The role of F9 fimbriae of uropathogenic *Escherichia coli* in biofilm formation

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Uropathogenic *Escherichia coli* (UPEC) is the primary cause of urinary tract infection (UTI) in the developed world. The major factors associated with virulence of UPEC are fimbrial adhesins, which mediate attachment to specific receptors, enhance persistence and trigger innate host responses. UPEC produce a range of fimbrial adhesins, with type 1 and P fimbriae of the chaperone-usher subclass being the best characterized. The prototype UPEC strain CFT073 contains ten gene clusters that contain genes characteristic of this class of fimbriae. However, only five of these gene clusters have been characterized in detail. In this study the F9 fimbrial gene cluster (*c1931–c1936*) from CFT073 has been characterized. The F9 fimbriae-encoding genes were PCR amplified, cloned and expressed in a K-12 background devoid of type 1 fimbriae. While F9 fimbrial expression was not associated with any haemagglutination or cellular adherence properties, a role in biofilm formation was observed. *E. coli* K-12 cells expressing F9 fimbriae produced a dense and uniform biofilm in both microtitre plate and continuous-flow biofilm model systems. In wild-type UPEC CFT073, expression of the F9 major subunit-encoding gene was detected during exponential growth in M9 minimal medium. F9 expression could also be detected following selection and enrichment for pellicle growth in a CFT073*fim foc* double mutant. The F9 genes appear to be common in UPEC and other types of pathogenic *E. coli*. However, their precise contribution to disease remains to be determined.

INTRODUCTION

Urinary tract infections (UTIs) are among the most common infectious diseases of humans and a major cause of morbidity. In the USA, UTI accounts for approximately $1.6 billion in medical expenditure each year. It is estimated that 40–50 % of healthy adult women have experienced at least one UTI episode in their lifetime (Foxman, 2002). The recurrence rate of UTI is high and often the infections tend to become chronic with many episodes. UTI usually starts as a bladder infection but often evolves to encompass the kidneys and ultimately can result in renal failure and may disseminate to the blood. Catheter-associated UTI accounts for 40 % of all nosocomial infections and is the most common source of Gram-negative bacteraemia in hospitalized patients. Most patients with an indwelling urinary catheter for 30 days or longer develop bacteriuria (Stamm, 1991).

Uropathogenic *Escherichia coli* (UPEC) is the cause of the majority (>80 %) of UTIs in humans. UPEC isolates exhibit a high degree of genetic diversity due to the possession of specialized virulence genes located on mobile genetic elements called pathogenicity islands (Oelschlaeger *et al.*, 2002b). Although no single virulence factor is uniquely definitive of UPEC, their ability to cause symptomatic UTI is enhanced by adhesins (e.g. type 1 and P fimbriae) and toxins (e.g. haemolysin) (Klemm & Schembri, 2000; Oelschlaeger *et al.*, 2002a). Adherence to the urinary tract epithelium is the first stage of UTI, enabling the bacteria to resist the hydrodynamic forces of urine flow and establish infection. Among the more common adhesins produced by UPEC are type 1, P, F1C, S and Auf fimbriae and the Afa/Dr adhesins (Oelschlaeger *et al.*, 2002a).

Fimbrial adhesins of UPEC are characterized by a conserved chaperone-usher pathway that underpins their assembly (Sauer *et al.*, 2004). Fimbriae assembled by the chaperone-usher pathway are typically encoded by

individual gene clusters that in addition to the chaperone- and usher-encoding genes also include genes encoding the major and minor pilin subunits. P and type 1 fimbriae are the best-characterized chaperone-usher class of fimbriae produced by UPEC. P fimbriae are most strongly associated with UTI in clinical studies. P fimbriae contribute to the establishment of bacteriuria by binding to the α-D-galacto-pyranosyl-(1-4)-β-D-galactopyranoside receptor epitope in the globoseries of glycolipids (Kallenius *et al.*, 1981; Leffler & Svanborg-Eden, 1981) and activate innate immune responses in animal models and in human infection (Svanborg *et al.*, 2006). Similarly, type 1 fimbriae enhance colonization and stimulate immune responses in the murine urinary tract (Connell *et al.*, 1996). Type 1 fimbriae confer binding to α-D-mannosylated proteins such as uroplakins, which are abundant in the uroepithelial lining of the bladder (Wu *et al.*, 1996). Both P and type 1 fimbriae recognize their receptor targets by virtue of organelle tip-located adhesins, namely PapG and FimH, respectively (Klemm & Schembri, 2000). F1C (Foc) are a third type of fimbriae that belong to the chaperone-usher subfamily and resemble type 1 fimbriae in their genetic organization and organelle structure (Klemm *et al.*, 1994, 1995; van Die *et al.*, 1991). F1C fimbriae mediate binding to galactosylceramide targets present on epithelial cells in the bladder and kidneys as well as globotriaosylceramide, present exclusively in the kidneys (Bäckhed *et al.*, 2002; Khan *et al.*, 2000). Other fimbriae of the chaperone-usher family that have been character-ized from UPEC include S fimbriae, which mediate adherence to sialic acid glycolipids or glycoproteins, and Auf fimbriae (Buckles *et al.*, 2004; Korhonen *et al.*, 1984).

Despite the vast array of adhesins that have been defined in UPEC, three recently sequenced UPEC genomes have revealed the existence of several additional gene clusters that display homology to genes from the chaperone-usher class of fimbriae (Brzuszkiewicz *et al.*, 2006; Chen *et al.*, 2006; Welch *et al.*, 2002). For example, the genome sequence of UPEC CFT073 contains ten different loci that may encode fimbriae that belong to this subfamily. Only half of these fimbrial gene clusters have been characterized to date and the functions of the remainder are unknown. Here we identify and characterize one of these fimbrial gene clusters (*c1931–c1936*) from UPEC CFT073. The fimbriae encoded by this gene cluster, termed F9 fimbriae, are homologous to type 1 and F1C fimbriae and promote biofilm formation on abiotic surfaces.

