly of TolC trimers (t) from monomers (m). Samples in SDS-PAGE loading buffer were incubated at 25 °C (non-denaturing; to distinguish monomers and trimers) or 100 °C (denaturing; total TolC present). **f,** Plots of the normalized relative intensity of the monomer band versus time (seconds) from data shown in panel 2c, solid line represents the fitted exponential decay, error bars are the SEM calculated from 4 biological replicates. **g,** Histogram of the fitted first order observed rate constants corresponding to the assembly of TolC in the different *E. coli* strains, error bars represent the 95 % confidence interval of fit.

Supplementary Figure S3. Peptide mapping of FimD fragments generated by Proteinase K digestion. a, MS1 chromatographic peak area-based quantitation where peptide coverage is plotted as a heat-map: red shows high abundance in peptide recovery, pink shows minimal detection of peptides, white shows complete absence of peptides. Note, sequence numbering is from the first translated methionine residue, no peptides were recovered corresponding to the signal sequence (residues 1-45) as expected. **b**, Peak areas of peptides shown in (a) were extracted by Skyline: SLNESGTNIQLVGYR (residues 466- 480), YSTSGYFNFADTTYSR (i, residues 481-496), DGVIQVKPK (residues 506-514),FTDYYNLAYNK (residues 515-525) and LQLTVTQQLGR (residues 529 - 539). Note that the C-terminal cleavage of DGVIQVKPK is non-tryptic, identifying one end of the Proteinase K cleavage site which was only identified in the C-terminal (~40 kDa) fragment. **c,** Annotated MS/MS spectra of the non-tryptic peptides

characterising cleavage events. Peptide DLYFNPR (left, residues 46-52) is the N-terminus of the mature FimD following signal peptide removal by signal peptidase, while peptide DGVIQVKPK (right, residues 506-514) identifies the initial peptide of the ~40 kDa fragment of FimD. **d,** Diagrammatic representation of the regions identified as the ~50 kDa N-terminal fragment, the ~40 kDa C-terminal fragment in TAM+ (wild-type) *E. coli*, and the ~45 kDa "*" fragment of FimD found in Δ*tamA E. coli*.

Supplementary Figure S4. Statistics on FimD assembly (in *bam* and *tam* mutants). a, Histogram of the fitted first order observed rate constants for the appearance of the ~50 kDa N-terminal FimD fragment (N), the ~45 kDa C-terminal fragment or the single, ~45 kDa FimD fragment found in the *tam* mutants (*) in different *E. coli* mutant strains. Error bars correspond to the 95% confidence interval of fit. **b,** Table of k_{obs} for the appearance of FimD fragments for all strains in the study. **c,** Plots of relative density of each of the bands (N), (C) and (*) versus time (seconds) for each of the *E. coli* strains in the study. Highlighted as solid red squares is the data for the ~45 kDa (*) band. The Δ*tamA* and Δ*tamB* mutants each accumulate much more of the ~45 kDa fragment FimD than any of the *bam* mutants. Error bars indicated SEM over 2 biological replicates and 2 technical replicates.

Supplementary Figure S5. Epitope mapping of the ~45 kDa fragment of FimD. a, Diagrammatic representation of the Strep-tag constructs of FimD. **b,** *E. coli* harbouring plasmids (pCJS51 or pCJS29) encoding either N_{term}-Strep or C_{term}-Strep forms of FimD were subject to a 32 minute assembly assay and treated with Proteinase K for 10 minutes, followed by TCA precipitation. Analysis was by SDS-PAGE and blotting using Streptactin-HRP (horse radish peroxidase) reagent. No 45 kDa fragment was observed.

