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A Newly Identified Group of P-like (PL) Fimbria Genes from Extraintestinal Pathogenic *Escherichia coli* (ExPEC) Encode Distinct Adhesin Subunits and Mediate Adherence to Host Cells

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ABSTRACT Fimbrial adhesins promote bacterial adherence and biofilm formation. Sequencing of avian pathogenic *Escherichia coli* (APEC) strain QT598 identified new fimbriae belonging to the π group, which we named PL (P-like) fimbriae since the genetic organization and sequence are similar to those of P and related fimbriae. Genes encoding PL fimbriae located on IncF plasmids are present in diverse *E. coli* isolates from poultry, human systemic infections, and other sources. As with P fimbriae, PL fimbriae exhibit divergence in adhesin-encoding genes and could be divided into 5 classes based on sequence differences in the PlfG adhesin. *plf* genes from two predominant PlfG adhesin classes, PlfG class I (PlfGI) and PlfGII, were cloned. PL fimbriae were visualized by electron microscopy, associated with increased biofilm, demonstrated distinct hemagglutination profiles, and promoted adherence to human bladder and kidney epithelial cells. The genes encoding hybrid fimbriae were comprised of genes from plf_{QT598}, wherein *plfG* was replaced by *papG*; the adhesin-encoding genes were also functional and mediated adherence to epithelial cells, demonstrating compatibility between the components of these two types of fimbriae. Deletion of *plf* genes did not reduce colonization of the mouse urinary tract in a single-strain infection model. In contrast, loss of *plf* genes significantly reduced competitive colonization in the mouse kidneys. Furthermore, *plf* gene expression was increased over 40-fold in the bladder compared to during *in vitro* culture. Overall, PL fimbriae represent a new group of fimbriae demonstrating both functional differences from and similarities to P fimbriae, which mediated adherence to host cells and improved competitive colonization of the mouse kidney.

IMPORTANCE Fimbriae are important colonization factors in many bacterial species. The identification of a new type of fimbriae encoded on some IncF plasmids in *E. coli* was investigated. Genomic sequences demonstrated these fimbrial gene clusters have genetic diversity, particularly in the adhesin-encoding *plfG* gene. Functional studies demonstrated differences in hemagglutination specificity, although both types of Plf adhesin under study mediated adherence to human urinary epithelial cells. A *plf* mutant also showed decreased colonization of the kidneys in a mouse competitive infection model. PL fimbriae may represent previously unrecognized adhesins that could contribute to host specificity and tissue tropism of some *E. coli* strains.

KEYWORDS fimbria, pili, pathogenic *E. coli*, hemagglutination, biofilm, adherence, urinary tract infection, poultry, adhesins, avian, extraintestinal diseases, fimbriae, pilus

Bacterial adherence to surfaces is an important survival mechanism. Attachment to host cells or extracellular matrix can provide access to specific niches and promote colonization of host tissues. Adhesins can also mediate biofilm formation through

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bacterium-bacterium associations and improve survival in the environment. Bacterial adhesins include hair-like appendages (fimbriae or pili) as well as other molecules, including proteins or polysaccharides, which are displayed on the cell surface (1). Many types of fimbriae (pili) in Gram-negative bacteria are assembled by the chaperone/usher pathway (CUP) (2, 3). In *Escherichia coli*, numerous types of CUP fimbriae have been identified and characterized. Pathogenic *E. coli* often can produce multiple types of fimbrial adhesins, and genomic analyses indicate that some strains may contain 10 or more fimbrial gene clusters (3, 4). The ability to produce a variety of adhesins can provide a fitness advantage by expanding potential host receptor targets or promoting adherence to environmental substrates.

Two main types of fimbriae, type 1 and P fimbriae, from *E. coli* have been extensively investigated to determine aspects of their roles in disease, particularly urinary tract infections (UTIs) (5–7), as well as molecular aspects of biogenesis and assembly of these structures (8). Type 1 fimbriae mediate adherence to mannose-containing receptors and have been shown to be critical for virulence of extraintestinal pathogenic *E. coli* (ExPEC), including *E. coli* strains causing urinary tract infections (9) and neonatal meningitis (10). P fimbriae were first described in uropathogenic *E. coli* (UPEC) and were named based on receptor affinities for P blood group oligosaccharides, and they were also described as pyelonephritis-associated pili (Pap), since these fimbriae were more associated with *E. coli* strains from cases of pyelonephritis (11). P fimbriae have also been identified in other ExPEC isolates, including *E. coli* associated with systemic infections in swine (12) and some strains of avian pathogenic *E. coli* (APEC) (13–16). The P fimbrial gene clusters are commonly located on horizontally acquired chromosomal regions, which have been termed pathogenicity islands (14, 17, 18).

The P fimbrial gene cluster comprises 11 genes, including regulatory genes (*papI* and *papB*) and genes dedicated to fimbrial assembly and structure (*papAHCDKJEFG*). The *papA* gene encodes the major fimbrial subunit and has been used to class P fimbriae into serological variants ($F7_1$ and $F7_2$ through F16) (19). The adhesin specificity of P fimbriae is mediated by the *papG* gene product. The G adhesins of P fimbriae were grouped into 3 major classes based on sequence differences and receptor specificity to different Gal(α1-4)Gal-containing glycolipids (20, 21). PapG class I (PapGI) adhesins recognize globotriaosylceramide (Gb3), PapGII adhesins recognize globotetraosylceramide (Gb4), and PapGIII or PrsGIII adhesins recognize galactosylgloboside (Gb5). These glycolipid receptors are usually found on the surface of red blood cells and on human bladder and kidney cells. A distinct variant allele, $papG_{BF31}$, which was termed class IV, was also reported, although receptor specificity for this fimbrial adhesin was not described (22).

P fimbriae are the archetype representatives of the π fimbrial family (2), which includes a number of other types of *E. coli* fimbriae, including Pix fimbriae present in some UPEC strains (23, 24) and Sfp fimbriae encoded on plasmids in some lineages of enterohemorrhagic *E. coli* (EHEC) (25, 26). This report describes a new type of *E. coli* fimbriae from the π group that we have named P-like (PL) fimbriae, since they share sequence similarity and genetic organization with P fimbriae. The PL fimbriae are distinct from other known members of the π fimbriae and are encoded on IncFIB plasmids containing numerous other virulence genes associated with ExPEC and APEC strains. As with P fimbriae (20), PL fimbriae have also diversified into a number of different G adhesin classes and major subunit variants, suggesting adaptive potential for host specificity and tissue tropism. Herein, we characterized two different types of PL fimbria genes encoding distinct G adhesins that were cloned from avian pathogenic *E. coli* strain QT598 and a urinary tract pathogenic *E. coli* (UPEC) strain, UMEA3703-1, and demonstrate these fimbriae can mediate adherence to host epithelial cells.

RESULTS

Genomic analysis identifies a new type of fimbria with a genetic organization similar to P fimbriae. Previously, we reported that ExPEC strain QT598, originally isolated from an infected turkey, contains a large ColV-type virulence plasmid, pEC598,

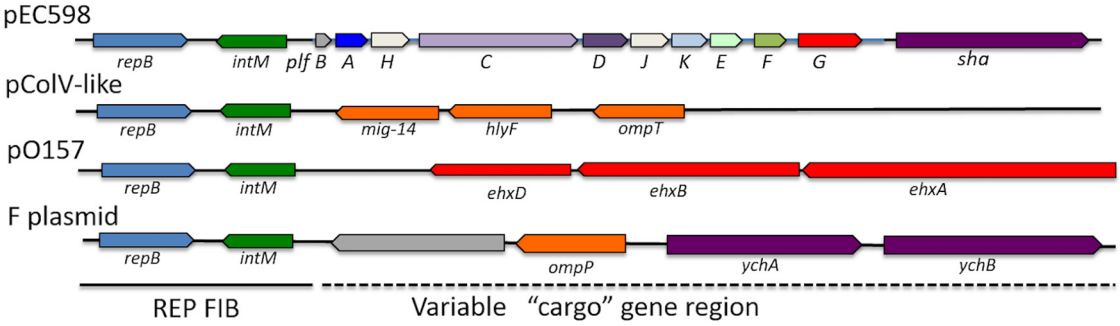

FIG 1 The P-like fimbrial (*plf*) gene cluster of plasmid pEC-598-1 is located adjacent to the RepFIB region. Comparison of the cargo genes adjacent to the RepFIB region on F and related conjugative plasmids. In other ColV-like plasmids, such as pAPEC-1 or pVM1, the cargo region contains *mig-14*, *hlyF*, and *ompT* virulence genes. In pO157 from *E. coli* O157:H7 strains, the genes encoding the Ehx hemolysin RTX toxin are within the cargo region. The F plasmid cargo gene region also contains virulence-associated genes, a gene coding for a protease, OmpP, that can degrade host defense peptides, and genes encoding self-associating AIDA-1-like autotransporters YchA and YchB. NCBI accession numbers: pEC598, NZ_KP119165.1; "pColV-like" pAPEC-1, CP000836; pVM01, NC_010409.1; pO157, AB011549; F plasmid, NC_002483.1.

that encodes a novel autotransporter protein, the serine-protease hemagglutinin autotransporter, Sha (27). The region adjacent to the *sha* gene on pEC598 contains a fimbrial gene cluster (Fig. 1). Due to the close protein identity and genetic organization with P fimbriae (see below), we have named these genes *plf* (for P-like fimbriae) and called these adhesins PL fimbriae. The *plf* gene cluster on the pEC598 plasmid is inserted beside a site-specific integrase gene located adjacent to the RepFIB region, which is one of the known replicons of IncF plasmids (Fig. 1). The RepFIB region and *intM* integrase genes are also present in most IncFII plasmids and are also commonly flanked by other predicted virulence genes, such as *mig-14*, *hlyF*, and *ompT*, in other APEC virulence plasmids, suggesting this conserved region may have led to acquisition of different genes through integration/recombination. Interestingly, this region of F and related plasmids is considered a "hot spot" for insertion of diverse virulence and antibiotic resistance genes, which have been termed cargo genes (Fig. 1) (28).

Fimbriae have been classed into specific groups by a variety of criteria, including comparison of usher, chaperone, and major fimbrial subunits (2, 29). Fimbria classification using the usher-encoding protein sequences has placed P fimbriae within the π fimbrial clade (2). Phylogenetic analysis using the usher proteins indicated that the PL fimbriae also belong to the π fimbrial clade and cluster with Pix, Sfp, and Pap fimbriae (Fig. 2A). Phylogenetic comparison based on the chaperone proteins also indicated PL fimbriae were most closely related to P, Pix, and Sfp fimbriae (see Fig. S1 in the supplemental material).

