F9 Fimbriae of Uropathogenic Escherichia coli Are Expressed at Low Temperature and Recognise Galβ1-3GlcNAc-Containing Glycans

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Abstract

Uropathogenic Escherichia coli (UPEC) is the leading causative agent of urinary tract infections (UTI) in the developed world. Among the major virulence factors of UPEC, surface expressed adhesins mediate attachment and tissue tropism. UPEC strains typically possess a range of adhesins, with type 1 fimbriae and P fimbriae of the chaperone-usher class the best characterised. We previously identified and characterised F9 as a new chaperone-usher fimbrial type that mediates biofilm formation. However, the regulation and specific role of F9 fimbriae remained to be determined in the context of wild-type clinical UPEC strains. In this study we have assessed the distribution and genetic context of the f9 operon among diverse E. coli lineages and pathotypes and demonstrated that f9 genes are significantly more conserved in a UPEC strain collection in comparison to the well-defined E. coli reference (ECOR) collection. In the prototypic UPEC strain CFT073, the global regulator protein H-NS was identified as a transcriptional repressor of f9 gene expression at 37°C through its ability to bind directly to the f9 promoter region. F9 fimbriae expression was demonstrated at 20°C, representing the first evidence of functional F9 fimbriae expression by wild-type E. coli. Finally, glycan array analysis demonstrated that F9 fimbriae recognise and bind to terminal Galβ1-3GlcNAc structures.

Introduction

Urinary tract infections (UTI) are among the most common infectious diseases of humans and a major cause of morbidity. In the USA, UTI account for approximately $1.6 billion in medical expenditures each year [1]. It is estimated that 40–50% of adult healthy women will experience at least one UTI episode in their lifetime. The recurrence rate of UTI is high and often the infections tend to become chronic with many subsequent episodes. UTIs usually start as cystitis but often evolve to encompass the kidneys and can ultimately result in dissemination into the bloodstream and/or renal failure. Catheter-associated UTIs are also very common and account for 40% of all nosocomial infections. Most patients with an indwelling urinary catheter for thirty days or more develop bacteriuria [2].

Uropathogenic Escherichia coli (UPEC) is the cause of the majority (>80%) of UTIs in humans. UPEC isolates contain numerous virulence factors, which allow for the successful colonisation of the urinary tract. Although no single virulence factor is uniquely definitive of UPEC, the ability to cause symptomatic UTI is enhanced by adhesins (e.g. type 1 and P fimbriae) and toxins (e.g. hemolysin) [3,4]. Adherence to the urinary tract epithelium is the first stage of UTI as it enables bacteria to resist the hydrodynamic forces of urine flow and establish infection. Among the best-described adhesins produced by UPEC are type 1, P, and F1C/S fimbriae of the chaperone-usher (CU) pathway [4].

The CU pathway is a highly conserved secretion system in Gram-negative bacteria that mediates the assembly of hair-like fimbrial polymers on the bacterial cell surface. CU fimbrial biogenesis requires a dedicated periplasmic chaperone and an outer membrane usher protein that functions as an assembly platform of the fimbrial organelle which is primarily composed of a helical array of 500 to 3,000 copies of major subunit protein [5,6]. The receptor-binding adhesin resides at the distal end of the fimbrial organelle and contains a C-terminal domain which connects the adhesin to the terminal major subunit protein sometimes aided by one or more minor subunits, and an N-terminal lectin domain which mediates binding to specific ligands [3]. The genes encoding the various components of CU fimbriae
are typically organised in an operon and transcribed as a single polycistronic mRNA molecule [7].

Genomic analysis of the E. coli pan genome has revealed 38 distinct chaperone-usher fimbrial types based on genomic locus position and usher phylogeny [8]. Type 1 and P fimbriae are primary contributors to the colonisation of the urinary tract by UPEC and have been the focus of extensive study (for a review, refer to [9]). Type 1 fimbriae confer binding to α-D-mannosylated proteins such as uroplakins, which are abundant in the uroepithelial lining of the bladder [10]. P fimbriae contribute to UTI by binding to the α-Gal(1–4)β-Gal receptor epitope in the globoseries of glycolipids found in the kidney [11,12]. F1C/S fimbriae also contribute to UTI through their ability to bind to GalNAcβ1-4Galβ glycolipids and sialyl galactoside glycoproteins present on epithelial cells in the bladder and kidneys [13–15].

We previously characterised F9 fimbriae as a new CU fimbriae type in UPEC [16]. F9 fimbriae are part of the y1 fimbrial subclade and are closely related to type 1 and F1C/S fimbriae in genetic organization and structural composition [8,17]. Low levels of expression of the F9 major subunit have been detected in enterohemorrhagic E. coli (EHEC) strain O157:H7 EDL933 and in a UPEC CFT073 fimb for null-mutant, however, to date there is no evidence of functional F9 fimbriae expression in any wild-type E. coli strain [16,18]. Cloning and expression of the β-fim genes in a recombinant E. coli strain revealed F9 fimbriae mediate strong biofilm formation, however F9 expression did not confer hemagglutination or cellular adherence properties. In this study, we have examined the distribution and conservation of the β-fim operon in E. coli, demonstrated the high frequency of β-fim genes in extant E. coli strains, and evaluated the conservation of the F9 adhesin lectin domain. Additionally, we have demonstrated that the β-fim fimbrial gene cluster is subjected to temperature-dependent repression by the global regulator H-NS. Repression was alleviated at lower temperatures, at which F9 fimbriae mediated significant biofilm formation on abiotic surfaces by wild-type E. coli. In order to study the ligand recognition properties of F9 fimbriae, we utilized a glycan array and identified Galβ1-3GlcNAc and lacto-N-tetraose glycans as novel receptor targets for these fimbriae.