METHODS

Bacterial strains, plasmids and growth conditions. The strains and plasmids used in this study are described in Table 1. *E. coli* CFT073 was isolated from the blood and urine of a woman with acute pyelonephritis (Mobley *et al.*, 1990). The *E. coli* Reference (ECOR) collection was obtained from the STEC Center, Michigan State University. Cells were routinely grown at 37 °C on solid or in liquid Luria–Bertani (LB) medium supplemented with appropriate anti-biotics unless otherwise stated. M9 minimal medium consisted of 42 mM Na_2HPO_4, 22 mM KH_2PO_4, 9 mM NaCl, 18 mM NH_4Cl, 1 mM $MgSO_4$, 0.1 mM $CaCl_2$ and 0.2 % glucose (Sambrook *et al.*, 1989), supplemented with 0.2 % arabinose and antibiotics where appropriate. For flow chamber biofilm experiments, the glucose concentration was reduced to 0.002 %.

Table 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristic(s)	Reference
***E. coli* strains**		
CFT073	UPEC reference strain	Mobley *et al.* (1990)
MG1655	K-12 reference strain	Bachmann (1996)
MS428	MG1655 *fim*	Kjaergaard *et al.* (2000b)
MS673	pBAD/Myc-HisA in MS428	This study
MS1129	pKC2 in MS428	This study
MS1417	CFT073 *fim*	This study
MS1418	CFT073 *f9* : : *kan*	This study
MS1432	CFT073 *fim foc* : : *kan*	This study
MS1433	CFT073 *fim foc*	This study
MS1436	CFT073 *fim foc f9* : : *kan*	This study
PK1097	CFT073 *fim* : : *kan*	Roos *et al.* (2006b)
Plasmids		
pGB2-24	*fimH* gene in pACYC184	Sokurenko *et al.* (1994)
pKD4	Template plasmid for *kan* gene amplification	Datsenko & Wanner (2000)
pKD46	λ-Red recombinase expression plasmid	Datsenko & Wanner (2000)
pCP20	FLP synthesis under thermal control	Cherepanov & Wackernagel (1995)
pKC1	*c1934–c1936* genes in pBAD/*Myc*-HisA	This study
pKC2	F9 gene cluster in pBAD/*Myc*-HisA	This study
pPKL52	*fimFGH* genes in pBR322	Klemm & Christiansen (1987)

DNA manipulations and genetic techniques. Plasmid DNA was isolated using the QIAprep Spin Miniprep kit (Qiagen). Restriction endonucleases were used according to the manufacturer's specifications (New England Biolabs). Chromosomal DNA was purified using the GenomicPrep Cell and Tissue DNA isolation kit (Amersham Pharmacia Biotech). PCR was performed using the Expand Long Template PCR system (for amplification of the *f9* gene cluster) or *Taq* polymerase (screening assays) according to the manufacturer's instructions (Roche). The primers used in this study are shown in supplementary Table S1, available with the online version of this paper. DNA sequencing was performed by the Australian Genome Research Facility.

Cloning of the F9 fimbriae-encoding gene cluster and PCR screening assay. The F9 fimbriae-encoding gene cluster (*c1931–c1936*) was amplified by PCR using primers 598 and 599. The PCR product was digested with *Xho*I and ligated to *Xho*I-digested plasmid pBAD/*Myc*-HisA (Invitrogen Life Technologies). In this construct, expression of the F9-encoding genes is under control of the arabinose-inducible *araBAD* promoter (Guzman *et al.*, 1995). Specific primers were employed to screen for the prevalence of the F9 chaperone- (596 and 597) and adhesin- (100 and 101) encoding genes.

Construction of CFT073*fim*, CFT073*fim foc* and CFT073*fim foc f9* deletion mutants. CFT073 deletion mutants were constructed using the λ-Red recombinase gene replacement system (Datsenko & Wanner, 2000). Briefly, the kanamycin resistance gene from plasmid pKD4 was amplified using primers containing 40–50 nt homology extensions to the start and end of the gene clusters to be deleted. The following primers were used: (i) 244 and 245 for deletion of the *fim* gene cluster; (ii) 495 and 496 for deletion of the *foc* gene cluster; (iii) 195 and 196 for deletion of the *f9* gene cluster. The primers were used to amplify a 1.6 kb PCR product from plasmid pKD4, representing the kanamycin resistance cassette from pKD4 and additional 40–50 bp overhang regions (underlined in Table S1) at the 5′ and 3′ ends of the PCR product complementary to the target genes in CFT073. CFT073*fim* was constructed by transforming CFT073(pKD46) with the PCR product containing homology to the *fim* gene cluster and selection of kanamycin-resistant colonies. The kanamycin resistance cassette was then removed using plasmid pCP20. The *foc* deletion was constructed in a similar manner using CFT073*fim*(pKD46) to generate CFT073*fim foc*. This strain was subsequently used to generate CFT073*fim foc f9* by the same procedure, except that in this case the kanamycin resistance cassette was not removed. All deletions were confirmed by PCR using k2 and kt primers (Datsenko & Wanner, 2000) in combination with 254 and 255 (*fim* deletion), 573 and 574 (*foc* deletion) and 252 and 253 (*f9* deletion) and subsequent DNA sequencing.

Agglutination and adherence assays. The capacity of F9-expressing *E. coli* MS1129 to agglutinate human, dog, horse or sheep red blood cells (RBC), or yeast cells, was assessed as previously described (Roos *et al.*, 2006a; Schembri *et al.*, 2000). Briefly, a suspension of washed bacterial cells (OD_{550} 0.5) was mixed with RBC or yeast cells on a glass slide and the time to agglutination was measured. Adherence of MS1129 to human HeLa epithelial cells was determined essentially as previously described (Ulett *et al.*, 2003).