Supplementary Figure S6. A system to measure rapid deployment of type I fimbriae. a, The UPEC reference strains CFT073 and UTI89 were employed. Each of these strains was engineered with a kmRExTET cassette using lambda red mediated-homologous recombination to incorporate a tetracycline-inducible PLtet0-1 promoter upstream of the *fimAICDFGH* operon, replacing the natural, phase variable *fimA* promoter (and the regulatory genes *fimB* and *fimE*). The modified strains were referred to as CFT073-P_{tet}-*fim* and UTI89-P_{tet}-*fim*, respectively. Anhydrotetracycline (AHT) was used to induce transcription of the *fim* genes in both strains. Initial validation experiments showed that 20 min was sufficient for gene induction, transcription, translation and targeting of the proteins encoded from the *fimAICDFGH* operon to produce functional fimbriae. Fimbrial activity was measured by agglutination of bacterial cells in the presence of polyvalent mannose particles: either synthesized in pure form[24] or provided as yeast cells[30]. **b,** CFT073-P_{tet}-*fim* and the CFT073-P_{tet}-*fim tamAB* mutant were induced for type 1 fimbriae expression with AHT. The time taken for agglutination of type 1 fimbriae expressing bacterial cells and yeast cells was measured at 20, 30, 40, 50 and 60 minutes post induction. Data was collected for CFT073-P_{tet}-*fim* (TAM+) (■),CFT073-P_{tet}-*fim*-Δ*tamAB* (•), and CFT073-P_{tet}-*fim*-Δ*tamAB* complemented with a plasmid encoding the *tamAB* genes [pTamAB] (⋀).All experiments were performed in triplicate; error bars indicate standard deviation. **c,** Equivalent data for UTI89-P_{tet}-*fim*, the UTI89-P_{tet}-*fim tamAB* mutant, and the UTI89-P_{tet}-*fim tamAB* mutant complemented with pTamAB following induction with AHT. Data was collected for UTI89-P_{tet}-*fim* (TAM+) (■),UTI89-P_{tet}-*fim*-Δ*tamAB* (•), and UTI89-P_{tet}-*fim*-Δ*tamAB* complemented with pTamAB (⋀). All experiments were performed in triplicate; error bars indicate standard deviation.

Supplementary Figure S7. Fimbrial operons and usher structures. a, Selected fimbrial operons found in *E. coli*, color-coded according to protein family designations. The usher gene names are shown, as well as the gene encoding FimH (the mannose-binding type 1 fimbrial

adhesin). PapC is a π-group usher required for the formation of P fimbriae and HtrE is an α-group usher required for the production of Yad fimbriae. YbgQ and YfcU are π-group ushers encoded by genes within fimbrial operons; Yfc fimbriae mediate biofilm formation when over-expressed[29], while the functional expression of Ybg fimbriae has not been described. **b,** Structural overlay of FimD and PapC depicts the overall high r.m.s.d. values (all atom superposition is 4Å) for the two structures except in the region of Loop 7. In PapC (red), the loop is structured with a tight turn that would be relatively insensitive to Proteinase K digestion. In FimD (yellow), the loop was readily degraded by trypsin, removing ~20 amino acid residues, by which treatment FimD crystals were able to form. This greater accessibility of Loop 7 for FimD relative to PapC is in direct accord with measurements of Proteinase K susceptibility in the usher assembly assays for FimD and PapC (Fig. 3 and Fig. 4).