**Materials and Methods**

**Phylogenetic inference, sequence alignment and diversity estimations**

The complete genomes of 42 E. coli strains representing the diversity of the species were investigated for presence of the β-fim operon using the NCBI BLAST2.2.25 program [19]. The β-fim operon and its genomic context were aligned and visualised using Easyfig [20]. The evolutionary relationship of the 42 E. coli strains was predicted by Multi-Locus Sequence Typing (MLST) of the ~9 kb concatenated nucleotide sequences of 7 housekeeping genes (adk, fumC, gpyB, tpi, mdh, purA, recA) as previously described [21]. Sequences were aligned in MEGA5 using the ClustalW DNA weight matrix under default settings. The Neighbour-Joining method of MEGA5 was used to infer the evolutionary history, with distances computed by the Jukes-Cantor method. The resulting phylogenetic tree was tested by a bootstrap test of 1000 replicates and visualised in iTOL [22] as a rooted phylogram. Diversity of the F9 adhesin protein was estimated with MEGA5. The mature adhesin (280 aa) and adhesin lectin domain (160 aa) sequences were aligned with ClustalW using the BLOSUM protein weight matrix under default settings; diversity was calculated using the Poisson model with a bootstrap confidence test for standard error measurements (1000 replicates).

**Bacterial strains, plasmids and culture conditions**

Strains and plasmids used in this study are listed in Table 1. E. coli CFT073 was isolated from the blood and urine from a woman with acute pylonephritis [23]. E. coli strains were routinely cultured at 37°C on solid or in liquid lysogeny broth (LB) medium [24] or liquid M9 minimal medium (42 mM Na2HPO4, 22 mM KH2PO4, 9 mM NaCl, 18 mM NH4Cl, 1 mM MgSO4, 0.1 mM CaCl2 and 0.2% (w/v) glucose). Where appropriate, media were supplemented with ampicillin (100 µg ml−1), kanamycin (100 µg ml−1) or chloramphenicol (25 µg ml−1). To induce expression of F9 fimbriae from plasmid pF9, culture media were supplemented with 0.2% (w/v) arabinose. Plasmid transformations into E. coli CFT073 were mediated by electroporation.

**DNA manipulations and genetic techniques**

Plasmid DNA was isolated using the QIAprep Spin Miniprep kit (Qiagen). Chromosomal DNA was purified using the GenomicPrep cell and tissue DNA isolation kit (GE Healthcare Life Sciences). PCR was performed using Taq DNA polymerase according to manufacturer’s instructions (Roche). Restriction endonucleases were used according to the manufacturer’s specications (New England Biolabs). Oligonucleotide primers used in this study were purchased from Sigma-Aldrich and are listed in Table S1. For sequencing, PCR products were amplified using the BigDye Terminator v3.1 Cycle DNA Sequencing Kit according to the manufacturer’s specifications (AB SCIEX), and analysed subsequently by the Australian Equine Genome Research Centre.

**Construction of plasmid pDW11**

To generate a Green Fluorescent Protein (GFP) expressing plasmid compatible with pF9, the gfp gene from plasmid pKEN2 [25] was amplified with primers 2319 and 2320 containing 5’ and 3’-Red mediated homologous recombination system as previously described [26]. Briefly, the FRT-flanked kanamycin resistance gene from pKD4 was amplified using primers containing 5’ BamHI and SalI sites, respectively (Table S1). The PCR product was digested by BamHI and SalI and directionally cloned into the corresponding sites of cloning vector pACYC184. Plasmid transformed E. coli strains were screened for GFP expression by fluorescence microscopy.

**Construction of CFT073 gene deletion mutants**

E. coli CFT073 gene deletion mutants were constructed using the λ-Red mediated homologous recombination system as previously described [26]. Briefly, the FRT-flanked kanamycin resistance gene from pKD4 was amplified using primers containing 5’ 50 bp regions homologous to the start and end sequence of the gene(s) to be deleted. The resulting approximately 1.6 kb PCR products were introduced by electroporation into appropriate strains expressing λ-Red recombinase from pKD4. Kanamycin resistant colonies were analysed by PCR and DNA sequencing to confirm deletion of the relevant gene. For the construction of E. coli CFT073 isogenic null-mutants, the kanamycin gene was removed using the pCP20 FLP-FRT site-specific recombination system, allowing for successive rounds of mutagenesis [26].

**Protein immunoblotting**

Rabbit immune serum against an F9 fimbriae over-expressing E. coli strain was previously generated [16]. Sera were absorbed against cell lysates of β-fim negative strains and β-fim null-mutants. For western blot analysis, bacterial EDTA heat-induced outer membrane vesicles (OMVs) were generated to enrich for the outer membrane-associated protein fraction, using a previously described method [27] with several modifications. Briefly, 50 ml LB or M9 medium was inoculated with 100 µl pre-culture (grown in the same medium) and incubated for 18 h at 37°C, 28°C or
20°C 250 rpm. Cells were harvested at 10,000 x g for 10 min at 4°C and washed in 25 ml 4°C PBS. The bacterial pellet was resuspended in 1 ml EDTA buffer (0.05 M Na₂HPO₄, 0.15 M NaCl, 0.01 M ethylenediaminetetraacetic acid (EDTA), pH 7.4) and incubated 30 min at 36°C, statically. Cells were centrifuged at 10,000 x g for 10 min at 4°C and the supernatant was filtered using a 0.22 μm PVDF low protein binding filter (Millipore). Trichloroacetic acid (TCA) was added to a final concentration of 20% (w/v) to precipitate proteins overnight at 4°C. Protein suspensions were separated according to electrophoretic mobility using SDS-PAGE and transferred to a polyvinylidene difluoride (PVDF) membrane, which was subsequently incubated in 1:500 rabbit serum albumin for 15 min at room temperature. DNA fragments were visualized by ultraviolet illumination.