Biofilm assays. Biofilm formation was monitored by using sterile non-treated 96-well round-bottom polystyrene microtitre plates (BD Falcon) essentially as previously described (Schembri & Klemm, 2001). Briefly, cells were grown for 24 h in M9 minimal medium (containing 0.2 % arabinose for induction of F9-encoding genes) at 37 °C, washed to remove unbound cells and stained with crystal violet. Quantification of bound cells was performed by addition of acetone/ethanol (20 : 80, v/v) and measurement of the dissolved crystal violet as absorbance at 595 nm. Flow chamber experiments were performed essentially as previously described (Kjaergaard *et al.*, 2000b; Schembri *et al.*, 2003), with the exception that cells were detected using BacLight green fluorescent stain (Molecular Probes). Briefly, biofilms were allowed to form on glass surfaces in a multi-channel flow system that permitted online monitoring of community structures. Flow cells were inoculated with OD_{600} standardized cultures pre-grown overnight in M9 medium. Glucose was used as the sole carbon source at a concentration of 0.002 % and BacLight green fluorescent stain was used at a concentration of 0.1 µM according to the manufacturer's instructions. F9 expression was induced with 0.2 % arabinose. Biofilm development was monitored by confocal scanning laser microscopy at 15 h after inoculation. All experiments were performed in triplicate. Biofilms analysed by scanning electron microscopy (SEM) were grown in 24-well cell culture plates on 15 mm sterile Thermanox plastic coverslip inserts (Nalgene Nunc International). Wells containing coverslip inserts were seeded with approximately 10^4 c.f.u. resuspended in 1 ml M9 medium and were incubated statically overnight at 37 °C. Coverslips were removed from the wells after 24 h, washed extensively with PBS and incubated in fresh medium for a further 24 h to allow biofilm maturation prior to processing for SEM analysis.

RNA isolation and RT-PCR. UPEC CFT073 was grown to exponential phase in M9 minimal medium and resuspended directly in an equal volume of ice-cold RNA*later* (Ambion). Total RNA was isolated using an RNeasy mini kit (Qiagen). RNA was treated with RNase-free DNase, repurified and stored at −80 °C. RNA was converted to cDNA using SuperScript II as described by the manufacturer (Invitrogen Life Technologies). A negative control of non-reverse-transcribed RNA was used to confirm the absence of contaminating genomic DNA. RT-PCR was performed for 25 cycles (94 °C 15 s, 50 °C 15 s, 72 °C 1 min) with primers 600 and 601. These primers were specific for F9 major subunit-encoding gene (*c1936*) and did not amplify the closely related *fimA* or *focA* genes.

Purification of F9 fimbriae and antibody production. A 500 ml culture of MS1129 cells was grown in LB to an OD_{600} of 1.0, induced for F9 fimbriae expression for 2 h with 0.2 % arabinose, harvested by centrifugation and resuspended in 10 ml PBS. Fimbriae were detached from the cells with a mini hand blender using five 30 s pulses essentially as previously described (Klemm *et al.*, 1998). The bacteria were pelleted and the detached F9 fimbriae present in the supernatant were precipitated with 4 vols ice-cold acetone. Proteins were collected by centrifugation, resuspended in PBS and stored at −80 °C until required. Fimbriae were prepared for SDS-PAGE analysis by boiling in acid (pH 2.0 adjusted by the addition of 2.0 M HCl) for 3 min. The preparation was then readjusted to pH 7.0 by the addition of 2.0 M NaOH and boiled for a further 3 min. SDS-PAGE analysis was performed as previously described (Ulett *et al.*, 2006). Polyclonal anti-F9 antiserum was raised in New Zealand White rabbits by intramuscular immunization as described elsewhere (Lam & Mutharia, 1994). The primary immunization dose was 200 µg in 0.5 ml Freund's Adjuvant Complete (Sigma)/PBS (1 : 1, v/v) followed by three booster doses at 2 week intervals using 200 µg in 0.5 ml Freund's Adjuvant Incomplete (Sigma)/PBS (1 : 1, v/v).

Electron microscopy. Cells for transmission electron microscopy (TEM) were prepared from freshly grown colonies or liquid suspensions resuspended in a drop of sterile ultra-pure water. A glow-discharged Formvar-coated copper grid was placed on the drop for 1 min to allow the cells to adsorb. Excess liquid was removed with filter paper before a drop of 1 % ammonium molybdate (negative stain) was placed on the grid. For immunoelectron microscopy bacteria were adsorbed onto glow-discharged carbon-coated copper grids for 5 min. Grids were placed on two drops of PBS (2 min) followed by 5 min on blocking buffer (PBS containing 0.2 % BSA, 0.2 % fish skin gelatin, 20 mM glycine). Samples were reacted with anti-F9 serum (1 : 100 diluted in blocking buffer) for 30 min and

washed four times in blocking buffer (5 min). Samples were then incubated with anti-rabbit IgG gold conjugate (10 nm diameter, diluted 1 : 60 in blocking buffer) for 30 min and washed four times (5 min) in PBS followed by four washes (2 min) in water. Some grids were dried and observed unstained while others were negatively stained with 1 % ammonium molybdate. Cells were examined under a JEOL JEM1010 TEM operated at 80 kV. Images were captured using an analySIS Megaview III digital camera. SEM was performed essentially as previously described (Webster *et al.*, 2004).

RESULTS

Identification of the F9 fimbriae-encoding gene cluster from UPEC CFT073

The UPEC strain CFT073 genome contains ten gene loci that share sequence identity with the chaperone-usher class of fimbriae (Table 2). Five of these gene clusters have been characterized to date. In this study, we chose to focus on the putative fimbrial gene cluster *c1931–c1936*, which encodes F9 fimbriae recently described from *E. coli* O157 : H7 (Low *et al.*, 2006a). The F9 gene cluster contains six open reading frames arranged in the same transcriptional orientation (Fig. 1A). The F9 gene cluster is flanked by two genes (*ydeP* and *hipA*) which are conserved in both the CFT073 and MG1655 genomes. However, part of the F9 gene cluster has been deleted in MG1655 (Fig. 1A). The F9 gene cluster is similar to other fimbrial operons of the chaperone-usher class in that it contains genes encoding putative major (*c1936*) and minor subunit proteins (*c1932, c1933*) as well as putative chaperone- (*c1935*), usher- (*c1934*) and adhesin- (*c1931*) encoding genes. The overall G + C content of the F9 gene cluster is 44.4 mol%. This is significantly lower than that normally found in *E. coli* (50.8 mol%) and suggests that the genes may have been acquired by horizontal transfer.