Girardeau et al. (29) also classified fimbriae based on amino acid sequence motifs within the major subunit proteins. The subfamily Ic (PapA-like) group, which included PapA variants, SmfA (*Serratia marcescens*), PmpA (*Proteus mirabilis*), and MrpA (*Proteus mirabilis*), also includes PixA, SfpA, and PlfA major subunit proteins that share a conserved sequence signature motif in segment S1 of the fimbrial subunits: GxG[KT]V[TS] FxG[TS]V[VI]DAP (Fig. 2B).

The PL fimbriae (*plf*) gene cluster contains 10 genes predicted to encode one regulatory protein and 9 structural/assembly proteins that share identity to equivalent proteins present in the *pap* gene cluster (Fig. 3). A predicted regulatory protein, PlfB, shares identity with members of the PapB regulatory family, which includes PapB (P fimbriae), PixB (Pix fimbriae), FocB/SfaB (F1C/S fimbriae), AfaA (Afa class III adhesin), Daa (F1845 fimbriae), and FanA/FanB (K99 pili) regulatory proteins (30). The highest identity was to PixB (57% identity, 76% similarity), followed by FanB (47% identity, 69% similarity) and PapB (45% identity, 67% similarity). No equivalent of the PapI regulatory gene was present. Some Plf proteins show higher identity to other *pap*-related fimbrial gene clusters, specifically from Pix fimbriae, identified in some *E. coli* urinary tract

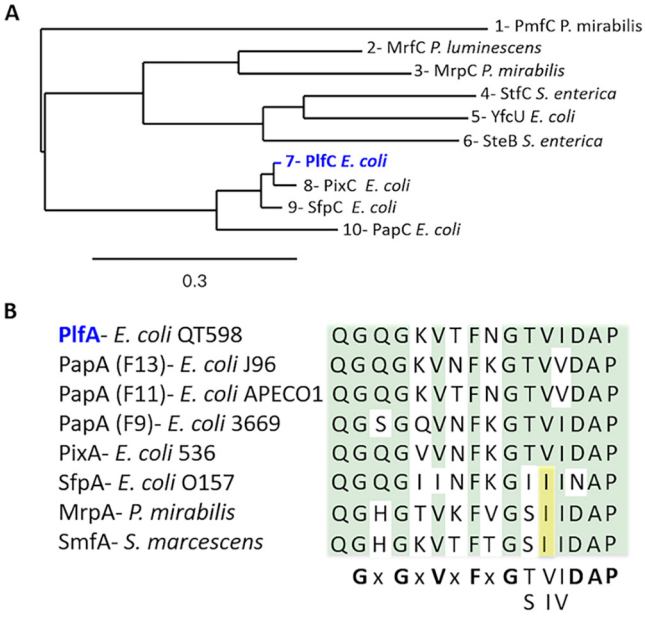

FIG 2 Phylogenetic relationship of PL fimbriae (Plf) with other types of fimbriae. (A) Phylogram using sequences of fimbrial usher proteins (FUPs) belonging to the π fimbriae clade based on the classification scheme of reference 2. The PlfC protein, shown in blue, clusters with other FUPs, Sfp and Pix, more closely related to P fimbriae. (B) Alignment of segment S1 of the major subunit proteins also places PlfA within the PapA-like subfamily (Ic) according to the scheme of Girardeau et al. (29). The alignment results in a consensus GxGxVxFxG[TS][VI][IV]DAP motif. Accession numbers for the sequences shown in panel A were from UniProt (uniprot.org) or GenBank: 1, P53514; 2, Q93MT4; 3, Q51904; 4, H9L4A4; 5, P77196; 6, A0A3U8KZY6; 7, AKG46878.1; 8, A0A454A7L3; 9, B8RHG0; 10, P07110. Accession numbers for the sequences shown in panel B were from UniProt or GenBank: PlfA, AKG46876.1; PapA (F13), X61239; PapA (F11), Q4FBG1; PapA (F9), M68059; PixA, A0A454A7E1; SfpA, W6JHT1; MrpA, Q03011; SmfA, P13421.

infection isolates (23, 24), and the plasmid-encoded Sfp fimbriae, present in sorbitol-fermenting diarrheagenic *E. coli* O157:H7 strains (25, 26) (Fig. 3). Despite demonstrating higher identity to certain proteins from these other fimbriae, only Plf demonstrates a complete set of structural/assembly protein genes equivalent to each in the *pap* gene

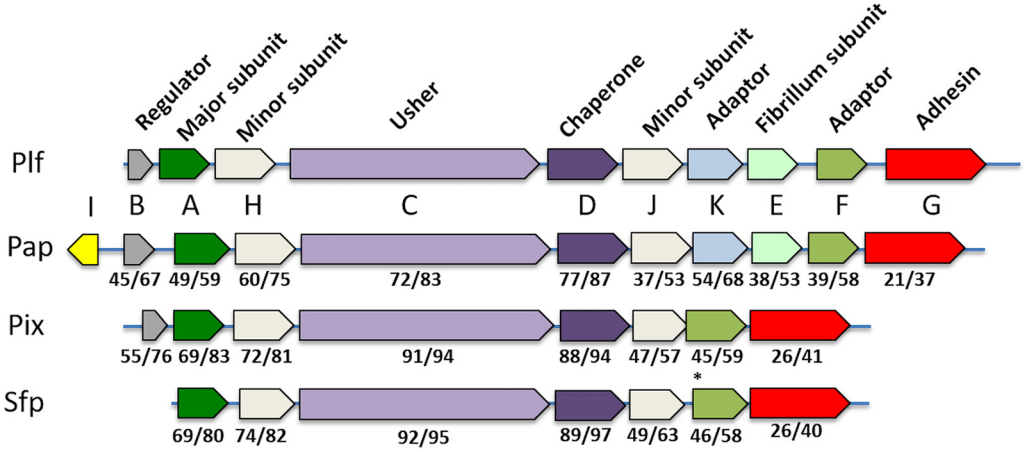

FIG 3 Genetic organization of the strain QT598 PL fimbrial (*plf*) gene cluster and comparison to related fimbrial gene clusters. The genes are labeled based on the *pap* gene cluster convention. The numbers below each gene are the percentage amino acid identity/similarity obtained using BLASTP (https://blast.ncbi.nlm.nih.gov/). The DNA sequences used were *plf* from strain QT598 (GenBank no. KP119165.1; bases 5500 to 14700), *pap* from UPEC 536 (57) (GenBank no. CP000247.1; region ECP_4533 to ECP_4543), *pix* from UPEC X2194 (23) (GenBank no. AJ307043), and *sfp* from pSFO157 (26) (AF401292.1; bases 10000 to 17000). *, SfpF was converted to a longer 186-amino-acid open reading frame based on the GenBank submission. Predicted functions of gene products are indicated above. Colors denote paralogs from each fimbrial gene cluster. The *sfp* and *pix* clusters lack genes encoding PapK and PapE paralogs, which encode an adaptor controlling fibrillum length (58) and minor fibrillum subunits (59), respectively, in P fimbriae.

cluster, as Pix and Sfp fimbriae both lack PapE or PapK protein paralogs, which are known to code for a fibrillum subunit and adaptor protein, respectively (Fig. 3). The gene products showing the greatest degree of diversity among these fimbrial protein paralogs were the G adhesins, which exhibited less than 30% identity. Taken together, the PL fimbrial system is highly similar to Pix and Sfp fimbrial systems, but shares a genetic organization more akin to that of P fimbriae, as it includes the PapK and PapE paralogous proteins PlfK and PlfE, which are predicted to be part of a thin fibrillum structure.

PL fimbrial gene clusters contain different types of G adhesins. To best identify potential fimbrial genes that are very closely related to the Plf system of strain QT598, alignment searches of the NCBI database were done against the predicted adhesin-encoding gene product PlfG using a cutoff of >90% amino acid identity. The search revealed 105 samples (104 *E. coli* strains and one *Escherichia albertii* strain) containing a *pflG* allele with high identity to $PflG_{QT598}$. Interestingly, among the sample sources, a majority were isolated from avian species as well as clinical isolates from urine or extra-intestinal infections in humans (Table S1). However, some samples were also from a variety of livestock, healthy human fecal samples, exotic zoo animal fecal samples, and environmental sources. BLAST analyses against $PlfG_{QT598}$ also identified a series of proteins demonstrating from 44% to 77% identity to PlfG that were all associated with fimbrial gene clusters belonging to the Plf family, since these fimbrial gene clusters shared the same genetic organization as the *plf* gene cluster and had highly conserved identity (>94%) to the $PlfD_{QT598}$ gene products (data not shown).

The initial similarity search of the NCBI database was based on specifically identifying *plf* gene clusters with PlfG adhesin similar to that of strain QT598. In order to determine the general prevalence of PL fimbrial genes distributed among different isolates, a protein BLAST search specific to the predicted PlfK protein was used. We chose this predicted open reading frame for the search as it is not present in the other closely related π fimbria Pix or Sfp and since identity to the PapK protein ortholog is only 54% (Fig. 3). The search (conducted in November 2021) revealed 686 distinct isolates/strain entries containing *plf* sequences, most of which contained complete *plf* gene clusters. This included 171 entries from human infections (mainly urinary isolates or blood infections) and 66 human fecal source isolates. As well, many of the entries were associated with infections in dogs, samples from turkeys, chickens, and other avian species, and a variety of environmental sources. Details of the sources of entries containing *plf* sequences are presented in the supplemental material (Table S2). Taken together, sequence survey analyses indicate that the PL fimbria-encoding sequences are present in *E. coli* strains associated with extraintestinal infections as well as a variety of other animal and environmental sources.

Based on sequences in the database and identification of entries containing enough sequence data to span the length of the fimbrial gene clusters, a phylogenetic analysis of distinct protein entries for different PlfG adhesins was determined. In all, 21 protein entries sharing identity with PlfG were identified (Fig. 4). Analysis determined 5 distinct clades of the PlfG adhesins, including a group (class V) specific to some *Cronobacter* spp. The number of individual entries from the sequence database indicated that PlfG class I and PlfG class II families were predominant, whereas PlfG classes III to V were represented by only a few individual strains in the sequence database (Fig. 4). All of the *plf* gene clusters identified from *E. coli* strains regardless of G adhesin class were inserted adjacent to a site-specific integrase and RepFIB region (data not shown), suggesting that these fimbrial systems are likely to be plasmid encoded. Taken together, these results suggest that *plf* gene clusters are present in numerous *E. coli* strains and that the G adhesins of these fimbriae have diversified into distinct alleles.