Table 1. Strains and plasmids used in this study.

| E.coli Strain or Plasmid | Relevant Characteristics | Reference |
|-------------------------|--------------------------|-----------|
| **Strain**               |                          |           |
| CFT073                  | Wild-type UPEC reference strain |           |
| CFT073f9                | CFT073 c1931::c1936:kant, Kan’ | Welch et al. 2002 [52] |
| CFT073hns               | CFT073 c1701::c1970::kan, Kan’ | Ulett et al. 2007 [16] |
| CFT073f9 hns            | CFT073 c1931::c1936, c1701::kan, Kan’ | Allsopp et al. 2012 [33] |
| CFT073f9 virF           | CFT073 c0421::kan, Kan’ | Allsopp et al. 2012 [33] |
| CFT073hns-like          | CFT073 c1699::kan, Kan’ | Allsopp et al. 2012 [33] |
| CFT073hns-like          | CFT073 c2411::kan, Kan’ | Allsopp et al. 2012 [33] |
| CFT073gfp               | CFT073 c0421::kan, Kan’ | Allsopp et al. 2012 [33] |
| CFT073luxS              | CFT073 c3244::kan, Kan’ | Allsopp et al. 2012 [33] |
| CFT073msdF             | CFT073 c3216::kan, Kan’ | Allsopp et al. 2012 [33] |
| CFT073papS             | CFT073 c4864::kan, Kan’ | Allsopp et al. 2012 [33] |
| CFT073virF             | CFT073 c5054::kan, Kan’ | Allsopp et al. 2012 [33] |
| **Plasmids**            |                          |           |
| pKD4                    | Template for kan gene amplification, Kan’ | Datsenko & Wanner 2000 [26] |
| pKD46                   | λ-Red recombinase expression vector, Amp’ | Datsenko & Wanner 2000 [26] |
| pCP20                   | Temperature sensitive FLP expression vector, Amp’ | Datsenko & Wanner 2000 [26] |
| pBAD30                  | Cloning vector with ara promoter, Amp’ | Guzman et al. 1995 [54] |
| pFLG                    | c1931::c1936 (F9G 5′T70) in pBAD30, Amp’ | Ulett et al. 2007 [16] |
| pBR322                  | Cloning vector, Amp’, Tet’ | Bolivar et al. 1977 [55] |
| pH-NS                   | c1701 (H-N5G 5′T70) in pBR322, Amp’ | Allsopp et al. 2012 [33] |
| pACYC184                | Cloning vector, Cam’, Tet’ | Chamber & Cohen 1978 [56] |
| pDW11                   | gfp (GFPpKEN2) in pACYC184, Cam’ | This study |

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5′ Rapid amplification of cDNA ends (5′ RACE)

The transcription start site of the f9 operon was determined using the 5′ RACE System v2.0 (Invitrogen) [28]. Experiments were performed according to manufacturer’s specifications except for the modifications listed below. Three gene specific primers were used for this assay: 4235, 4236 and 4237 (Table S1). To verify that the first nucleotide of the mRNA was a guanine, the cDNA was dA-tailed with a dATP substitution. PCR amplification of dA-tailed cDNA was performed using the (dT)17-adaptor primer 4296 (Table S1). Amplified cDNA was sequenced by the Australian Equine Genome Research Centre.

Electrophoretic mobility shift assay

Gel shift assays were performed as previously described [29]. Briefly, a DNA mixture containing an equimolar ratio of the 251 bp PCR amplified f9 promoter region and TaqI-SphI digested pBR322 was incubated with native purified H-NS protein in 30 μl H-NS binding buffer (40 mM HEPES pH 7, 60 mM potassium glutamate, 8 mM magnesium aspartate, 5 mM diethiothreitol, 10% glycerol, 0.1% octylphenoxy polyethoxyethanol, 0.1 mg/ml bovine serum albumin) for 15 min at room temperature. DNA fragments and DNA-protein complexes were resolved by gel electrophoresis (0.5 × Tris/Borate/EDTA buffer, 3% agarose MS gel, ran at 50 V, 4°C), stained with ethidium bromide and visualized by ultraviolet illumination.
Microtitre plate biofilm formation assay

Bacterial biofilm formation was assessed on sterile non-coated 96-well polyvinyl chloride (PVC) microtitre plates (BD Falcon) as previously described [30]. Briefly, cells were cultured with aeration at various temperatures for 24 hours in 150 μL M9 medium containing 0.2% (v/v) glucose. After incubation, cells were washed, stained with 0.1% crystal violet for 30 min at 4°C, and washed three additional times. Bound bacterial cells were quantified by adding ethanol-acetone (80:20 v/v) and measurement of the dissolved crystal violet at an optical density of 595 nm.

F9 Immunogold labelling and electron microscopy

Cells for immunogold labelling and transmission electron microscopy (TEM) were prepared from liquid cultures grown overnight at 20°C in M9 minimal medium. A glow-discharged carbon-coated Formvar copper grid was placed on a drop of the bacterial suspension for 1 min to allow the cells to adsorb. Grids were washed twice on drops of water (1 min), and blocked for 30 min in blocking buffer (PBS containing 0.2% BSA, 0.2% fish skin gelatin, 20 mM glycine). Samples were exposed to 1:25 anti-F9 rabbit immune serum for 30 min and washed four times in blocking buffer (5 min) before incubation with Protein A-gold conjugate (10 nm diameter, diluted 1:60 in blocking buffer) for 30 min and four washes (5 min) in PBS. Cells were fixed with 4% paraformaldehyde in PBS (5 min) and grids were washed four times (2 min) in sterile ultrapure water before examination under a JEOL 1010 TEM operated at 80 kV. Images were captured using an analySIS Megaview III digital camera.