Cloning and expression of the F9 fimbriae-encoding gene cluster

The F9 gene cluster was amplified by PCR from UPEC CFT073 and cloned as a transcriptional fusion behind the tightly regulated *araBAD* promoter in the pBAD/*Myc*-HisA expression vector to generate plasmid pKC2. To determine if the F9 gene cluster encodes the capacity to produce fimbriae we transformed *E. coli* MS428 with pKC2 to create MS1129. *E. coli* MS428 contains a deletion in the type 1 fimbrial gene locus and therefore does not produce any fimbriae (Kjaergaard *et al.*, 2000b). When MS1129 cells were induced with 0.2 % arabinose we observed 0.2–2 µm long fimbrial organelles on the bacterial cell surface by TEM (Fig. 1C). The fimbriae were purified and examined by SDS-PAGE. A major protein of approximately 15 kDa was obtained (Fig. 1B) and this preparation was used to raise an anti-F9 polyclonal antiserum in rabbits. Immunogold electron microscopy with this antiserum reacted strongly against MS1129 cells induced for F9 fimbriae expression (Fig. 1D). Thus, the F9 gene cluster is functional and its expression results in the production of fimbriae in a defined K-12 host background.

### Agglutination and adherence properties of F9 fimbriae

The agglutination of erythrocytes is the classical assay for monitoring interactions between fimbrial adhesins and species-specific glycoproteins. MS1129 cells induced for F9 expression did not agglutinate erythrocytes from humans, dog, horse or sheep. Since the F9 genes display strong similarity to type 1 fimbrial genes we also tested their ability to agglutinate yeast cells. Yeast cell agglutination is the most highly conserved binding property for type 1 fimbriae. However, MS1129 cells were also unable to agglutinate yeast cells, indicating that unlike its FimH counterpart, the putative F9 adhesin does not bind to D-mannose. Arabinose-induced MS1129 cells were also unable to bind to human HeLa epithelial cells.

F9 fimbriae mediate biofilm formation

MS1129 cells were tested for their ability to form biofilms in standard microtitre plate assays as well as a continuous-flow chamber system. F9 fimbriae mediated strong biofilm formation in the hydrodynamic microtitre plate assay after growth in M9 medium (Fig. 2A). Interestingly, we did not

Table 2. Fimbriae-encoding genes in CFT073

Description	c number	Genes	Receptor target
Putative chaperone-usher fimbrial operon	*c0166–c0172*	*yadN-ecpD-htrE-yadMLKC*	Unknown
F1C	*c1237–c1245*	*sfaCB focAICDFGH*	Galactosylceramide
F9	*c1931–c1936*	*c1936-34-ydeSRQ*	Unknown
Putative chaperone-usher fimbrial operon	*c2635–c2638*	*yehABCD*	Unknown
Putative chaperone-usher fimbrial operon	*c2878–c2884*	*yfcOPQRSUV*	Unknown
P fimbriae	*c3583–c3593*	*papIBAHCDJKEFG*	α-D-Gal-(1-4)-β-D-Gal
Putative chaperone-usher fimbrial operon	*c3791–c3794*	*ygiLGH-c3794*	Unknown
Auf fimbriae	*c4207–c4214*	*aufABCDEFG*	Unknown
P fimbriae (2)	*c5179–c5189*	*papIBAHCDJKEFG*	α-D-Gal-(1-4)-β-D-Gal
Type 1 fimbriae	*c5391–c5399*	*fimBEAICDFGH*	D-Mannose
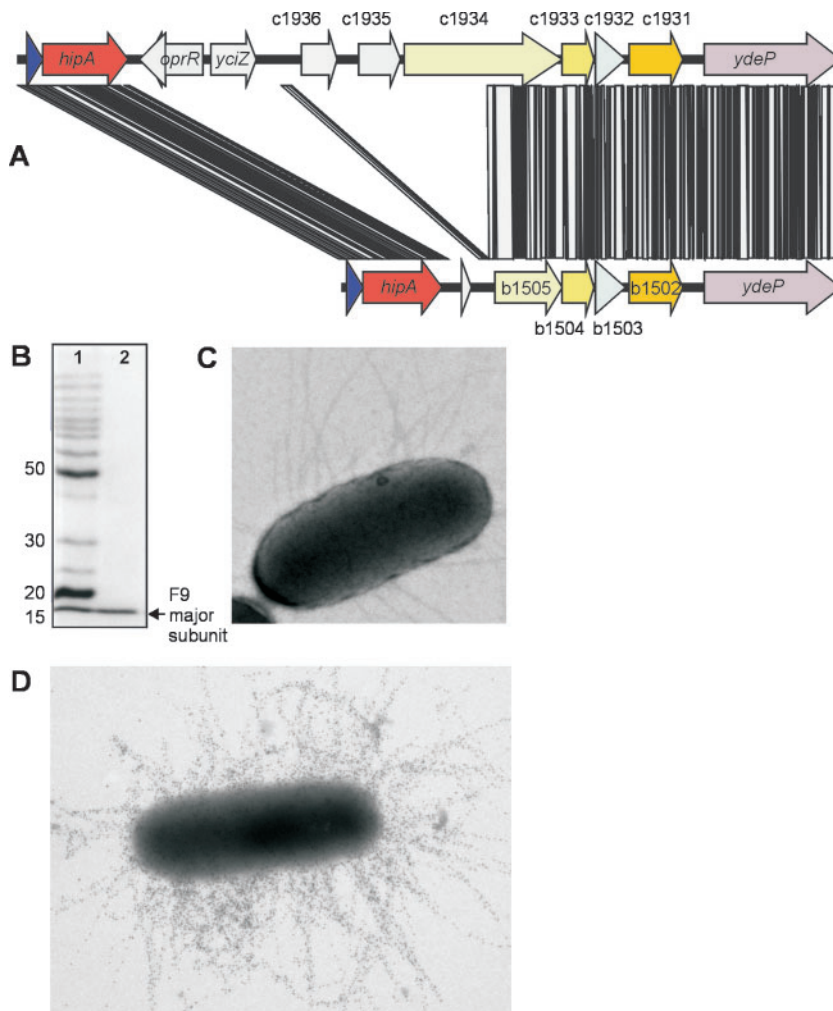
Fig. 1. (A) Physical map of the F9 gene cluster from *E. coli* CFT073 indicating the arrangement of the genes (including adjacent genes). A comparison of the CFT073 F9 gene cluster is shown together with the corresponding gene cluster in MG1655. Highly homologous regions are shaded black. The F9 genes are indicated by their c number and are predicted to encode the following proteins: *c1936*, major subunit; *c1935*, chaperone; *c1934*, usher; *c1933*, minor subunit; *c1932*, minor subunit; *c1931*, adhesin. (B) Coomassie blue stain of F9 fimbriae purified from *E. coli* MS1129 and separated on a 15 % SDS-PAGE gel. (C) Transmission and (D) immunogold electron microscopy demonstrating expression of F9 fimbriae in recombinant *E. coli* K-12 MS1129. F9 fimbrial expression was induced in MS1129 by the addition of arabinose. Immunogold labelling was performed with an F9-specific antiserum.