Phylogenetic analysis based on the comparison of the PlfG adhesin sequences in the database demonstrated that two main classes of PlfG adhesins, class I and class II, are predominant in sampled genomes. Specific BLAST comparisons of the PlfG class II adhesin from *E. coli* strain QT598 with a representative gene encoding the class I

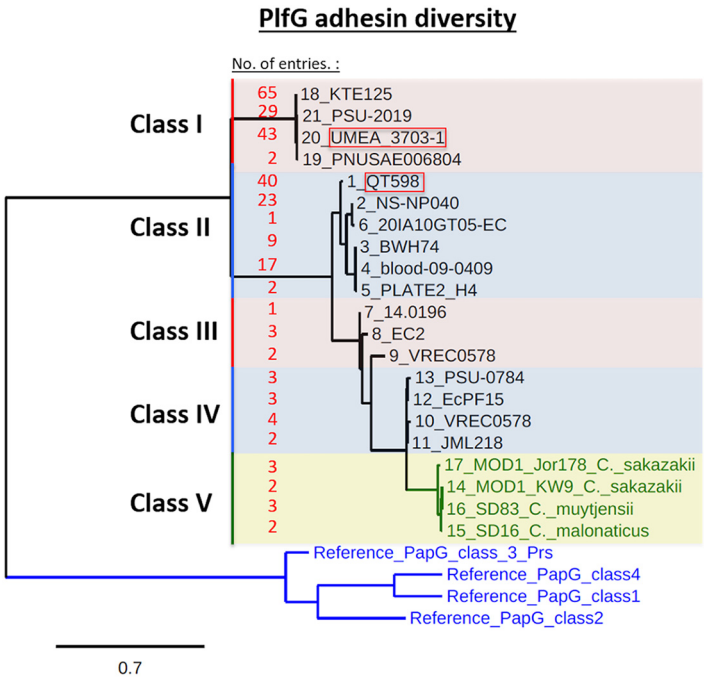

FIG 4 Phylogenetic analysis of different PlfG adhesin proteins. Predicted PlfG proteins from individual isolates were obtained from the sequence database at NCBI (https://www.ncbi.nlm.nih.gov) and one individual isolate sequence was selected based on sequence diversity and association with a complete *plf* fimbrial gene cluster. The total number of protein accessions for each group (no. of entries) at time of submission are listed on the left in red. Multiple-sequence alignment (MUSCLE) and phylogeny (PhyML) were generated using Phylogeny.fr (www.phylogeny.fr). Analysis determined 5 distinct clades of PlfG adhesins. Included is a group (class V) specific to some *Citrobacter* spp. (indicated in green). The PapG reference adhesins from P fimbriae clustered as a distinct group from all of the PlfG adhesin proteins. The *plf* gene clusters from two strains, UMEA-3703-1 (PlfGI) and QT598 (PlfGII), both boxed in red, were cloned for further analysis. The total numbers of protein accessions for each group, at time of submission, are listed on the right. Twenty-one different nonredundant entries were used: 1, WP_059331527.1; 2, WP_137488293.1; 3, WP_097732425.1; 4, WP_033555940.1; 5, WP_201475228.1; 6, EGW8442016.1; 7, MBB8123006.1; 8, WP_029305610.1; 9, WP_112039355.1; 10, WP_096965282.1; 11, WP_137504062.1; 12, WP_176323703.1; 13, EFO1491433.1; 14, WP_133116004.1; 15, WP_158696804.1; 16, WP_158685756.1; 17, WP_105536056.1; 18, WP_001523394.1; 19, EFB9349400.1; 20, WP_016233112.1; 21, WP_033549358.1. Alignment also included reference entries for the 4 established PapG alleles under the following accession numbers: PapGI (strain J96), CAA43570.1; PapGII (strain IA2), AAA24293.1; PapGIII (strain J96), P42188; PapGIV (AAK08949.1) strain BF31.

adhesin from strain UMEA-3703-1, showed 45% identity and 65% similarity. This sequence divergence is similar to the difference between P fimbriae class I and class II G adhesins (46% identity, 64% similarity). As such, and since these two PlfG classes are the most common in the database, we cloned both of them for further investigation.

The *plf* class I and II gene clusters encode fimbriae with distinct hemagglutination activity. To demonstrate that the *plf*-containing clones produced fimbriae, the plasmids encoding *plf* genes were transformed into the afimbriated *E. coli* K-12 strain ORN172. Transmission electron microscopy (TEM) demonstrated that both plf_{QT598}- and $plf_{UMEA\text{-}3703\text{-}1}$-containing plasmids produced peritrichous fimbrial filaments at the surface of cells of strain ORN172 (Fig. 5; Fig. S2). In contrast, ORN172 containing the empty vector did not produce any fimbriae, as expected.

P fimbrial adhesins are known to be mannose-resistant hemagglutinins, and they demonstrate lectin activity specific to Gal(α1-4)Gal-containing glycolipids present on the surface of erythrocytes and other host cells. To compare the hemagglutination activity of P fimbria reference clones with that of clones producing PL fimbriae, we tested hemagglutination activity of fimbriae expressing clones in the nonfimbriated *E. coli* strain ORN172 for a variety of erythrocytes from different species (Fig. 6). The reference clone encoding P fimbriae with the PapG class I adhesin from *E. coli* J96/pPap5

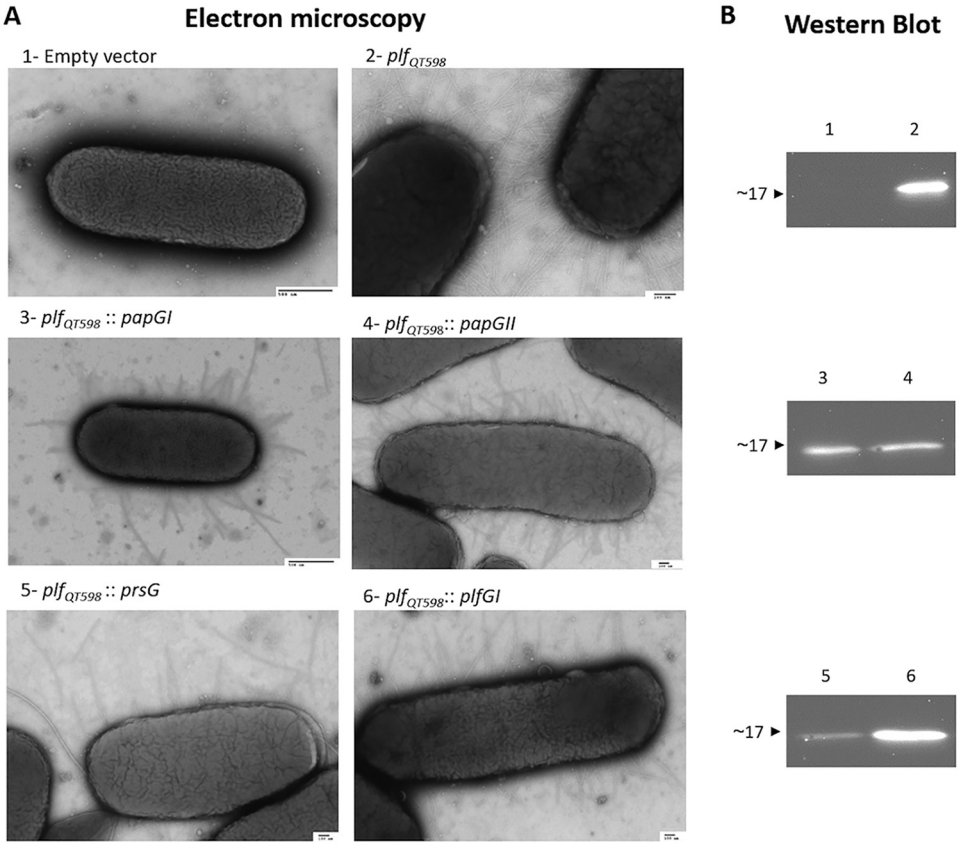

FIG 5 Cloning and expression of PL and Pap chimeric fimbriae. (A) Chimeric clones expressing different classes of PapG/PlfG were visualized by electron microscopy. The Plf class II clone was used as a template to generate chimeric clones. (B) Western blot analysis of heated surface protein extracts from *plf* and chimeric clones. The antibodies used were polyclonal rabbit antibodies raised against a peptide corresponding to the fimbrial major subunit protein PlfA.

demonstrated strong hemagglutinin activities with human, pig, dog, and rabbit erythrocytes. The reference clone encoding P fimbriae containing the Pap class II adhesin from *E. coli* IA2/pDC5 strongly agglutinated pig and human erythrocytes and, to a lesser extent, sheep and chicken erythrocytes. The reference clone encoding Prs fimbriae with the PapG class III (PrsG) adhesin from uropathogenic *E. coli* J96/pJFK102 agglutinated dog, pig, and sheep erythrocytes. The clone encoding PL fimbriae containing a Plf class I adhesin from *E. coli* UMEA-3703-1 agglutinated a broad range of erythrocytes from all species tested, except dog blood, although hemagglutinin titers were higher for human and sheep blood. Interestingly, the clone encoding the Plf class II adhesin from *E. coli* QT598 only strongly agglutinated human and turkey blood (Fig. 6). Taken together, these results indicate that, as with the P fimbrial classes of adhesins, PL fimbriae are hemagglutinins and that the PlfG class I and class II adhesins demonstrate distinct hemagglutination activities compared to the P fimbrial adhesins.