Glycan array analysis

Glycan array slides and whole-cell binding assays were essentially performed as previously described [31,32]. Glycan arrays comprised 120 unique carbohydrates (Table S2) printed on super epoxy slides (ArrayIt). All array experiments consisted of a minimum of three independent biological repeats. E. coli strains MS428 (pF9, pDW11) and MS428 (pBAD, pDW11) were cultured overnight at 37°C with gentle agitation in M9 minimal medium containing ampicillin (100 μg mL⁻¹), chloramphenicol (25 μg mL⁻¹) and 0.2% arabinoose. Post-incubation, cells were diluted to an OD of 0.6, representing approximately 1 x 10⁶ CFU mL⁻¹. A volume of 125 μL of cells was hybridised to a pre-blocked (0.1% BSA in PBS with 2 mM MgCl₂ and CaCl₂, 5 mins) glycan array for 20–30 mins at room temperature in the dark. Glycan array slides were placed in a 50 mL tube and washed in filter sterilized buffer 1 (PBS with 2 mM MgCl₂ and CaCl₂) for 5 mins, buffer 2 (buffer 1 with 0.01% tween-20) for 2 mins, and finally rinsed in fresh buffer 1. Cells were fixed for 10 mins in PBS with 10% formaldehyde and dried by centrifugation for 5 mins at 2000 xg. The array slide was scanned using a ProScan Array microarray scanner (Perkin Elmer) using a 488 nm argon laser. Images were attained and analysed using the ScanArray Express software package (Perkin Elmer). To determine minimum binding concentration, glycans were printed in serial dilutions from 5 mM to 5 nM on an array slide. For competition assays, cells were pretreated with the glycan of interest for 15 mins prior to hybridisation on the slide.

Statistical analyses

The frequency of intact f9 operons in different E. coli strain collections and between different E. coli phylogenetic groups was compared using Fisher’s exact test with a two-tailed P value. Biofilm formation was compared between f9 encoding strains and their isogenic f9 null mutant using a two-tailed t test. P values < 0.05 were considered significant. For glycan array analysis, binding was classified as RFU (relative fluorescence units) above average background (defined as background mean plus 3 standard deviations) and was tested for statistical significance using a two-tailed t test with a P value < 0.001.

Results

Genetic organisation and distribution of f9 fimbrial operons in E. coli

In order to investigate the distribution and genetic location of the f9 operon in E. coli, we examined the genome sequence of 42 diverse E. coli strains available on the NCBI database, including representatives of all E. coli lineages and various pathotypes (Table 2). The E. coli species exhibits extensive genetic substructure and can be divided into 5 major monophyletic clades (phylogroup A, B1, B2, D and E) [21]. To evaluate the conservation and evolutionary history of F9 fimbriae among E. coli phylogroups, a phylogenetic tree based on multi-locus sequence typing (MLST) of 7 concatenated housekeeping genes (~9 kB) was constructed and combined with f9 genomic context alignments (Figure 1). The f9 operon consists of six structural genes, encoding, from 5’ to 3’: the major subunit, chaperone, usher, two minor subunits and an adhesin. f9 operons containing deletions, truncations and/or insertion elements were considered disrupted. Comparative genomic analysis revealed that all strains possessed at least part of the F9 encoding DNA sequences. In 60% (25/42) of E. coli strains the f9 operon appeared intact (Figure 1 and Table 2). Among E. coli phylogenetic groups, the f9 operon was conserved in the majority of B1 and E strains, and to a lesser extent in B2 and D strains. The f9 operon was disrupted in all phylogroup A strains. In a pathotype context, the intact f9 operon was highly prevalent in intestinal pathogenic E. coli, including adherent-invasive E. coli (AIEC; 3/3), enterohaemorrhagic E. coli (EHEC; 2/2), enteroaggregative E. coli (EAEC; 2/2), and enteropathogenic E. coli (EPEC; 3/3) and enterohemorrhagic E. coli (EHHEC; 7/8), but not in enterotoxigenic E. coli (ETEC; 0/2) (Table 2). The f9 encoding genes were not detected in genome sequences from other bacterial genera available in the NCBI database (except for Shigella, a subgenus of Escherichia).

The f9 operon is flanked by the highly conserved ydeP gene involved in acid resistance (downstream) and the relatively well-conserved hipA1 cell persistence gene cluster (upstream). The immediate 5’ region is variable, and contains a range of different insertions and/or deletions, including three hypothetical transcriptional regulators (Figure 1). Currently, there is no evidence linking these putative regulators to the transcriptional control of f9 genes.