observe any biofilm formation when the same experiment was performed using LB medium (data not shown). SEM of the biofilms confirmed these results (Fig. 2B). No significant biofilm was formed by the *E. coli* MS673 vector control strain (Fig. 2C). The ability of F9 fimbriae to promote biofilm formation in dynamic conditions was examined using a continuous-flow chamber system. F9-expressing cells produced a dense and uniform biofilm that covered the entire surface of the flow cell with an average depth of 20 μm (Fig. 2D). Taken together, these data demonstrate that F9 fimbriae mediate strong biofilm formation in *E. coli* K-12 cells employing two different model systems.

Analysis of F9 fimbriae expression by UPEC CFT073

In order to determine whether the F9 genes are expressed by wild-type UPEC CFT073, transcription of the F9 major subunit-encoding gene (*c1936*) was analysed by RT-PCR. RNA was extracted from exponentially growing cells, converted to cDNA and a PCR was performed using *c1936*-specific primers. We detected a *c1936* transcript from cells grown in minimal medium (Fig. 3). No PCR products were obtained from RNA samples prior to cDNA synthesis. These results demonstrate that the *c1936* gene in CFT073 is transcribed during exponential growth in M9 minimal medium. However, despite these results, we were unable to detect expression of the corresponding protein by Western blotting using F9-specific antiserum on crude lysates of CFT073 grown under the same conditions.

In *E. coli*, the expression of some types of fimbriae is enhanced under aerated static liquid growth conditions (Old & Duguid, 1970; Stentebjerg-Olesen *et al.*, 2000). Static growth results in the formation of a pellicle at the air–liquid interface that can be promoted by either type 1 or F1C fimbriae (Snyder *et al.*, 2005). Before attempting to induce F9 expression in CFT073 by static growth we therefore deleted the type 1- and F1C-encoding fimbrial genes in CFT073 by λ-Red recombination of linear DNA (Datsenko & Wanner, 2000). The correct deletions were confirmed by PCR and sequencing of the resultant strain (Fig. 4A, B), which was designated CFT073*fim foc*. To determine if CFT073*fim foc* could produce a pellicle at the
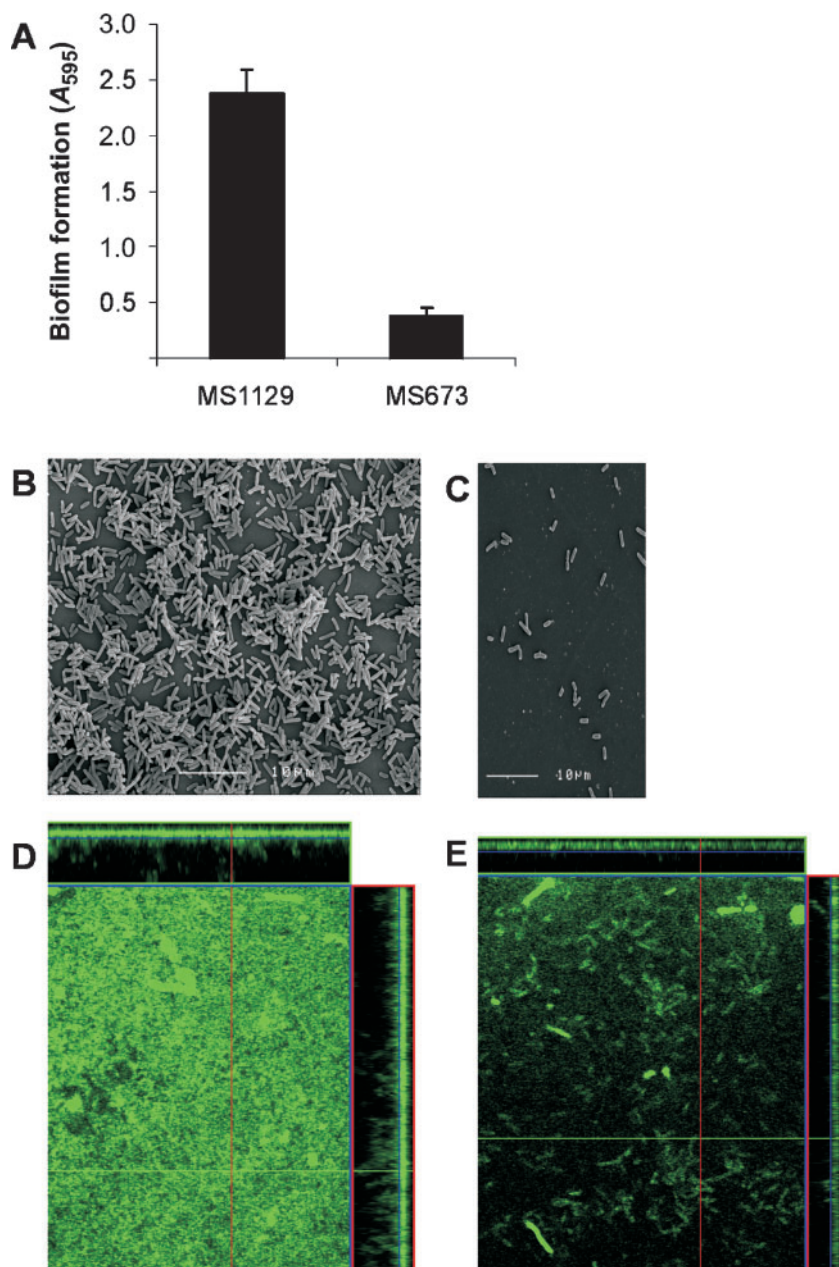
Fig. 2. Biofilm formation by *E. coli* MS1129 in the microtitre plate assay. (A) MS1129 cultures were grown overnight in M9 minimal medium containing 0.2 % arabinose, washed to remove unbound cells and stained with crystal violet to measure biofilm formation (plotted as mean ± SD of three independent determinations). Significantly increased biofilm formation by the F9-induced MS1129 cells in comparison to MS673 vector control cells is demonstrated. (B, C) Similar experiments were also performed using biofilms grown on plastic coverslip inserts in 24-well plates. SEM of these cells was used to visualize the biofilm produced by MS1129 cells (B) and MS673 control cells (C). (D, E) Flow chamber biofilm formation of (D) *E. coli* MS1129 (F9 positive) and (E) *E. coli* MS673 (vector control) cells. Biofilm development was monitored by confocal scanning laser microscopy 15 h after inoculation. Micrographs represent horizontal sections. Depicted to the right and below are vertical sections through the biofilm collected at the positions indicated by the lines.