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METHODS

Pulse chase assays in E. coli

Details of plasmids, primers and strains used in this study are available on request. The mutants were constructed in *E. coli* BL21 star (DE3) using the gene gorging technique[31], by cloning a kanamycin resistance cassette in place of the gene of interest: either *bamB, bamC, bamE, tamA,* or *tamB*. Plasmid constructs for *fimD (pKS02), phoE*(pKS07), *tolC* (pMB11) and *ybgQ* (pCJS40) from *E. coli* K-12 strain MG1655 were cloned in pET15b(+) (Novagen). *papC* (pCJS32) from UPEC CFT073 was cloned in pET22b(+) (Novagen); wereas, *htrE* (pCJS49) and *yfcU* (pCJS33) from UPEC CFT073 were cloned in pET15b(+). The *fimD* containing a C-terminal *strep*-tag II (engineered sequence: ASWSHPQFEK) from a modified pAN2 vector[6] was further modified to include a TEV site (ENLYFQG) and two cysteine substitutions, replacing amino acid residues 452-458, 88 and 756 of the mature protein (Waksman, G., unpublished data). This modified *fimD* was sub-cloned into pET15b(+) to generate pCJS29. To generate the N-terminal *strep*-tagged *fimD*, the *fimD* signal peptide (amino acid residues 1-45) was replaced by *strep*-tag II and subsequently inserted into pET22b(+) to incorporate the *pelB* signal sequence, generating pCJS51. The accession numbers for the sequences used for each substrate protein are as follows: FimD (NP_418737), PhoE (NP_414776), TolC (NP_417507), PapC (NP_755465), HtrE (NP_752120), YbgQ (NP_415246) and YfcU (NP_754765).

Expression was performed in *E. coli* BL21 star (DE3) cells and the five isogenic mutant strains. Cells were incubated at 37 °C on an orbital platform rotating 200 strokes per minute and routinely grown in LB or M9 Minimal Medium (M9+S) also containing 0.031% (w/v) thiamine, 0.4% (w/v) glucose as carbon source and instead of ammonia as nitrogen source, the 0.1% (w/v) drop-out mix comprised of only the amino acids was used. A second formulation of M9 Minimal

Media, but without sulphur (referred to as M9-S; i.e. no methionine, no cysteine, and $MgCl_2$ replaces $MgSO_4$), was used. When required, antibiotics were used at the following final concentrations: ampicillin, 100 μg mL^{-1}; kanamycin, 30 μg mL^{-1}. Samples for pulse chase experiments were from cells incubated in LB to mid-log phase and then resuspended in M9-S. After 30-minute incubation, 20% (v/v) glycerol was added and aliquots of cells were snap frozen in liquid nitrogen, and stored at -80 °C. Each biological replicate was a batch of cells prepared independently, whereas aliquots of a batch were considered technical replicates.

Cells were thawed on ice, and resuspended in M9-S. Addition of 0.5 mg mL^{-1} rifampicin followed by 37 °C incubation for 60 min was used to inhibit the *E. coli* RNA polymerase. T7 RNA polymerase-based transcription was induced with 0.2 mM IPTG for 5 min (25 °C for TolC, 30 °C for fimbrial ushers, 37 °C for PhoE). Cells were 'pulsed' with 21 μCi mL^{-1} [^{35}S]-Methionine and –Cysteine (MP Biomedicals) for 40 sec and then 'chased' by resuspension in M9+S. Where indicated, aliquots were removed and incubated on ice for 10 min with or without 50 μg ml^{-1} Proteinase K (PK solution, Promega) or incubated at 30 °C for 30 min with or without 100 μg mL^{-1} TEV supplemented with 1 mM DTT. Cells were incubated for a further 5 min on ice with 10% (v/v) trichloroacetic acid (TCA), before centrifugation. Following at least one acetone wash, pellets were resuspended in SDS-loading buffer and boiled for 3 min.

Samples were separated by electrophoresis using SDS-PAGE gels or, for TolC and PhoE experiments, 4-16% continuous semi-native (SN) gels. The SN gels were prepared according to the SDS-PAGE protocol, except that SN buffer (0.5 mM EDTA, 375 mM Tris, pH 8.8) was used in place of Separating and Stacking SDS-Buffers, and electrophoresis was performed using MES Running Buffer (50 mM MES, 2 mM EDTA, 0.2% w/v SDS, 50 mM Tris, pH 7.3) in place of SDS Running Buffer. In all cases, proteins were transferred to 0.45 μm nitrocellulose

membranes. Radiation was captured using a storage phosphor screen (GE Health sciences) and analysed using Typhoon Trio (detection set to 320 nm).

Mass Spectrometry Analysis

Samples were prepared using the pulse-chase method above, except that 35S-label was omitted, the induction time was doubled, and the chase time was 15 min. Samples were separated as above and bands corresponding to the fractions were excised for mass spectrometry.