Prevalence of f9 genes in E. coli

Based on the genomic analysis described above, primers were designed in conserved regions of the f9 gene cluster to screen for the major subunit, usher and adhesin genes in two large E. coli strain collections. These included fifty-one UPEC isolates collected from patients presenting with urosepsis at the Princess Alexandra Hospital (Brisbane, Australia) as well as seventy-two strains of the well-defined and diverse ECOR reference collection. In the UPEC collection, 80% (41/51) of strains screened positive by PCR for all three f9 genes (Figure 2A). A further 16% (8/51) screened positive for at least one f9 gene, while 4% (2/51) of strains screened negative for all genes. In the ECOR collection, 61% (44/72) of strains screened positive for all three f9 genes, 29% (21/72) screened positive for at least one f9 gene and 11% (8/72) did not yield any positive PCR results. F9 operon prevalence (as judged by screening positive for the major subunit, usher and adhesin genes)
| E. coli Strain | Phylogroup | F9 Status* | Reference                  |
|---------------|------------|------------|----------------------------|
| UPEC          |            |            |                            |
| CFT073        | B2         | +          | Welch et al. 2002 [52]     |
| NA114         | B2         | +          | Avasthi et al. 2011 [57]   |
| UTI89         | B2         | D          | Chen et al. 2006 [58]      |
| 536           | B2         | D          | Hochhut et al. 2006 [59]   |
| IA139         | D          | D          | Touchon et al. 2009 [60]   |
| UMN026        | D          | +          | Touchon et al. 2009 [60]   |
| ABU           |            |            |                            |
| 83972         | B2         | +          | Zdziarski et al. 2010 [61] |
| NMEC          |            |            |                            |
| IHE3034       | B2         | +          | Moriel et al. 2009 [62]    |
| S88           | B2         | D          | Nash et al. 2010 [66]      |
| CE10          | D          | D          | Lu et al. 2011 [63]        |
| APEC          |            |            |                            |
| APEC01        | B2         | +          | Johnson et al. 2007 [64]   |
| AIEC          |            |            |                            |
| LF82          | B2         | +          | Miquel et al. 2010 [65]    |
| NRG 857C      | B2         | +          | Nash et al. 2010 [66]      |
| UM146         | B2         | +          | Krause et al. 2011 [67]    |
| EAEC          |            |            |                            |
| S5989         | B1         | +          | Touchon et al. 2009 [60]   |
| 042           | D          | +          | Chaudhuri et al. 2010 [68] |
| EPEC          |            |            |                            |
| O127:H6 E2348/69 | B2     | +          | Iguchi et al. 2009 [69]    |
| O55:H7 CB9615 | E          | +          | Zhou et al. 2010 [70]      |
| O55:H7 RM12579| E          | +          | Kyle et al. 2012 [71]      |
| ETEC          |            |            |                            |
| O78:H11 H10407| A          | D          | Crossman et al. 2010 [72]  |
| E24377A       | B1         | D          | Rasko et al. 2008 [73]     |
| EHEC          |            |            |                            |
| O26:H11 11368 | B1         | +          | Ogura et al. 2009 [74]     |
| O103:H2 12009 | B1         | D          | Ogura et al. 2009 [74]     |
| O111:H- 11128 | B1         | +          | Ogura et al. 2009 [74]     |
| O157:H7 EDL933| E          | +          | Perna et al. 2001 [75]     |
| O157:H7 Sakai | E          | +          | Hayashi et al. 2001 [76]   |
| O157:H7 EC4115| E          | +          | Eppinger et al. 2011 [77]  |
| O157:H7 TW14359| E         | +          | Kulasekara et al. 2009 [78]|
| O157:H7 Xuzhou21| E         | +          | Xiong et al. 2012 [79]     |
| Environmental |            |            |                            |
| SMS-3-5       | D          | D          | Fricke et al. 2008 [80]    |
| Commensal     |            |            |                            |
| ATCC 8739     | A          | D          | Joint Genome Institute [81]|
| HS            | A          | D          | Rasko et al. 2008 [73]     |
| IA11          | B1         | +          | Touchon et al. 2009 [60]   |
| SE11          | B1         | +          | Oshima et al. 2008 [82]    |
| W             | B1         | +          | Archer et al. 2011 [83]    |
| ED1a          | B2         | D          | Touchon et al. 2009 [60]   |
| SE15          | B2         | +          | Toh et al. 2010 [84]       |
| Laboratory    |            |            |                            |
| BL21(DE3)     | A          | D          | Jeong et al. 2009 [85]     |
was significantly higher ($P<0.05$) in UPEC isolates compared to the ECOR collection (Figure 2A). PCR data from the two E. coli collections were merged to evaluate $f\beta$ prevalence relative to strain phylogenetic group (Figure 2B). Consistent with the genome-sequenced strains, the frequency of intact $f\beta$ operons in phylogroup A strains was significantly lower in comparison to strains belonging to other phylogroups ($P<0.05$). The $F9$ operon was detected in 100% of phylogroup B1 strains and the majority (>70%) of strains from phylogroup B2, D or E.

Transcription of the $f\beta$ operon is repressed by H-NS

We previously detected very weak expression of $F9$ fimbriae in UPEC strain CFT073$\text{fim}^+$ cultured at 37°C [16]. However, to date there have been no reports of functional $F9$ fimbriae expression in wild-type UPEC. To investigate the genetic basis of $f\beta$ gene regulation in UPEC, we employed a previously described panel of CFT073 isogenic single gene deletion mutants lacking a selection of defined/putative regulatory genes (c0421 $\text{virF}_\text{like}$, c1699 $\text{tphS}$, c1701 [hrs], c2091 [varF$_\text{like}$], c2411 [hrs$_\text{like}$], c3218 [rpoA], c3244 [luxS-A2], c3744 [varF$_\text{like}$], c4864 [spaR] and c5054 [soxR]) [33]. Expression of the $F9$ fimbrial major subunit protein was assessed by western blot analysis of CFT073 deleted for gene clusters encoding type 1, F1C, P1 and P2 fimbriae could be the result of alleviation of repression of an $F9$ related gene. Since H-NS negatively controls the operon is repressed by H-NS [36,37]. To evaluate whether temperature had an effect on the expression of $F9$ fimbriae, CFT073 and the isogenic $\text{hns}$ null mutant strain, but not CFT073$\text{hns}$ double mutant signal was absent in a CFT073$\text{hns}$ double mutant. Interestingly, a faint band similar in size to the $F9$ major subunit signal was observed in CFT073$\text{hns}$, suggesting some non-specific cross reactivity of the $F9$ antiserum with a similar sized protein. Since H-NS negatively controls the expression of various distinct fimbrial operons, this observation could be the result of alleviation of repression of an $F9$ related fimbrial type [34]. We addressed this by constructing a mutant deleted for $\text{virF}_\text{like}$ encoding type 1, F1C, P1 and P2 fimbriae (referred to as CFT073A$\text{fim}$, and a CFT073A$\text{fim}$ strain deleted for the $f\beta$ genes (CFT073A$\text{fim}^+$). Indeed, mutation of the $\text{hrs}$ gene in CFT073A$\text{fim}$ and CFT073A$\text{fim}^+$ resulted in the complete loss of this cross-reacting band (Figure 3B). Combined, these results demonstrate that H-NS represses the expression of $F9$ fimbriae in CFT073.

H-NS binds to the promoter region of the $f\beta$ operon

In order to determine whether H-NS influences $f\beta$ gene transcription by directly binding to the promoter region, the $f\beta$ promoter was characterised using 5′-RACE and investigated for H-NS interaction by electrophoretic mobility shift assays. The $f\beta$ transcription start site was identified as a guanine residue, 251 nucleotides upstream of the $f\beta$ major subunit gene start codon (Figure 4A). The transcription start site was preceded by a strong −10 promoter consensus sequence (CATATA) and a moderate −35 promoter consensus sequence (TAGTCG) with an 18 bp spacer region. In silico analysis of the promoter region discerned a ribosomal binding site (RBS) directly upstream of the translation initiation site, and identified six putative H-NS binding motifs at positions −111, −105, +8, +14, +57 and +89 (Figure 4A) [35]. To investigate $f\beta$ promoter/H-NS interactions, the 251 bp promoter region was amplified by PCR and mixed with TaqI-SspI-digested pBR322 DNA (containing the H-NS recognised bla promoter). The DNA mixture was incubated with increasing concentrations of purified H-NS protein and analysed by mobility shift electrophoresis. The $f\beta$ promoter region and the positive control $\text{bla}$-promoter fragment were equally impeded in gel migration in the presence of increasing concentrations of H-NS (Figure 4B). In contrast, the mobility of pBR322 fragments lacking the $\text{bla}$-promoter sequence was not altered in the presence of H-NS. These results demonstrate that H-NS binds to the $f\beta$ promoter region.