air–liquid interface it was grown under static culture conditions for 72 h. A sample from the air–liquid interface was then removed and used to inoculate a second culture. After three successive rounds of selection and enrichment for pellicle growth, the CFT073*fim foc* mutant produced a thick pellicle. These cells were then collected, blended to remove any fimbriae and the proteins present in the supernatant were precipitated with acetone. When assessed by SDS-PAGE one prominent band (~18 kDa) as well as a minor band (~16 kDa) was observed (Fig. 4C). These protein sizes correlate with the predicted size of major subunit proteins from many fimbriae of the chaperone-usher class. Western blotting employing our F9-specific antiserum demonstrated the presence of the major F9 subunit as part of the weaker band (Fig. 4D). We also attempted to identify these proteins by N-terminal sequence analysis. The predominant band (~18 kDa) was confirmed to be the PapA2 major subunit protein of P fimbriae. We also identified the PapA protein as part of the minor band. However, we were unable to conclusively detect the F9 major subunit protein sequence as a minor component from the background trace of this N-terminal sequence; the background trace was at 10 % of the overall signal intensity. We also note that in experiments with plasmid-encoded *pap* gene clusters we did not observe any cross-reaction between our F9-specific antiserum and P
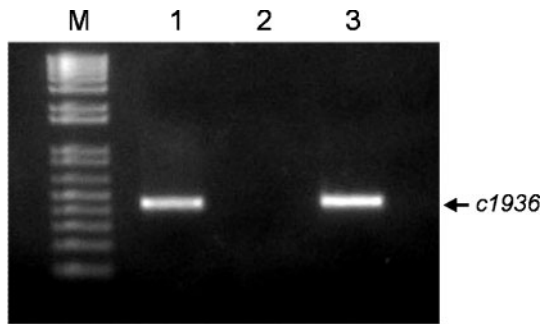
Fig. 3. RT-PCR analysis of *c1936* expression by CFT073. Total RNA was extracted from CFT073 during exponential growth in M9 minimal medium. Lanes: 1, *c1936*-specific PCR product (450 bp) obtained using cDNA as the template; 2, negative control using RNA prior to cDNA synthesis; 3, positive control using CFT073 genomic DNA as the template; M, 1-kb Plus DNA Ladder (Invitrogen).

fimbriae (data not shown). To confirm that the positive reaction in the Western blot was due to the presence of F9 fimbriae, we deleted the F9 genes from CFT073*fim foc* to construct CFT073*fim foc f9*. This strain produced a thick pellicle after three successive rounds of selection and enrichment by static growth. However, when the fimbriae of pellicle-enriched CFT073*fim foc f9* were purified and probed by Western blot analysis using the F9 antiserum, no reaction was observed (Fig. 4D). Taken together, these results demonstrate that the F9 major subunit-encoding gene (*c1936*) is transcribed in CFT073 and that F9 expression can be induced by static growth in a CFT073*fim foc* background, albeit at very low levels. However, under these

growth conditions, P fimbriae are the major fimbriae induced in the CFT073*fim foc* mutant.

## Biofilm formation by CFT073 wild-type and fimbrial deletion mutants

To test the ability of CFT073, CFT073*f9*, CFT073*fim*, CFT073*fim foc* and CFT073*fim foc f9* to form a biofilm we employed both static and dynamic biofilm assays. The strains were grown in M9 minimal medium and biofilm formation was assessed using sterile non-treated polystyrene microtitre plates. There was no difference in biofilm formation between any of the strains under either of the two conditions (Fig. 5). These results are consistent with our observations of a very low level of F9 expression in CFT073.