To determine whether lectin-based hemagglutination by PL adhesins was similar to those of Pap and related fimbriae, we tested whether globoseries glycolipids known to be recognized by P fimbriae (Gb3, Gb4, or Gb5) were able to inhibit PL fimbria-mediated hemagglutination. For the hemagglutination tests, we used a macroagglutination assay using human and turkey erythrocytes, since these were both recognized by the PlfG class I and class II fimbriae. In this assay, the PlfG class I and II adhesins strongly agglutinated human and turkey erythrocytes, and addition of either Gb3, Gb4, or Gb5 did not inhibit or reduce this agglutination. For the test controls, none of the PapG adhesins agglutinated turkey erythrocytes, but all strongly agglutinated human erythrocytes. For

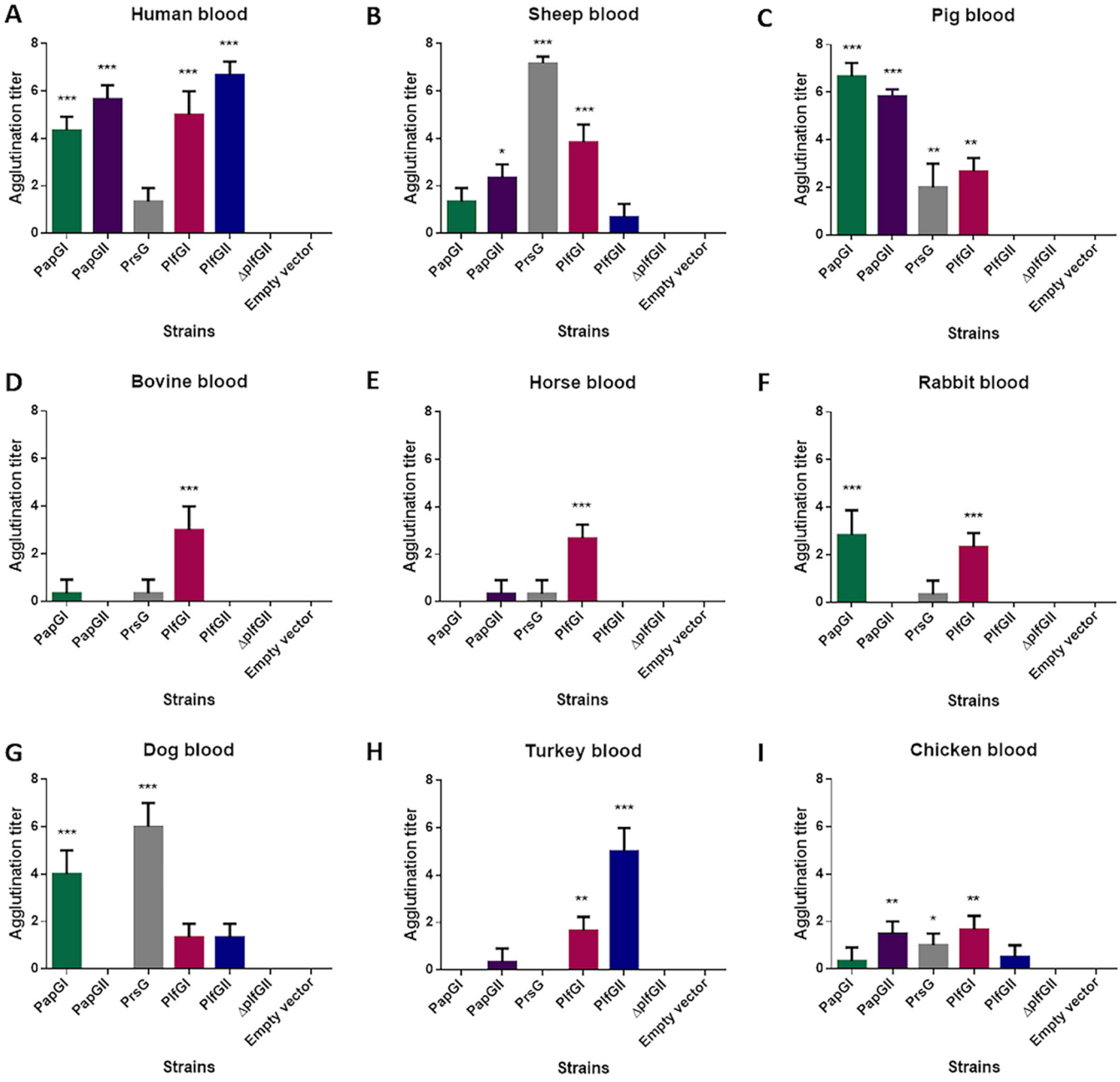

FIG 6 Hemagglutination activity of different clones expressing P or PL fimbriae. Clones were *E. coli* afimbriated *fim*-negative strain ORN172 expressing P or PL fimbriae. Cells were adjusted to an OD_{600} of 0.6 and then cells were centrifuged and concentrated 100-fold and then 2-fold serial dilutions were made in 96-well plates containing a final concentration of 3% erythrocytes from different species. Titers are the average maximal dilution showing agglutination. Erythrocytes tested were: (A) human, (B) sheep, (C) pig, (D) bovine, (E) horse, (F) rabbit, (G) dog, (H) turkey, and (I) chicken. Both human A and O blood types gave similar titers. Reference clones showed different hemagglutination activity. However, the Δ*plfG* clone as well as the empty vector showed no hemagglutination activity of any of the erythrocytes tested. *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$, versus empty vector by one-way ANOVA. The plasmids used were pPap5 (*papGI*), pDC5 (*papGII*), pJFK102 (*prsG*), pIJ523 ($plf_{UMEA\text{-}3703\text{-}1}$::*plfI*), pIJ507 ($plf_{QT598}$::*plfII*), and pIJ598 ($plf_{QT598}$ Δ*plfG*).

PapG class I, Gb3 and Gb4 reduced this agglutination, but not Gb5. For PapG class II, only Gb4 was able to inhibit agglutination. For PapG class III (Prs), Gb4 and Gb5, but not Gb3, were able to inhibit agglutination (Table 1). Taken together, these results indicate that the PL fimbriae belonging to either class I or class II recognize receptors on both human and turkey erythrocytes, but these receptors are distinct from those recognized by P fimbrial adhesin classes.

Different Plf and Pap G adhesin alleles can be expressed by PL fimbriae. Since the PlfG class I and II adhesin sequences from *E. coli* strains QT598 and UMEA-3703-1,

TABLE 1 Effect of globosides on hemagglutination of turkey or human (O) erythrocytes by PL fimbriae or Pap fimbrial adhesins expressed on plasmids in *E. coli* fimbria-negative K-12 strain ORN172

	Hemagglutination result for[a]:					
	Globotriaosylceramide (Gb3)		Globotetraosylceramide (Gb4)		Galactosylgloboside (Gb5)	
Erythrocytes and strain	Present	Absent	Present	Absent	Present	Absent
Turkey erythrocytes						
QT5722 (PapGI P fimbriae)	−	−	−	−	−	−
QT5723 (PapGII P fimbriae)	−	−	−	−	−	−
QT5724 (PapGIII P fimbriae)	−	−	−	−	−	−
QT4741 (PlfGI PL fimbriae)	+++	+++	+++	+++	+++	+++
QT5725 (PlfGII PL fimbriae)	+++	+++	+++	+++	+++	+++
QT5727 (*plfB–F* Δ*plfG*II)[b]	−	−	−	−	−	−
QT5726 (empty vector)	−	−	−	−	−	−
PBS	−	−	−	−	−	−
Human (O) erythrocytes						
QT5722 (PapGI P fimbriae)	+	+++	+/−	+++	+++	+++
QT5723 (PapGII P fimbriae)	+++	+++	−	+++	+++	+++
QT5724 (PapGIII P fimbriae)	+++	+++	+/−	+++	−	+++
QT4741 (PlfGI PL fimbriae)	+++	+++	++	++	+++	+++
QT5725 (PlfGII PL fimbriae)	+++	+++	+++	+++	+++	+++
QT5727 (*plfB–F* Δ*plfGII*)[b]	−	−	−	−	−	−
QT5726 (empty vector)	−	−	−	−	−	−
PBS	−	−	−	−	−	−

[a]Macroagglutination level: +++, strong; ++, moderate; +, weak; +/−, very weak; −, absent. Tests were repeated twice. See Materials and Methods for details of the agglutination assay.
[b]Strain QT5727 contains all plf_{QT598} fimbrial genes, except *plfG*, predicted to encode the adhesin.

respectively, are quite distinct from each other and as the *plf* gene clusters share close genetic organization with *pap* gene clusters, we generated chimeric fimbrial gene clusters encoding different G adhesin alleles. These chimeric clones were based on the plf_{QT598} gene cluster by generating a clone lacking the *plfG* gene (pIJ598) and then cloning the $plfG_{UMEA-3703-1}$ or *papG* alleles from each of the three PapG adhesin classes (Fig. S3A and B). Electron microscopy demonstrated that each of the five chimeric clones introduced to nonfimbriated *E. coli* ORN172 produced fimbriae on the surface of the cells (Fig. 5A). The PlfA subunit protein was also detected from cell surface extracts, as shown in immunoblots, although the level of protein present was decreased when the *papG* recombinant alleles were expressed compared to the $plfG_{QT598}$- and $plfG_{UMEA-3703-1}$-expressing clones (Fig. 5B). We also verified the hemagglutination capacity of hybrid fimbrial systems with some types of blood. The PL hybrid fimbriae with Pap class I or Pap class II adhesin could agglutinate human and pig blood, but were no longer able to agglutinate turkey blood. The PL fimbria hybrid with Pap class III Prs adhesin showed agglutination of sheep and human blood, but was also unable to agglutinate turkey erythrocytes (Table S3). This indicates that replacement of the PlfG allele with a Pap adhesin resulted in production of hybrid fimbriae with an altered binding specificity. It was also possible to replace the PapG adhesin in reference clone pDC1 (31) with the PlfG class II adhesin, resulting in production of fimbriae, as shown by electron microscopy (Fig. S3). However, this hybrid fimbrial system was unable to agglutinate either human or turkey erythrocytes. These results indicate that the PlfG adhesin can complement loss of the PapG adhesin for biogenesis of fimbriae, but suggest that an altered conformation of the adhesin in the hybrid system results in loss of hemagglutination capacity. As such, the Pap and PL fimbrial systems, although they share some characteristics, are not fully interchangeable with regard to adhesin function and specificity.

PL fimbriae mediate adherence to human epithelial cells. The adherence of bacteria to host epithelial cells such as bladder and kidney cells is an important step in colonization of the urinary tract. To investigate whether PL fimbriae can mediate adherence to host

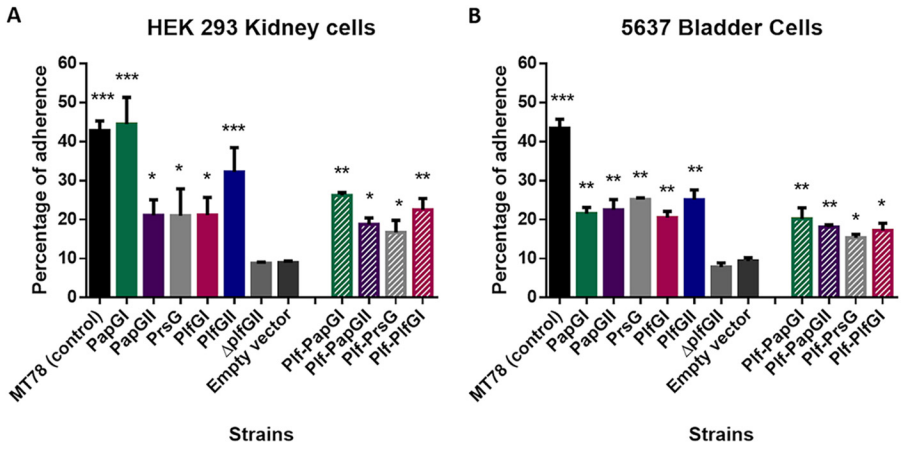

FIG 7 Reference and chimeric clones promote adherence to human kidney (strain HEK-293) and bladder (strain 5637) epithelial cells. Cell monolayers were infected with *E. coli fim*-negative ORN172 expressing P and PL fimbrial proteins at a multiplicity of infection (MOI) of 10 as described by Matter et al. (55) and incubated at 37°C at 5% CO_2 for 2 h. Adherent bacteria were enumerated by plating on LB agar. Empty vector was used as a negative control and APEC MT78 as a positive control for adherence to cell lines. All the clones encoding P or PL fimbrial adhesins (reference and chimeric clones) demonstrated increased adherence to cells of the human bladder 5637 and kidney HEK-293 epithelial cell lines compared to the strain containing the empty vector. The Δ*plfG* clone also did not adhere to human epithelial cells. Data are the averages from three independent experiments. Error bars represent standard errors of the means. *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$, versus empty vector by one-way ANOVA.

cells, we used clones expressing PlfG class I, PlfG class II, or PapG class I, II, or III fimbrial adhesins.