Expression of $F9$ fimbriae in UPEC CFT073 is temperature-dependent

The global regulator H-NS modulates the expression of a large subset of genes in response to external stimuli such as temperature [36,37]. To evaluate whether temperature had an effect on the expression of $F9$ fimbriae, CFT073 and the isogenic $\text{hns}$ null mutant were cultured at various temperatures and examined by western blot analysis employing an $F9$ specific antiserum. No protein bands were detected when CFT073 was cultured at 37°C or 28°C, but at 20°C an 18.3 kDa band corresponding to the mature $F9$ major subunit was observed (Figure 5). This band was not detected in the CFT073$\text{hns}$-null mutant at all temperatures examined, confirming the identity of the band as the $F9$ major subunit protein (Figure 5). To strengthen these findings we also examined $F9$ fimbriae expression on the cell surface by immunogold electron microscopy. We detected $F9$ fimbriae on the surface of CFT073A$\text{fim}$ but not CFT073A$\text{fim}^+$ following culture at 20°C (Fig. 5B and 5C). These data represent the first evidence of $F9$ fimbrial expression by UPEC, and based on the temperature expression profile suggest a role for $F9$ fimbriae outside the mammalian host.

F9 fimbriae mediate biofilm formation in UPEC strain CFT073

We previously demonstrated that $F9$ fimbriae mediate a strong biofilm on abiotic surfaces using a plasmid-based system in a recombinant E. coli strain [16]. To determine whether $F9$ fimbriae expressed by wild-type UPEC are involved in biofilm formation.
we investigated CFT073 and CFT073 Δf9 for biofilm formation at 20°C using a microtitre plate assay. Consistent with our F9 fimbrial expression findings using western blot analysis and immunogold-TEM, the expression of F9 fimbriae at 20°C by CFT073 correlated with significant biofilm formation compared to CFT073 Δf9 under these growth conditions (P<0.001; Figure 6). Complementation of CFT073 Δf9 with the F9 fimbrial expression plasmid pF9 restored the strong biofilm phenotype. These data confirm that F9 fimbriae promote significant biofilm growth on abiotic surfaces by wild-type CFT073 at 20°C.

The F9 fimbrial adhesin is highly conserved and displays receptor specificity to Galβ1-3GlcNAc terminating glycans

The predicted F9 adhesin is encoded by the last gene in the f9 operon and contains a characteristic two-domain structure comprising a C-terminal fimbrial integration domain and an N-terminal receptor-binding region. Comparison of the amino acid sequence of the full-length F9 adhesin among the 25 E. coli strains that contained an intact f9 operon revealed a high degree of conservation, with a mean diversity of 0.013±0.004 amino acid

Figure 1. Conservation and genetic organisation of the E. coli f9 fimbrial operon in an evolutionary context. Left: The phylogeny of 42 E. coli strains is displayed as inferred by the Neighbour-Joining method on the concatenated nucleotide sequence of 7 housekeeping genes (9,015 nt over an equal number of positions). E. coli strains are colour-coded according to phylogroup (A, B1, B2, D and E). The scale on the phylogenetic tree represents the number of nucleotide substitutions per site. Closely related strains with identical f9 genetic context are collapsed and included E. coli K-12 (n=3; strains MG1655, DH10B, BW25113), E. coli B (n=2; strains BL21(DE3), B REL606), E. coli O55 (n=2; strains CB9615, RM12579), E. coli O157 (n=5; strains EDL933, Sakai, EC4115, TW14339, Xuzhou21). Right: Alignment of the f9 genes (blue) and their flanking genes. The f9 operon is flanked 3' by the highly conserved ydeP gene (grey) and 5' by the hipBA operon (red). The direct 5' region of the f9 operon is variable, and involves three distinct hypothetical transcriptional regulators (green, purple, and lilac). The percentage DNA sequence identity is indicated in grey. The scale on the aligned genetic context represents DNA length in kilobase pair.

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substitutions per site over 280 positions. More specific interrogation of the receptor-binding domain of the F9 adhesin revealed even greater amino acid sequence conservation, with a mean diversity of 0.003 ± 0.002 substitutions per site over 160 positions.

The above analysis demonstrates that the F9 adhesin sequence is highly conserved, and indicates that the F9 adhesin from CFT073 can be used to define the overall receptor-binding characteristics of F9 fimbiae. We therefore employed a glycan array in combination with a F9 over-expressing E. coli strain labelled with GFP (MS428[pF9, pDW11]) to evaluate the binding specificity of F9 fimbiae to different carbohydrates. In this assay, F9 fimbiae mediated specific binding to Galβ1-3GlcNAc terminating structures, including lacto-N-tetraose (Galβ1-3GlcNAcβ1-3Galβ1-4Glc), globotriose (Galβ1-4galβ1-4Glc) and the globotriose terminal disaccharide (GalNAcβ1-3Gal) (P < 0.05). The presence of fucose in Galβ1-3GlcNAc glycans eliminated or reduced affinity by at least 100-fold (data not shown). Of the glycans that were bound by F9 fimbiae, lacto-N-tetraose displayed the highest affinity. A glycan competition analysis using 50 μM free lacto-N-tetraose resulted in no observable F9 fimbiae-mediated binding to any of the glycans on the array. Taken together, these data provide the first evidence for Galβ1-3GlcNAc glycans as specific receptors for F9 fimbiae, and identify lacto-N-tetraose as a high affinity glycan.