The FimH adhesin cannot be incorporated into the F9 structural organelle

Previous studies have demonstrated that reciprocal exchange of components between type 1 and F1C fimbriae can result in the expression of functional chimeric organelles (Klemm *et al.*, 1994; Sokurenko *et al.*, 2001). Given the strong similarity between the F9 and type 1 fimbrial proteins (Table 3), we attempted to exchange the minor components of the *fim* system with those of the F9 system. The F9 gene cluster was truncated by removal of the region encoding *c1931–c1933* to generate plasmid pKC1 (containing *c1934–c1936*). *E. coli* MS428 cells harbouring pKC1 were then transformed with a plasmid containing the *fimFGH* genes (pPKL52). When these cells were mixed with yeast cells no agglutination occurred, indicating that the FimH adhesin was not incorporated
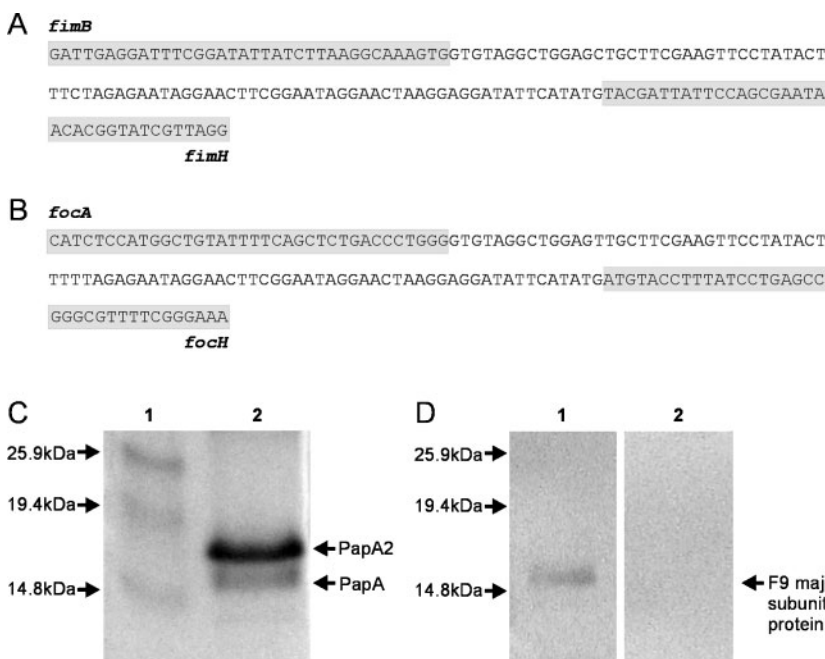
Fig. 4. (A) DNA sequence of the type 1 fimbrial (*fim*) deletion in CFT073*fim foc*; the *fimB* and *fimH* sequences are highlighted and the 84 bp scar is also shown. (B) DNA sequence of the F1C (*foc*) fimbrial deletion in CFT073*fim foc*; the *focH* and *focA* sequences are highlighted and the 84 bp scar is also shown. (C) SDS-PAGE analysis of purified fimbrial proteins from the CFT073*fim foc* pellicle. The major and minor bands were identified by N-terminal amino acid sequencing as PapA2 and PapA, respectively (lane 2). Molecular size markers are indicated (lane 1). (D) Western blot of purified fimbrial proteins from CFT073*fim foc* (lane 1) and CFT073*fim foc f9* (lane 2) reacted with F9-specific antiserum. The F9 major subunit protein was only observed in fimbrial preparations from CFT073*fim foc* (lane 1).
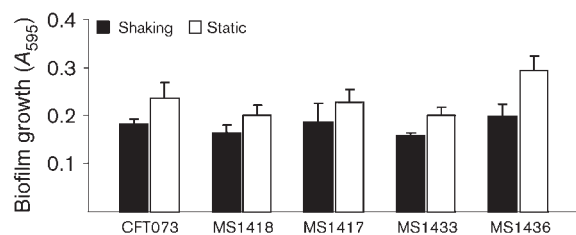
Fig. 5. Biofilm formation by CFT073, MS1418 (CFT073*f9*), MS1417 (CFT073*fim*), MS1433 (CFT073*fim foc*) and MS1436 (CFT073*fim foc f9*) strains. Biofilm formation was examined in polystyrene microtitre plates using static and dynamic growth conditions. Cells were grown at 37 °C for 24 h in M9 minimal medium supplemented with 0.2 % glucose. Adhered cells were stained with crystal violet. The quantification of results after the determination of A_{595} readings is shown as the mean ± SD of three independent experiments.

into the F9 organelle. We also combined a plasmid encoding the entire F9 cluster (pKC2) with a plasmid encoding the *fimH* gene alone (pGB2-24) or the *fimFGH*-containing plasmid (pPKL52), in MS428. However, both of these strains also failed to agglutinate yeast cells. Taken together, these results indicate that although the type 1 and F9 genes display a high degree of sequence conservation, the minor components (FimF, FimG) and adhesin (FimH) of type 1 fimbriae cannot be incorporated into the F9 fimbrial organelle.

Prevalence of F9 fimbriae-encoding genes in other *E. coli*

We screened 20 UPEC isolates from our laboratory collection as well as the 72 strains from the ECOR collection to determine the prevalence of the F9 gene cluster amongst a diverse population of *E. coli* isolates. Primers were designed from regions predicted to be highly conserved within the gene cluster, i.e. the putative chaperone- (*c1935*) and adhesin- (*c1931*) encoding genes. We found that the *c1935*

Table 3. Comparison of the F9 protein and nucleotide sequences with the corresponding Fim and Foc sequences

F9 protein	Putative function	Amino acid identity (and nucleotide sequence conservation) to corresponding	
		Fim protein	Foc protein
C1936	Major subunit	59 % (59 %)	54 % (54 %)
C1935	Chaperone	56 % (61 %)	60 % (62 %)
C1934	Usher	56 % (62 %)	51 % (56 %)
C1933	Minor subunit	43 % (54 %)	28 % (48 %)
C1932	Minor subunit	56 % (61 %)	36 % (51 %)
C1931	Adhesin	46 % (54 %)	32 % (47 %)

and *c1931* genes were present in 80 % (16/20) of clinical isolates in our collection and 56 % (40/72) of strains from the ECOR collection. *E. coli* K-12 MG1655 contains the *c1931* gene and a truncated version of *c1934* but does not contain *c1935* (Fig. 1A). The high frequency of both *c1935* and *c1931* in the UPEC isolates tested here suggests that the deletion found in the K-12 MG1655 strain is not representative of the clinical UPEC isolates in our collection. We also screened the strains in the ECOR collection for the expression of F9 fimbriae using slide agglutination reactions with our F9 antiserum. Ten positively reacting strains were identified (data not shown). However, these ten strains also reacted positively in yeast cell agglutination reactions, suggesting the possibility of cross-reaction between some FimA variants of type 1 fimbriae and our F9 antiserum. In this respect we note that our type 1 fimbriae antiserum (raised against purified type 1 fimbriae from MG1655) did not cross-react with F9 fimbriae produced by MS1129. The precise characterization of these strains with respect to this phenotype is in progress.

DISCUSSION

In order to establish infection in the urinary tract UPEC must be able to adhere to host cells and avoid elimination. Virtually all *E. coli* strains are able to express adhesins in the form of fimbriae, long thread-like surface organelles that enable bacteria to recognize and attach to receptor molecules on specific host cell surfaces (Klemm & Schembri, 2000). Most UPEC isolates are able to express several different adhesins that mediate specific binding to the uroepithelium with different receptor specificities. The most common fimbriae on UPEC are type 1 and P fimbriae, and >80 % of the strains are able to express either or both fimbrial types (Gander & Thomas, 1987; Nowicki *et al.*, 1984); 14–30 % are able to express F1C fimbriae (Pere *et al.*, 1987). In the present study we have characterized F9 fimbriae from UPEC CFT073.