All the clones encoding P or PL fimbrial adhesins (reference and chimeric clones) demonstrated increased adherence to the bladder 5637 and kidney HEK-293 epithelial cell lines compared to the strain containing the empty vector (Fig. 7). The chimeric clones containing the *plf* gene cluster with hybrid *pap* or *plfG* adhesin-encoding genes also promoted adherence to epithelial cells. However, the clone containing a *plf* gene cluster, lacking the *plfG* or *papG* gene showed no appreciable adherence compared to the empty vector-containing clone (Fig. 7). These results demonstrate that PL fimbriae producing distinct types of G adhesins can mediate adherence to urinary tract epithelial cells and suggest that these fimbriae may potentially play a role during host colonization.

PL fimbriae promote biofilm production. Since fimbriae can contribute to biofilm production, we tested for biofilm formation in polystyrene microtiter plates at different temperatures (25, 37, and 42°C) (Fig. 8). The clone expressing PL fimbriae with the PlfG class II adhesin showed a high level of biofilm production at all tested temperatures, even above that of a positive-control biofilm-forming *Serratia liquefaciens* reference strain. The clone expressing PL fimbriae with the PlfG class I adhesin also produced biofilm at 25 and 37°C at moderate levels compared to the clone producing the PlfG class II adhesin. However, biofilm production was very low at 42°C. The Pap class I- and III-producing reference clones were also able to form significantly more biofilm at 25, 37, and 42°C than the negative control. The Pap class II-expressing reference clone produced biofilm at higher temperatures (37 and 42°C), but biofilm levels were reduced at 25°C.

The chimeric clones that expressed different Pap or Plf adhesins fused to the plf_{QT598} gene cluster were all able to produce appreciable levels of biofilm at both 25 and 37°C, although biofilm was much reduced at 42°C. The clone expressing the plf_{QT598} gene cluster lacking a *papG* or *plfG* adhesin-encoding gene (Δ*plfG* clone) as well as clone containing the empty vector were not able to produce biofilm at all the tested temperatures (Fig. 8). Taken together, these results indicate that PL and P fimbriae expressing different types of G adhesins can mediate biofilm production in *E. coli* K-12 and that the PlfG class II adhesin in particular can contribute to strong biofilm formation.

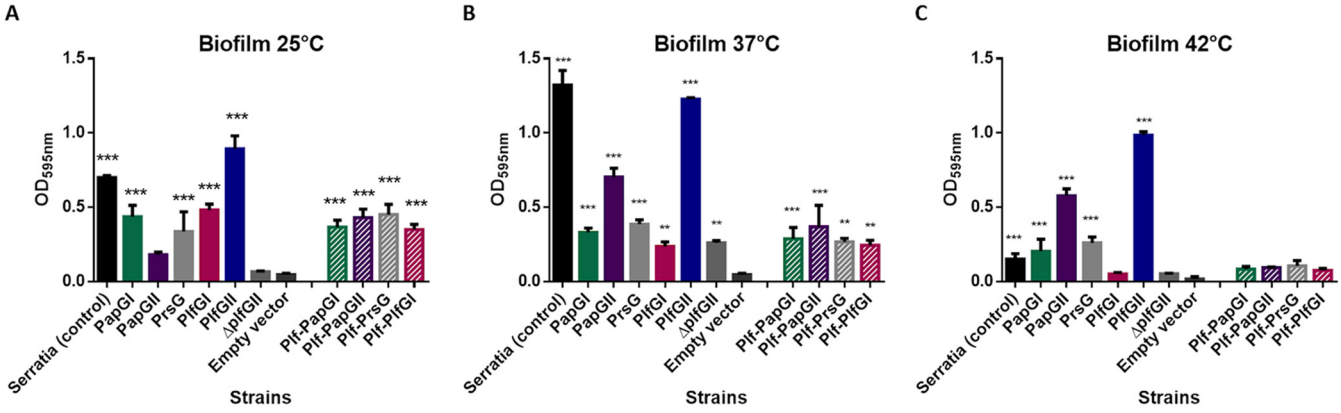

FIG 8 Biofilm production by clones expressing Plf_{QT598} and $Plf_{UMEA-3703-1}$ and reference and chimeric clones at different temperatures. Clones of *E. coli* strain ORN172 expressing P and P-like fimbriae proteins were grown at different temperatures (25, 37, and 42°C) in polystyrene plate wells for 48 h and then stained with crystal violet. The remaining crystal violet after washing with acetone was measured as absorbance at 595 nm. Data are the means from three independent experiments, and error bars represent standard errors of the means. *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$, compared to empty vector using one-way ANOVA. Empty vector was used as a negative control and *S. liquefaciens* strain was used as a positive control for biofilm production.

PL fimbrial genes are upregulated in the bladders of infected mice and contribute to competitive colonization in a mouse urinary track model. To investigate the potential role of the PL fimbriae in virulence in the UTI model, 6-week-old female CBA/J mice were infected with wild-type strains QT598 and UMEA-3703-1 or with mutant Δ*plf* strains lacking the genes encoding PL fimbriae. In the mouse infection model, loss of PL fimbriae did not have a significant effect on colonization of the bladder or kidneys by strain QT598 (Fig. S4). Strain UMEA-3703-1 and its Δ*plf* mutant also showed no significant differences in colonization. However, UMEA-3703-1 was only able to colonize at lower levels (10^2 to 10^3 CFU/g) compared to strain QT598 (10^5 to 10^6 CFU/g) (Fig. S4). Interestingly, the expression level of plf_{QT598} was upregulated by more than 40-fold in the bladder of infected mice compared to expression following growth *in vitro* in LB medium (see Fig. 9 below). This suggests that the expression of these fimbriae is favored by environmental cues during infection in the urinary tract.

We also conducted a competitive infection model using the *plf* mutant and a wild-type *lac*-negative derivative of strain QT598. In this infection model, there was no significant difference in colonization of the mouse bladders (Fig. 10A). In contrast, the bacterial numbers of the *plf* mutant were significantly lower than those of the *plf*-expressing strain ($P = 0.0084$) and showed a median decrease of more than 16-fold

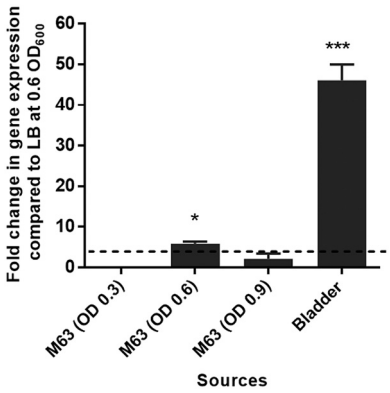

FIG 9 RT-PCR analysis of *plf* expression by strain QT598. QT598 was grown in LB medium to OD_{600} of 0.6 and used as a standard to compare it with growth in M63 minimal medium (with glycerol as carbon) at different growth phases (OD_{600} values of 0.3, 0.6, and 0.9). RNAs were also extracted from infected bladders. Transcription of *plf* was significantly upregulated in the mouse bladder. Error bars indicate standard deviations. *, $P < 0.05$, and ***, $P < 0.001$, by Student's *t* test. The dashed line corresponds to the cutoff for a significant difference in expression.