**Discussion**

Bacterial adhesins mediate attachment to host tissues and abiotic surfaces and provide the first step in colonisation and biofilm formation. Despite the large repertoire of CU fimbriae encoded by UPEC [8], there are only a few well-studied examples of fimbiae that are directly associated with pathogenesis or mediate tissue tropism. Many UPEC fimbiae are cryptic in nature and have not been thoroughly characterised. We previously described F9 fimbiae in UPEC as a functional CU fimbral type promoting formation of E. coli biofilms [16] and have recently demonstrated that they are closely related to the type I and F1C/S fimbiae [38], which are both involved in colonisation of the human urinary tract [3]. In this study, the distribution and conservation of F9 fimbiae in diverse E. coli lineages was investigated and evaluated in an evolutionary and pathotype associated context. Evolutionary diversity analysis of the F9 adhesin sequence revealed a high conservation of the receptor recognising lectin domain. Furthermore, H-NS was identified as a temperature dependent negative regulator of F9 expression by binding directly to the f9 promoter region. F9 fimbiae were expressed by CFT073 at 20°C and mediated significant biofilm formation at this temperature. This is the first report of functional F9 expression in wild-type E. coli, and provides the first evidence that F9 fimbiae specifically recognise Galβ1-3GlcNAc and lacto-N-tetraose glycans.

E. coli population genetics have identified five major monophyletic clades (phylogenetic A, B1, B2, D and E) [21]. Despite the high frequency of f9 DNA sequences in the E. coli species, the conservation of the f9 operon between E. coli phylogenetic groups varied significantly. A genomic comparison of the f9 operon from 42 E. coli genomes showed that intact f9 operons were particularly prevalent in phylogenetic group B1 and E, and to a lesser degree in phylogroups B2 and D. In strains from phylogenetic group A, all f9 operons were disrupted. Variation was also observed among E. coli pathotypes, with the f9 fimbral genes particularly conserved in intestinal pathogenic isolates representing AIEC, EAEC, EPEC and EHEC, but not ETEC, suggesting a potential role in the pathogenic lifestyle of these bacteria. Indeed, signature-tagged mutagenesis screens using EHEC strains of serotype O157:H7 and O26:H1 have previously identified insertion mutants in the f9 gene cluster that were significantly impaired for intestinal colonisation in young calves [39,40]. The f9 operon was moderately conserved in UPEC genomes. A PCR screen of the 51 isolates in our UPEC collection suggested that the f9 operon is intact in 80% of the strains, significantly higher than the 61% prevalence of intact f9 operons in the 72 strains of the diverse and well defined ECOR reference collection. In a phylogenetic context, the results from the f9 gene prevalence screen of the two collections were consistent with the genomic data. F9 encoding sequences were not found in any other species (except for Shigella, a subgenus of Escherichia), indicating this fimbral operon is unique to E. coli. The ubiquity of f9 genes in extant E. coli strains suggests that the f9 operon is ancient and was present in the E. coli common ancestor.
H-NS is a histone-like DNA-binding protein that shows affinity for A-T rich and bent nucleation sites on DNA [41]. In this study, several lines of evidence demonstrated a role for H-NS in the regulation of F9 fimbrial expression. In a CFT073\textit{hns} mutant background, F9 expression was de-repressed, and this effect could be reversed through the introduction of a plasmid containing the\textit{hns} gene. In addition, H-NS bound to a 251 bp DNA fragment containing the mapped\textit{f9} promoter region and a positive control\textit{bla}-promoter fragment with equal affinity. H-NS has been shown to repress multiple other virulence-associated genes in UPEC, including genes encoding alpha-hemolysin, iron uptake systems, fimbriae and autotransporter proteins [33,41–43]. In \textit{E. coli} K-12, several cryptic chaperone-usher fimbrial genes are also repressed by H-NS [34]. The data presented here is the first direct demonstration that H-NS represses F9 fimbriae, and is consistent with a role for H-NS in the regulation of multiple UPEC virulence factors.

F9 fimbriae expression by UPEC CFT073 also displayed a temperature-dependent profile. At 20°C, we detected expression of the F9 major fimbrial subunit protein by western blot and F9

**Figure 3. H-NS is a negative regulator of F9 fimbriae expression.** (A) Western blot analysis of CFT073 and 10 isogenic defined/putative regulatory gene deletion mutants using an F9 specific antiserum. A strong-reacting band consistent with the size of the mature F9 major subunit (~18.3 kDa, indicated by an arrow) was observed in CFT073\textit{hns} (CFT073\textit{c1701}) but not in the other regulator deletion mutants. (B) Western blot analysis of F9 fimbriae expression in CFT073\textit{f9} and \textit{hns} null mutants cultured at 37°C. The F9 specific antiserum reacted strongly with the mature F9 major subunit (F9 MS indicated by an arrow, ~18.3 kDa) in over-expressing strain CFT073\textit{f9} (pF9). Repression of the \textit{f9} operon is alleviated in the CFT073\textit{hns} mutant. This signal is lost again in isogenic null mutant CFT073\textit{f9} or in the H-NS over-expressing complemented strain CFT073\textit{hns} (pH-NS), demonstrating F9 fimbriae expression is negatively regulated by H-NS. The faint band in CFT073\textit{f9 hns} suggests cross reactivity with a related fimbrial subunit, and is indeed lost in the isogenic \textit{fim, foc, pap1, pap2} null mutant CFT073.D4f9\textit{hns}.