The four bands corresponding to FimD (full length FimD and the N-, *- and C-fragments) were excised, and subjected to in-gel tryptic digestion[32]. The resulting tryptic peptides were resolved by reverse-phase chromatography performed on a Dionex Ultimate 3000 UHPLC system (Thermo Scientific) connected to a 50-cm Acclaim PepMap RSLC analytical nano-column (C18, 2μm, 100 Å (Thermo Scientific)). The peptides were eluted at a flow rate of 0.25 μl/min using linear gradient of 2 to 34% ACN in 0.1% FA over 30 min. Mass spectrometry was performed using a Q Exactive Plus mass spectrometer (Thermo Scientific) in positive mode using repetitively full MS scan followed by higher-energy collisional dissociation of the 10 most dominant ions selected from the first MS scan.

For analysis of the LC-MSMS data, Thermo LC-MSMS data files from analysis of tryptic digests of FimD-containing gel slices were analysed with Morpheus[33]. Up to 400 peaks per spectrum were matched against a UniProt E.coli database containing 4304 target sequences (and an equivalent number of "on-the-fly" decoy sequences) using tryptic enzyme specificity with up to 3 missed cleavages. Mass tolerances were 10 ppm for both precursor and fragments, with monoisotopic precursor correction applied of +/-1. A 1% FDR threshold was applied to the results, yielding 4125 unique target peptide sequences from 476 target protein groups, with 31

peptides matching the canonical FimD sequence (P30130). A subsequent non-tryptic search yielded 69 peptides from FimD (including 29 of the 31 previously identified peptides), most of which were low abundant potential degradation products.

Label-free peptide-level quantitation was achieved using Skyline software[34] (version 3.1.0.7382, University of Washington). Briefly, the search results and mzML spectral files (generated with msconvert) were used to construct a Bibliospec spectral library in Skyline. Precursor chromatographic peak areas for 3-5 isotopes of identified peptides from FimD (UniProt accession P30130) were extracted from the raw MS data. Retention times, isotope dot product values and mass errors of detected peaks were manually verified to correlate with the observed and expected values respectively. Peaks with isotope dot products below 0.9 or average absolute mass errors >10ppm were removed from further analysis. Protein coverage maps were created by calculating the total peak area assignable to each amino acid in the protein, removing peaks less than 2% of the sample's maximum to remove noise/carryover and normalising across samples to remove bias against poorly ionising peptides.

Fimbrial function measured by cell adhesion assays

Mannose functionalized hyperbranched poly(amido amine)s were prepared as previously described[24]. *E. coli* strains were grown in LB until an $OD_{600\,nm}$ of 1.0. Cells were harvested by centrifugation at 4,000 g, and resuspended to $OD_{600\,nm}$ of 1.0 in PBS (pH 7.2) with 0.1 mM $MnCl_2$ and 0.1 mM $CaCl_2$. The functionalized polymer was added to the cell suspensions at a final concentration of 10 mg/ml mixed and the reduction in $OD_{600\,nm}$ in response to the agglutination was monitored over 1 hour. Alternatively, because of the high-content of mannosylated proteins in yeast cell walls, yeast cells were used in cell adhesion assays as described[30].

In order to measure type 1 fimbriae biogenesis in CFT073-P_{tet}-*fim*, UTI89-P_{tet}-*fim* and their *tamAB* mutant derivatives, we measured the capacity of these strains to mediate agglutination of yeast cells following induction with AHT. Overnight cultures of each strain were standardized to an OD_{600nm} of 0.5 before inducing with 0.5 mM AHT and incubation at 37°C with 250rpm shaking. At 10 minute intervals (timepoints 10, 20, 30, 40, 50 and 60 minutes post induction), samples of the cultures were taken and assayed for their capacity to mediate yeast cell agglutination as previously described[30]. The time to cause visible agglutination was monitored.

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