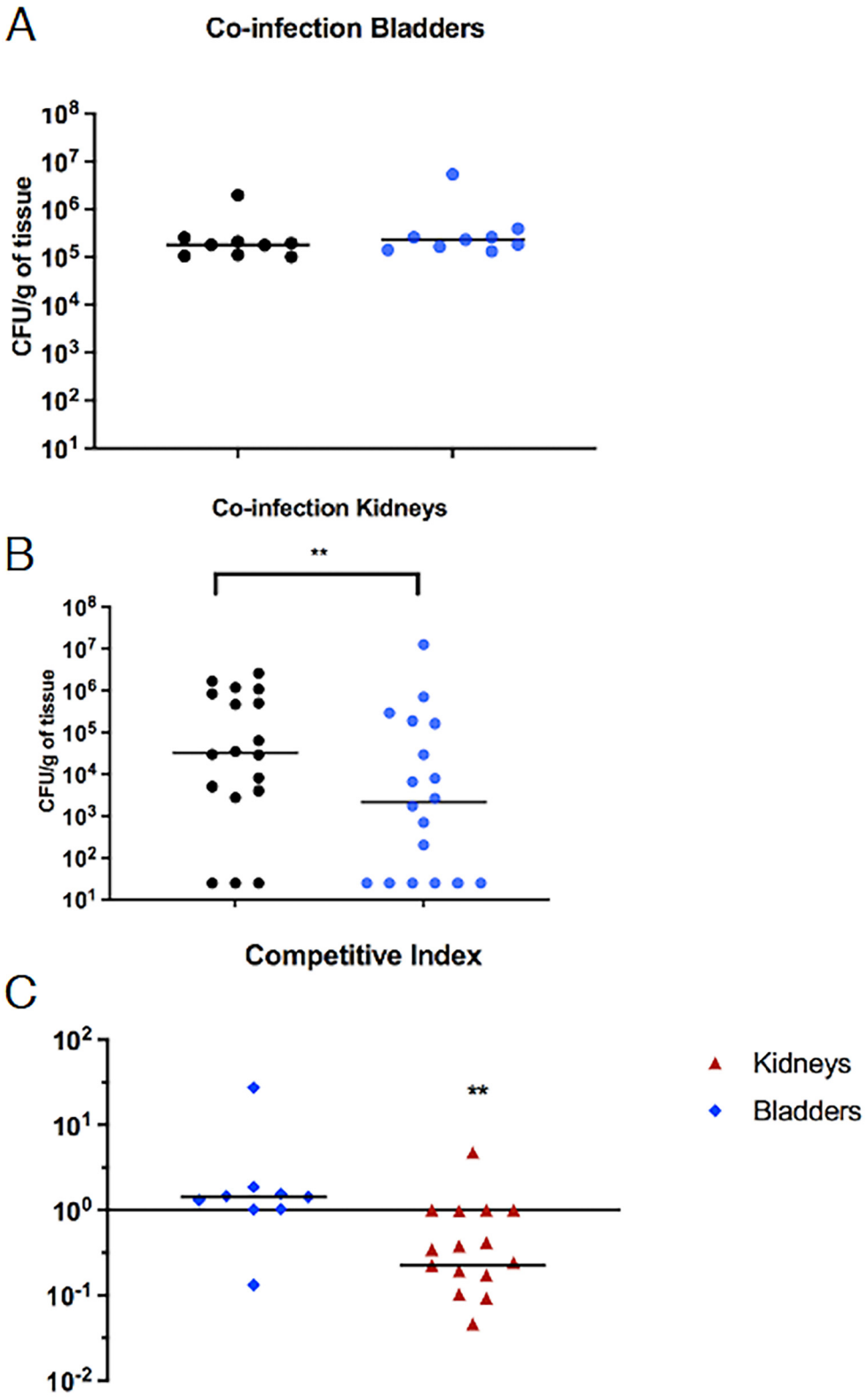

FIG 10 A *plf* mutant shows decreased colonization in mouse kidneys in a coinfection model. An equal number of cells each of the two strains was administered to CBA/J mice. Mice (n = 9) were euthanized after 48 h, and bladder and kidneys were harvested for colony counts. (A) Bacterial numbers in the bladders; (B) bacterial numbers in the kidneys. Mice were infected with the QT598 Δ*lacZYA* strain QT4567 (black circles) and the QT598 Δ*plf* mutant QT4420 (blue circles). The horizontal lines indicate the median numbers of bacterial colonies in tissues. (C) Competitive index comparing the colonization in these organs by the QT598 (*lac*-negative) strain and the QT598 Δ*plf* mutant. The Wilcoxon matched-pairs test was used for statistical analysis. *, $P < 0.05$; **, $P < 0.01$.

(Fig. 10B) and an overall competitive index of −0.65 (Fig. 10C) in the kidneys. Taken together, these results indicate that the *plf* fimbrial gene expression is increased in the mouse urinary tract and that in competitive infection, loss of the PL fimbriae decreases competitive fitness in the mouse kidneys.

DISCUSSION

A novel plasmid-carried fimbrial gene cluster was identified on the large colicin V plasmid of avian pathogenic *E. coli* strain QT598 (serotype O1:K1, sequence type ST1385). Strains from this and related sequence types such as ST91 are commonly associated with extraintestinal infections in poultry and urinary tract infections in humans (27) (http://enterobase.warwick.ac.uk/). The *plf* genes were shown to be adjacent to the RepFIB, and *intM* genes on plasmid pEC598. The is a common integration site for a diversity of genes on F and related plasmids, and collectively this region has been named the cargo gene region (28). The cargo gene region has been found to encode a diversity of accessory genes, insertion sequences, and integrons known to carry genes for resistance to antimicrobials and metals, microcins, and virulence genes (28, 32). It is therefore likely that the *plf* fimbrial gene cluster along with other genes was inherited by certain *E. coli* strains through a recombination/integration event and that it has since disseminated or been transferred into a diversity of *E. coli* isolates associated with different host or environmental sources (highlighted in Tables S1 and S2 in the supplemental material). As with the P fimbriae, PL fimbriae have also diversified considerably, and there has been notable divergence in the PlfG adhesin-encoding sequences into 5 distinct PlfG adhesin classes (Fig. 4). Such changes may have occurred to promote adherence and colonization to a variety of surfaces or host cell receptors in different niches or environments.

The PL fimbriae are new members of the π fimbrial family, which contains P-fimbria-like operons present in some *Betaproteobacteria* and *Gammaproteobacteria* (2). More specifically, based on comparison of the fimbrial usher proteins, the PL fimbriae are part of a subgroup which includes true P fimbriae, as well as closely related Sfp and Pix fimbriae (2) (Fig. 2), all of which have been shown to mediate mannose-resistant hemagglutination (MRHA) of erythrocytes from humans in addition to some distinct MRHA profiles for erythrocytes from other species. Pix fimbriae, which have been identified in some uropathogenic *E. coli* strains, were shown to agglutinate human erythrocytes, but not sheep or goat erythrocytes, and do not recognize the Gal-Gal sugars recognized by P fimbriae (23). Sfp fimbriae also mediate MRHA of human erythrocytes, which was dependent on the *sfpG* gene (26). However, to our knowledge, no tests for MRHA for erythrocytes from other species have been reported. Interestingly, the G adhesin proteins from Pix and Sfp fimbriae share amino acid homology between them (63% identity and 81% similarity), suggesting these G adhesin proteins are more closely related to each other than to PlfG or PapG adhesins, which share no more than 25% amino acid identity. Herein, we demonstrated that PL fimbriae producing the class I adhesin mediated MRHA for erythrocytes from different species, including equine, ovine, bovine, rabbit, and human erythrocytes, whereas PL fimbriae producing the class II adhesin mediated MRHA only to human and turkey erythrocytes (Fig. 6). Taken together, this subgroup of π fimbriae (true P fimbriae, Sfp, Pix, and PL fimbriae) have developed important differences in adhesin protein sequences that have expanded the capacity to adhere to a variety of receptors on erythrocytes and host cells from different species. It will be of interest to more specifically determine the lectin receptor specificity of this family of fimbriae.

The genetic organization of the *plf* gene cluster includes 9 predicted fimbrial subunit genes, which is the number of predicted structural genes encoding P fimbriae (Fig. 3). In contrast, both the Sfp and Pix fimbrial gene clusters comprise 7 structural genes and lack the genes corresponding to the *papK* and *papE* genes predicted to encode an adaptor and a minor fimbrial subunit (Fig. 3). From this standpoint, overall, PL fimbriae are most similar to true P fimbriae.

To further demonstrate potential complementarity between P and PL fimbriae, we also generated hybrid fimbrial gene clusters, wherein the $plfG_{QT598}$ gene was replaced

by PapG adhesin-encoding genes belonging to class I, class II, or class III adhesins. Each of these clones was able to produce a functional fimbrial structure that also increased adherence to human urinary tract epithelial cells. This also further indicates that the PL fimbriae, despite having adhesins that are quite distinct in amino acid sequence from P fimbriae, also produce mannose-resistant hemagglutinins that can mediate adherence to human bladder and kidney cells and that the bioassembly of these fimbriae is compatible with that of P fimbrial G adhesins. It is interesting, however, that the production of the hybrid fimbriae from bacterial cells was substantially reduced compared to that of the PL fimbrial clones, suggesting that efficiency of biogenesis of the hybrid fimbriae is reduced.

As with the *plf* gene cluster, the location of the *sfp* genes is also on IncF plasmids, in close proximity to RepFIB of the pSFO157 plasmid (26). However, it is flanked on both sides by insertion sequences that are distinct from the region adjacent to *plf* genes on pEC598. The Sfp fimbriae were initially found not to be expressed by EHEC strains under normal laboratory conditions, and properties of these fimbriae were first determined using cloned fimbrial genes in *E. coli* K-12 (26). The *sfp* genes encoding a fimbrial system with mannose-resistant hemagglutinin activity have been identified on a subgroup of sorbitol-fermenting EHEC/Shiga-toxigenic *E. coli* (STEC) strains and some EHEC O165:H25/NM strains from humans and cattle, but are absent from most other types of *E. coli* (25, 26). This suggests that the *sfp* genes were likely acquired independently by horizontal transfer to both a nonmotile sorbitol O157 strain and independently to an O165:H25/NM strain and have since remained in these branches of EHEC (25). This is clearly in contrast to the *plf* gene cluster, which is present in a diversity of *E. coli* strains from multiple sources and has likely been transferred either through multiple conjugation and/or recombination events and has also diversified, since distinct G adhesin classes have emerged among strains.

DNA sequence comparisons of gene clusters that are highly similar to the *plf* fimbrial system of *E. coli* QT598 from nucleotide databases provided a means to identify subgroups of PL fimbria genes encoding 5 distinct classes of PlfG adhesins (Fig. 4). Since the PlfG class I- and class II-encoding alleles were predominant among isolates that notably included strains associated with human extraintestinal infections as well as infections from poultry, we focused our attention on functional characterization of one of each of the PL fimbriae belonging to these classes. It was also interesting to identify some variant alleles of the PlfG adhesin in other *E. coli* strains as well as a subgroup that was identified in some strains of *Cronobacter sakazakii* and other *Cronobacter* spp. (Fig. 4). Although *Cronobacter* strains containing the *plf* fimbrial clusters were sampled from spices, *C. sakazaki* and related *Cronobacter* spp. are important foodborne pathogens that can contaminate dehydrated milk and other products and cause serious extraintestinal infections, particularly in neonates (33, 34).

The capacity of PL fimbriae to form biofilms at different temperatures was also investigated, and both the class I and class II PL fimbriae promoted biofilm formation, with PlfG class I producing more biofilm at 25 and 37°C, but not 42°C. In contrast, the PlfG class II adhesin produced very high levels of biofilm at all temperatures tested. The presence of the PlfG adhesin was important for high-level biofilm production for PlfG class II, since the absence of the *plfG* adhesin gene greatly reduced biofilm formation. Notably, after growth at 37°C, the level of biofilm produced by the Δ*plfG* clone was significantly higher than that with the empty vector and comparable to levels of biofilm produced by Pap reference clones and the PlfG class I clone. This suggests that other factors in addition to the G adhesin may also contribute to increased biofilm formation associated with expression of *plf* or *pap* fimbrial genes.

Since both types of PL fimbriae conveyed increased adherence to human epithelial bladder and kidney cells (Fig. 7), we investigated the potential of these fimbriae to contribute to urinary tract colonization in a murine model. Deletion of the *plf* genes from either *E. coli* strain QT598 or strain UMEA-3703-1 did not have an appreciable effect on colonization of either the bladder or the kidneys. Furthermore, despite being isolated

from a human UTI, strain UMEA-3703-1 was not a strong colonizer in the mouse UTI model.