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**Figure 4. The H-NS protein binds to the \textit{f9} promoter region.** (A) Nucleotide sequence and features of the F9 promoter region of uropathogenic \textit{E. coli} CFT073. 5' RACE analysis identified the transcription start site as a guanine residue (labelled as +1), 251 nucleotides upstream of the start codon of the \textit{f9} major subunit (+252). The predicted ribosomal binding site (RBS), –10 and –35 promoter elements are highlighted in boldface. Six putative H-NS binding sites (positions –111, –103, +8, +14, +57 and +89) were identified with the Virtual Footprint bacterial promoter analysis tool [35]. (B) Electrophoretic band shift of the amplified 251 bp \textit{f9} promoter and TaqI-SspI digested pBR322 DNA in the presence of various concentrations H-NS (0 \textmu M, 1 \textmu M, 2 \textmu M, 3 \textmu M, 4 \textmu M and 10 \textmu M). Similar to the \textit{bla} promoter positive control, the signal of the \textit{f9} promoter diminishes as its gel migration is impeded by increasing H-NS concentrations, demonstrating that H-NS binds directly to the \textit{f9} promoter sequence. Migration of \textit{bla}-negative pBR322 fragments was not affected by H-NS.

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Figure 5. Expression of F9 fimbriae is temperature-dependent. (A) Western blot analysis of wild-type CFT073 and isogenic f9 null-mutants cultured at various temperatures. The F9 specific antiserum reacts with the F9 mature major subunit protein (~18.3 kDa) in over-expressing strain CFT073(pF9). No expression is observed in wild-type CFT073 when cultured at 37°C or 28°C. F9 expression is observed in CFT073 at 20°C, and lost again in isogenic null-mutant CFT073/f9, illustrating the temperature-dependent regulation of F9 fimbriae in UPEC. The mature F9 major subunit (MS) is indicated by an arrow. The 22 kDa higher molecular weight cross-reacting band detected from CFT073 following growth at 20°C is consistent with the size of the unprocessed F9 major subunit protein. TEM micrograph of CFT073/f9 (B) and CFT073/f9/f9 (C) labelled with immunogold anti-F9 serum after growth at 20°C. Scale bars (500 nm).

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F9 Fimbriae of UPEC

been shown that the expression of the F9 major subunit is increased at 20°C compared to 37°C, however expression levels were too low to detect F9 fimbriae by immunofluorescence [18]. While F9 fimbrial expression in a recombinant E. coli K-12 strain promoted enhanced binding to bovine rectal epithelial cells, there were no significant differences in colonization of the terminal rectum of cattle by a wild-type and F9 mutant strain, suggesting that F9 fimbriae are not responsible for EHEC O157 rectal tropism in cattle [18]. Combined, these data suggest that F9 fimbriae contribute to the E. coli lifestyle outside the mammalian host, potentially involving colonization of epithelial surfaces and persistence in the environment through biofilm formation.

Two other types of E. coli adhesins that are expressed strongly at 20°C have also been described, namely Mat (or ECP) fimbriae and curli fibres [44,45]. Mat fimbriae mediate biofilm formation by neonatal meningitis E. coli and UPEC at low temperature [46,47]. The expression of Mat fimbriae has also been observed more generally in E. coli following cultivation in DMEM, suggesting that temperature-mediated regulation is linked to specific growth conditions [44]. Curli are also strongly expressed at 20°C and associated with biofilm formation [48], however to the best of our knowledge CFT073 has not been shown to produce curli. In our experiments, although the reduction in biofilm formation at 20°C between wild-type CFT073 and the CFT073/f9 mutant was significant, CFT073/f9 still formed a reasonable biofilm (Figure 6). This suggests that CFT073 produces other biofilm formation mechanisms under these conditions, which may include Mat fimbriae. It remains to be determined whether Mat fimbriae are produced by CFT073 at 20°C under the conditions used in our experiments, whether F9 and Mat fimbriae can be co-expressed at 20°C, and if there are additional layers of regulatory control in E. coli strains that have the capacity to express both of these fimbriae.

The sequence of the F9 adhesin lectin domain was shown to be highly conserved in E. coli strains from different phylogenetic lineages. In order to examine the receptor binding specificity of F9 fimbriae, a glycan array containing 120 structures was utilized. The glycans on the array represented host cell surface glycocalyx including terminal galactose, mannose, fucosylated and sialylated structures and glycans, with lacto-N-tetraose identified as a high affinity glycan. The glycans were too low to detect F9 fimbriae by immunofluorescence [18]. While F9 fimbrial expression in a recombinant E. coli strain over-expressing F9 fimbriae to human exfoliated urothelial cells, human T24 bladder epithelial cells, human A498 kidney epithelial cells, human Caco-2 intestinal epithelial cells, or human type A red blood cells (data not shown). Thus, the Galβ1-3GlcNAc glycan-containing target cells bound to by F9 fimbriae in the mammalian host remain to be identified.

In conclusion, we have shown that the F9 adhesins are common to many different E. coli lineages and pathotypes and are regulated by H-NS and temperature. F9 fimbriae bind with high affinity to Galβ1-3GlcNAc glycans, including lacto-N-tetraose. Finally, UPEC CFT073 expresses F9 fimbriae at 20°C which
correlates with strong biofilm formation on abiotic surfaces. Further characterisation of F9 fimbriae is now required to identify its potential role in the colonisation of specific biotic surfaces.

Supporting Information

Table S1 Primers used in this study.
(DOCX)

Table S2 Glycans screened in this study.
(DOCX)

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Author Contributions

Conceived and designed the experiments: DJW MT MPJ MAS. Performed the experiments: DJW MT LPA LEH CJD KMP SS JY JT. Analyzed the data: DJW MT CJD JT MPJ MAS. Contributed reagents/materials/analysis tools: GCU RAS MPJ MAS. Wrote the paper: DJW MT GCU MPJ MAS.

Figure 6. F9 fimbriae mediate biofilm formation in UPEC CFT073 at 20°C. PVC microtitre plate biofilm formation assay of CFT073 and isogenic f9 null mutants cultured in M9 medium at 20°C. The optical density (OD) at 595 nm (mean ± SD) is an indication of biofilm formation. CFT073 formed a significantly stronger biofilm in comparison to the isogenic f9 null mutant. The strong biofilm phenotype was restored by complementation of the f9 deletion mutant with the F9 expression vector pF9 but not with the empty vector pBAD. * Statistically significant (P<0.001).
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