The genes encoding F9 fimbriae are highly similar to those of type 1 and F1C fimbriae (Table 3) and are arranged in a similar structural unit. A gene encoding a putative LysR-family transcriptional regulatory protein (*ycjZ*) lies 1620 bp upstream of *c1936*; however, at present there is no evidence to indicate that it plays a role in the regulation of F9 expression. The expression of other chaperone-usher class fimbriae in UPEC is phase variable, although the underlying regulatory systems are very different. Type 1 fimbriae expression is based on DNA inversion (Klemm, 1986), whereas phase variation of P fimbriae depends on a combination of Dam methylation and the PapB and PapI regulators (Braaten *et al.*, 1994; Forsman *et al.*, 1989). F1C and S fimbrial expression is thought to be similar to that of P fimbriae although the exact regulatory mechanisms involved remain to be elucidated. We did not find any evidence based on DNA sequence analysis that F9 expression could be phase variable. There are no recombinase-encoding genes

upstream of the F9 genes and the nearest GATC site lies 558 bp upstream of the predicted F9 major subunit start site.

Among the genome-sequenced *E. coli* strains available on the NCBI database, several, including F11 (ExPEC), O42 (EAEC), B171 (EPEC), B7A (ETEC), E110019, E22, E2348/69 (EPEC), EDL933 and RIMD 0509952 (EHEC), contain an intact F9 gene cluster. The deletion encompassing *c1934–c1936* in MG1655 is common to W3110 (K-12) and HS (commensal strain). The recently sequenced UPEC strain UTI89 contains a unique deletion in the F9 cluster, which includes part of *c1932*, *c1933* and part of *c1934*. Taken together, these genome sequence data suggest that the intact F9 gene cluster is found in many types of *E. coli*. These data also correlate with our observation that the *c1931* and *c1935* genes were highly prevalent in our UTI clinical strains. Our PCR strategy of screening for the *c1931* and *c1935* genes would have successfully identified strains that contained the MG1655 deletion. However, the newly identified smaller deletion in UTI89 would not have been identified by this approach. Thus, a more detailed analysis of the F9 genes is required to accurately determine the prevalence of the intact gene cluster in UPEC.

Previous work has demonstrated that the expression of one fimbrial type can repress the expression of other fimbriae, suggesting there may be a hierarchical structure associated with coordinated fimbrial expression (Holden & Gally, 2004). For example PapB, the regulator of P fimbrial expression, can repress the expression of type 1 fimbriae (Xia *et al.*, 2000). Given the strong homology between type 1, F1C and F9 fimbriae, we deleted the type 1 and F1C fimbrial gene clusters in CFT073. Static growth of this strain (CFT073*fim foc*) induced the formation of a pellicle that consisted primarily of cells that produced P fimbriae derived from both *pap* gene clusters. To our knowledge, this is the first report to demonstrate that P fimbriae can promote pellicle growth. When the purified fimbriae present in the pellicle were tested by Western blotting a positive reaction was also obtained with our F9-specific antiserum, indicating the presence of the F9 major subunit protein. No F9 fimbriae were detected from pellicle preparations of the CFT073*fim foc f9* triple mutant. Thus, although P fimbriae were the major fimbriae induced in CFT073*fim foc* static cultures, a low level of F9 fimbriae expression was also evident and made up a minor part of the total fimbriae purified from the cell pellicle.

UPEC are capable of forming biofilm-like structures within the superficial umbrella cells of the bladders of mice (Anderson *et al.*, 2003). These structures are associated with the expression of type 1 fimbriae and Ag43 and are thought to promote persistence in the urinary bladder (Eto *et al.*, 2006). In *E. coli* K-12, several cell-surface factors, including type 1 fimbriae (Kjaergaard *et al.*, 2000a; Schembri & Klemm, 2001; Schembri *et al.*, 2003), flagella (Pratt & Kolter, 1998), Ag43 (Kjaergaard *et al.*, 2000a; Schembri *et al.*, 2003) and curli (Vidal *et al.*, 1998), have

been implicated in biofilm formation. It is likely that many of these factors also contribute to catheter-associated UTIs caused by UPEC. Here we demonstrate that F9 fimbriae mediate strong biofilm growth in a recombinant *E. coli* strain. The environmental conditions that activate the expression of F9 fimbriae in wild-type UPEC remain to be determined. However, it is noteworthy that several studies have reported increased expression of F9 genes in a variety of *E. coli* pathotypes. A recent microarray study identified the F9 genes as being upregulated in CFT073 isolated directly from the urine of infected mice (Snyder *et al.*, 2004). In *E. coli* O157 : H7, the F9 gene cluster is analogous to the *loc8* locus (Dziva *et al.*, 2004; Low *et al.*, 2006a). Expression of the F9 genes was analysed by chromosomally integrated *lacZ* promoter fusions and shown to be enhanced during biofilm growth and during growth at 28 °C (as opposed to 37 °C) (Low *et al.*, 2006b). Here we demonstrate that F9 fimbriae promote biofilm growth. Consistent with our findings, Low *et al.* (2006b) also did not find any evidence of phase variation from the F9 promoter. In other studies, the F9 genes have been reported to be important for the colonization of calves by *E. coli* O157 : H7 and O26 (Dziva *et al.*, 2004; van Diemen *et al.*, 2005) and F9 fimbriae were shown to mediate adherence to bovine epithelial cells (Low *et al.*, 2006a). However, in contrast to the bundle-like arrangement of F9 fimbriae at the cell pole observed when the *E. coli* O157 : H7 F9 genes were expressed in *E. coli* K-12 (Low *et al.*, 2006a), the F9 fimbriae produced by our recombinant strain MS1129 were arranged peritrichously on the cell surface, with a length varying between 0.2 and 2 μm. Furthermore, we did not observe a difference in the amount of F9 major subunit protein produced following static growth of the CFT073*fim foc* mutant at 28 °C and 37 °C (data not shown).

In summary, the genes encoding F9 fimbriae are commonly found in UPEC and other types of pathogenic *E. coli*. Several studies, including the present work, have now demonstrated expression of the F9 genes in *E. coli* (Dziva *et al.*, 2004; Low *et al.*, 2006a, b; Snyder *et al.*, 2004; van Diemen *et al.*, 2005). One function of F9 fimbriae is their ability to mediate strong biofilm growth. Further molecular characterization of the F9 genes is now required to investigate how their expression is regulated in relation to other fimbriae and to assess their function in wild-type *E. coli*.

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