The mouse UTI model may not be as representative of a human infection when using certain bacterial strain backgrounds or when investigating specific mechanisms of virulence such as adherence and fimbrial adhesins. P fimbriae have been shown to play a role in urinary infection, particularly for pyelonephritis in cynomolgus monkeys (35), and these fimbriae alone can confer an asymptomatic *E. coli* urinary strain the capacity to elicit strong regulatory modulation in humans by acting as an IRF-7 agonist and reprogramming the immune response in the urinary tract (5). In the case of the murine model, it has been demonstrated that P fimbriae can reduce the immune response in the kidney by decreased production of polymeric Ig receptor and reduced secretion of IgA (36). However, the role of P fimbriae in bacterial colonization in the UTI mouse model has been less evident. Initially, *pap* genes cloned into an avirulent *E. coli* K-12 strain or an intestinal commensal *E. coli* strain were shown to increase colonization of the mouse kidney (37, 38). In contrast, deletion of *pap* from different UPEC strains did not alter colonization of the urinary tract in CBA/J mice (39). Reasons why PL fimbriae as much as P fimbriae may not play as critical role in the mouse UTI model may be due to differences in lectin receptor target specificity present on murine cells and/or the potential redundancy of adherence mechanisms due to production of multiple fimbrial adhesins in UPEC strains. Since a slight increase in colonization of the plf mutant of strain UMEA-3703-1 in the bladder was observed (Fig. S4), it is also possible that loss of the *plf* genes may alter expression of other types of fimbriae, since regulatory cross talk can occur and loss of one type of fimbriae can enhance expression of other types of fimbriae. The expression of *plf* was also upregulated more than 40-fold by strain QT598 in the bladder and was increased by 5-fold in minimal medium compared to rich medium (Fig. 9). This indicates that the expression of these fimbriae can be increased by cues during infection, which may include host factors or decreased nutrient availability. In the current study, we used an acute infection model with an endpoint of 48 h, and our detection of increased expression of *plf* in the urinary tract was only determined at this time point. As such, these fimbriae may potentially play a more important role in more prolonged infections, and it may be of interest to investigate colonization at later time points using other models, such as catheter-associated infections.

Since the competitive coinfection model demonstrated a potential fitness advantage in the mouse kidneys, this nevertheless supports a potential advantage for production of these fimbriae by strain QT598 during UTI in the mouse model (Fig. 10). The prevalence of *plf* genes was associated with strains from human infections, as well as canine infections and from samples in poultry, including turkeys and chickens. In future studies, it will be of interest to determine whether PL fimbriae or specific PlfG adhesin classes may contribute to infection in other animal models such as poultry and to further investigate PL fimbria receptor specificity, potential role in modulation of host immune response and the regulation of production of this newly identified group of fimbriae.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 2. ExPEC strain QT598 (a passaged derivative of strain MT156 [40]) is an O1:K1 sequence type ST1385 strain originally isolated from a turkey suffering from colibacillosis in France (27). UPEC strain CFT073 was isolated from the blood and urine of a woman suffering from urinary tract infection (41), and UMEA-3703-1 (NCBI Biosample no. SAMN01885978) was isolated from the urine of a human with bacteremia. *E. coli* K-12 laboratory strains DH5α and ORN172 (type 1 fimbria *fim*-negative strain) were used for cloning and protein expression. Reference clones expressing fimbria genes encoding different PapG adhesin classes were used as controls, including plasmids pPap5 ($papG_{J96}$ class I) (42, 43), pDC5 ($papG_{IA2}$ class II) (31), and pJFK102 ($prsG_{J96}$ class III) (44, 45).

Bacteria were routinely grown at 37°C on solid or liquid Luria-Bertani (LB) medium (Alpha Bioscience, Baltimore, MD). When required, antibiotics were added to a final concentration of 100 μg/mL of ampicillin, 30 μg/mL of chloramphenicol, or 50 μg/mL of kanamycin.

Bioinformatics analysis. Identification and comparison of sequences in the databases were achieved by accessing data on completed genomes and BioProjects publicly available in the NCBI database (www.ncbi.nlm.nih.gov). Analyses included BLAST against both nucleotide and protein entries. Figures presenting the organization and comparison of genes and gene clusters were generated from the nucleotide

TABLE 2 Plasmids and strains used in this study

Plasmid, strain, or clone	Characteristic(s)	Source or reference(s)
Plasmids		
pKD4	Plasmid used for amplification of *kan* cassette	50
pKD13	Plasmid used for amplification of *kan* cassette	50
pKD46	λ Red plasmid; Amp^r	50
pUCmT	Cloning vector; Amp^r	Bio Basic, Inc., Markham, ON, Canada
pBC sk+	Cloning vector; Cm^r	Stratagene, La Jolla, CA
pPap5	P fimbriae expressing PapGI from J96	42, 43
pDC5	P fimbriae expressing PapGII from IA2	31
pJFK102	P fimbriae expressing PrsG (PapGIII) from J96	44, 45
pIJ507	pUCmT::*plf*$_{QT598}$, PL fimbriae PlfGII	This study
pIJ523	pBC sk+::*plf*$_{UMEA-3703-01}$, PL fimbriae PlfGI	This study
pIJ594	pUCmT::*plf*$_{QT598}$::*papGI*, PL chimera *papGI*	This study
pIJ595	pUCmT::*plf*$_{QT598}$::*papGII*, PL chimera *papGII*	This study
pIJ596	pUCmT::*plf*$_{QT598}$::*prsG*, PL chimera *papGIII*	This study
pIJ597	pUCmT::*plf*$_{QT598}$::*plfGI*, PL$_{QT598}$ hybrid PlfGI	This study
pIJ598	pUCmT::*plf*$_{QT598}$ Δ*plfGII*, clone lacks *plfG* gene	This study
Strains		
ORN172	*fim*-negative strain; *thr-1 leuB thi-1*Δ*(argF-lac)U169 xyl-7 ara-13 mtl-2 gal-6 rpsL tonA2 supE44*Δ(*fimBEACDFGH*)::Km *pilG1*	60
MT78	APEC O2:H$^+$:K1, ST95	61
QT2799	*Serratia liquefaciens*	ATCC 27592
QT598	APEC O1:K1, ST1385	27, 40
QT4420	QT598 Δ*plf* Km^r	This study
UMEA-3703-1	UPEC strain from urine of patient with bacteremia	NCBI Biosample no. SAMN01885978
QT4598	UMEA-3703-1 Δ*plf* Km^r	This study
Clones		
QT5722	ORN172/pPap5 (reference clone expressing P fimbriae with PapGI adhesin)	This study
QT5723	ORN172/pDC5 (reference clone expressing P fimbriae with PapGII adhesin)	This study
QT5724	ORN172/pJFK102 (reference clone expressing P fimbriae with PrsG (PapGIII adhesin)	This study
QT4741	ORN172/pIJ523 (reference clone expressing PL fimbriae with PlfGI adhesin)	This study
QT5726	ORN172/pIJ507 (reference clone PL fimbriae with PlfGII adhesin)	This study
QT5727	ORN172/pIJ598 (reference clone expressing *plf*$_{QT598}$ Δ*plfGII*)	This study
QT5732	ORN172/pUCmT empty vector	This study
QT5728	ORN172/pIJ594 (chimeric clone expressing *papGI*)	This study
QT5729	ORN172/pIJ595 (chimeric clone expressing *papGII*)	This study
QT5730	ORN172/pIJ596 (chimeric clone expressing *prsG*)	This study
QT5731	ORN172/pIJ597 (chimeric clone expressing *plfGI*)	This study

accession numbers and entries using SnapGene (version 5.2.1) (www.snapgene.com). For comparison of the protein sequences, entries were obtained from either the NCBI or the Universal Protein Resource (UniProt) (www.uniprot.org) website. Phylogenetic analyses of protein sequences were done with the platform at Phylogeny.fr (http://www.phylogeny.fr) using the default ("one click") parameters (46). Analyses consisted of multiple-sequence alignment with MUSCLE (47), alignment curation with GBlocks (48), maximum likelihood phylogeny analysis using PhyML 3.0 (49), and TreeDyn for generation and editing of trees (www.treedyn.org). Specific parameters are described at the Phylogeny.fr web site platform.

Construction of plasmids. Cloning of the *plf* gene clusters and *plfG* and *papG* genes encoding the different classes of adhesins was performed by PCR amplification using specific primers (Table 3) and Q5 high-fidelity DNA polymerase (New England Biolabs [NEB]). The A-Tailing kit (NEB) was then used to add additional deoxyadenosine (A) to the 3′ end of the PCR products. The insert possessing the additional A at the 3′ end was ligated to the linearized vector with additional deoxythymidine (T) residues using T4 DNA ligase (NEB). The *plf* gene cluster from strain QT598 (*plf*$_{QT598}$) was amplified using primers CMD1847_F and CMD1900_R and cloned into vector pUCm-T (Bio Basic, Markham, ON, Canada), generating plasmid pIJ507. This plasmid contains the full *plf* gene cluster with a PlfG class II adhesin. The *plf* gene cluster from strain UMEA-3703-1 (*plf*$_{UMEA}$) was amplified using primers CMD2119_F and CMD2120_R and cloned into vector pBC sk+, generating plasmid pIJ523. This plasmid contains the full *plf*$_{UMEA}$ gene cluster with a PlfG class I adhesin. To generate chimeric gene clusters comprised of *plf*$_{QT598}$ with different types of G adhesin-encoding genes, pIJ507 was used as a template. *plfG*$_{QT598}$ was deleted using an inverse-PCR method with primers CMD2168_F and CMD2169_R, which introduced PmeI sites and amplified a linear fragment lacking the *plfG*$_{QT598}$ gene. The linearized product was then treated with DpnI endonuclease (NEB) to cleave any methylated template sequence. The linear fragment was either ligated using T4 DNA ligase (NEB) to generate pIJ598, which contains *plfBAHCDJKEF* (*plf*$_{QT598}$ Δ*plfG*), or

TABLE 3 Primers used in this study

Primers	Characteristic(s)[a]	Sequence
CMD1847	Plf_{QT598}_cloning_F	AGCTTAGCGGCCGCATCCGCACAAACGGTCTTAC
CMD1900	Plf_{QT598}_cloning/screening_R	ATGAACGGGCCCACCCGACATGAACATTCTCC
CMD2119	$Plf_{UMEA-3703-01}$_cloning_F	TCCCCCGGGCTGCAGGAATTCGAGGGAGGGCGTGAATTCTG
CMD2120	$Plf_{UMEA-3703-1}$_cloning_R	GGCGAATTGGGTACCGGGCCCTCTGCAGATGTCACCG
CMD2171	$PapGI_{J96}$_cloning_F	CGATGATGTAAGGTTTATGAAAAAATGGTTCCCTGCTT
CMD2172	$PapGI_{J96}$_cloning_R	CATAATAAAAATGTTTTCAGGGGAAACTCAGAACCA
CMD2174	$PapGII_{CFT073}$_cloning_F	CGATGATGTAAGGTTTATGAAAAAATGGTTCCCAGCTTTG
CMD2175	$PapGII_{CFT073}$_cloning_R	CATAATAAAAATGTTTTTATGGCAATATCATGAGCAGC
CMD2177	$PrsG_{J96}$_cloning_F	CGATGATGTAAGGTTTATGAAAAAATGGCTCCCTGC
CMD2178	$PrsG_{J96}$_cloning_R	CATAATAAAAATGTTTTTATGGCAATATCATGAGCAGCG
CMD2180	$PlfGI_{UMEA-3703-1}$_cloning_F	CGATGATGTAAGGTTTATGAAAAGAGTTATCCTTTTGCTATTG
CMD2181	$PlfGI_{UMEA-3703-1}$_cloning_R	CATAATAAAAATGTTTTCAATTAATATCAACCTTTAAAACAGCGC
CMD2168	Delete $PlfGII_{QT598}$_KO_F	ACGCTAACTCACGTTTAAACATTTTTATTATGATGTTAAAATATTTGTGTCGCCTTTTG
CMD2169	Delete $PlfGII_{QT598}$_KO_R	AAACGTGAGTTAGCGTTTAAACCTTACATCATCGGATCATAAAAAAACGCACGCGTGAC
CMD2112	$Plf_{QT598/UMEA-3703-1}$_KO_F	AGTAATAACTGACAGGATATTTTAACTATAATCAGGAGGTTATTTCCATGGTGTAGGCTGGAGCTGCTTC
CMD2113	Plf_{QT598}_KO_R	CCCGACATGAACATTCTCCAGACTATATTACAGGAGAATTCACCAGTTCCATGGGAATTAGCCATGGTCC
CMD2114	$Plf_{UMEA-3703-1}$_KO_R	TGCTACGTGCCATCTCATGCTCTTTACTTTCTCTCTTGGTTAATATCAAAATGGGAATTAGCCATGGTCC
CMD1849	Plf_{QT598}_screening_F	AGATGGGATCCACAAACACAAGGTCGCTCAGGGC
CMD2115	$Plf_{UMEA-3703-1}$_screening_F	ATGTCCGTTGAGCACTTTCG
CMD2116	$Plf_{UMEA-3703-1}$_screening_R	TGAAATCACCCCTATGCACA
CMD2186	qPCR PlfA_F	CGGATCAGGGACAAGGTAAAG
CMD2187	qPCR PlfA_R	CAGCCAGATGAGCTTTGG

[a]F, forward; R, reverse; KO, knockout.

used as a template to generate chimeric fimbrial gene clusters containing different G adhesin genes using the T4 DNA ligase (NEB). PCR fragments containing G adhesin genes were obtained using primer pairs CMD2171_F and CMD2172_R (for *papG* class I from strain J96), CMD2174_F and CMD2175_R (for *papG* class II from strain CFT073), CMD2177_F and CMD2178_R (for *prsG* [*papG* class III] from strain J96), and CMD2180_F and CMD2181_R (for $plfG_{UMEA-3703-1}$::*plfGI* from strain UMEA-3703-1). Cloning experiments to generate recombinant plasmids or subclones were first achieved using *E. coli* strain DH5α. The plasmids were extracted using a Miniprep kit according to the manufacturer's recommendations (Bio Basic, Inc.) and then transformed into *E. coli fim*-negative strain ORN172 for phenotypic testing. Strains containing the following reference plasmids that contain full P fimbrial gene clusters were used as reference controls: pPap5 (encoding P fimbriae PapG class I from *E. coli* J96) (42, 43), pDC5 (encoding P fimbria PapG class II from strain IA2) (31), and pJFK102 (encoding Prs fimbria PrsG [PapG class III] from *E. coli* J96) (44, 45).

Deletion of the *plf* genes from strains QT598 and UMEA-3703-1. A *plf* knockout mutant of APEC strain QT598 was obtained by the lambda red recombinase method (50). First, plasmid pKD46 expressing lambda red recombinase was transformed into QT598 by electroporation, and then the kanamycin resistance cassette was amplified from plasmid pKD4 by PCR with primers CMD2112_F and CMD2113_R and transformed into QT598 carrying plasmid pKD46 by electroporation. Mutants were selected at 37°C, and then the loss of genes was confirmed by PCR using screening primers CMD1849_F and CMD1900_R to obtain the QT598 Δ*plf* strain (QT4420). The same method was used to create the Δ*plf* deletion mutation in UMEA-3703-1 using primers CMD2112_F and CMD2114_R to generate strain QT4598 (UMEA-3703-1 Δ*plf*). The deletion was confirmed using primers CMD2115_F and CMD2116_R.

Extraction of fimbriae and Western blotting. Fimbriae were extracted using the heat extraction method as described previously by (51), with some modifications. Briefly, overnight cultures were incubated at 56°C for 1 h and harvested by centrifugation at 4,000 rpm for 15 min. Supernatants were incubated in 10% trichloroacetic acid (TCA) to precipitate proteins. Proteins were then concentrated by centrifugation at 12,000 rpm for 20 min, washed twice with Tris-EDTA (0.05 M) at pH 12 and 8.5, and resuspended in 0.1 mL of Tris-EDTA (0.05 M) at pH 8.5. Western blotting was performed as previously described (52), with some modifications. Proteins were separated using 15% polyacrylamide gel, transferred onto a nitrocellulose membrane (Bio-Rad Laboratories, CA, USA), and blocked with 15 mL of blocking buffer TBST (0.15 M NaCl, 0.025 M Tris, 0,05% Tween, 3% skim milk) for 1 h at 4°C. Fimbrial major subunit protein was detected using rabbit polyclonal antibodies provided by New England Peptide (1:1,000) against a peptide corresponding to part of the PlfA major fimbrial subunit (Ac-CAHLAADGISVKKD-amide) for 45 min at room temperature. The membrane was then washed 3 times with wash buffer (0.15 M NaCl, 0.025 M Tris, 0,05% Tween) and incubated with an anti-rabbit-conjugated secondary antibody (1:20,000) for 45 min at room temperature. After four washes with TBST, proteins were detected using SuperSignal West Pico chemiluminescent substrate (Pierce) according to the manufacturer's instructions.

Transmission electron microscopy. Bacteria for electron microscopy were grown overnight at 37°C. Cultures were then adsorbed onto a glow-discharged Formvar-coated copper grid for 1 min and stained with 1% phosphotungstic acid. The excess of liquid was removed with a filter paper. Samples were then dried and observed under a Hitachi H700 transmission electron microscope.

Hemagglutination assays. Hemagglutination was performed in 96-well round-bottom plates as described in reference 53. Briefly, different types of blood were tested for this assay, human (A and O), horse, bovine, sheep, pig, rabbit, chicken, turkey, and dog red blood cells (RBCs) were suspended in phosphate-buffered saline (PBS) at a final concentration of 3% and added to 96-well plates. Clones expressing different classes of PapG were grown in LB broth at 37°C and centrifuged at 3,000 × *g* for 15 min, and pellets were suspended in PBS (pH 7.4) and adjusted to an optical density at 600 nm (OD_{600}) of 0.6, centrifuged, and then concentrated 100-fold in PBS (pH 7.4). The agglutinating titer was determined as the most diluted well with agglutination after 30 min of incubation on ice.

To investigate potential inhibition of hemagglutination by carbohydrates known to be recognized by P fimbriae, a macroagglutination assay was performed. Overnight bacterial cultures were centrifuged and concentrated 10× in PBS. Thirty microliters of 6% human O blood group or turkey erythrocyte suspensions were deposited into each well of a 10-well glass plate. Two microliters of the different inhibitors was added to the erythrocytes. Finally, 30 μL of the different control and test strains was mixed using toothpicks. Samples were left on ice for 30 min followed by gentle agitation, then hemagglutination levels were noted. The carbohydrates used for inhibition tests were ceramide trihexosides (CTH) (globotriaosylceramides [Gb3]) and globotetrahexosylceramide-globosides (Gb4) obtained from Matreya (State College, PA, USA), and Forssman antigen pentaose (Gb5) from Biosynth Carbosynth (San Diego, CA, USA). Stock solutions of the carbohydrates were prepared in dimethyl sulfoxide (DMSO). Final concentrations used for hemagglutination assays were as follows: Gb3, 32 μg/mL or 1.8 nmol per well; Gb4, 161 mg/mL or 7.5 nmol per well; and Gb5, 161 μg/mL or 11 nmol per well. Samples were tested in duplicate and gave similar results.

Biofilm assays. Biofilm formation in 96-well microtiter plates was examined as previously described (54). Fimbrial clones were grown statically in LB at 25, 30, 37, and 42°C for 48 h. After 48 h of incubation, the liquid was discarded, and plates were washed and stained with 0.1% crystal violet (Sigma) for 15 min. Ethanol-acetone solution (80:20) was used to dissolve biofilm, and the optical density was measured at 595 nm to determine the production of biofilm.

Bacterial adherence to epithelial cell lines. Cells of the human bladder 5637 (ATCC HTB-9) and kidney HEK-293 (ATCC CRL-1573) epithelial cell lines were grown to confluence in 24-well plates in RPMI 1640 or Eagle's minimal essential medium (EMEM) (Wisent Bio Products, St-Bruno, Canada) supplemented with 10% fetal bovine serum (FBS) at 37°C in 5% CO_2. Fimbrial clones expressing different classes of PapG or PlfG adhesins were grown in LB medium at 37°C, cultures were then centrifuged, resuspended in RPMI 1640 or EMEM with 10% FBS, and added to cells at a multiplicity of infection (MOI) of 10 for 2 h, as described previously (55). After 2 h, cells were washed three times with PBS, lysed with 1% Triton X-100, diluted, and plated onto LB agar plates supplemented with selective antibiotics.

Murine urinary tract infection models. To determine the potential role of PL fimbriae in virulence, wild-type strains QT598 and UMEA-3703-1 as well as the QT598 Δ*plf* (QT4420) and UMEA-3703-1 Δ*plf* (QT4598) mutants were tested in 6-week-old CBA/J female mice using an ascending UTI model adapted from reference 38. A total of 5 mice in each group were infected with 10^9 CFU/mL of bacteria. After 48 h, the infected mice were euthanized, and bladders and kidneys were harvested aseptically for the bacterial count on MacConkey's agar plates. To study the expression of *plf in vivo*, bladder samples after necropsy were homogenized with TRIzol LS reagent (Thermo Fisher Scientific) for RNA extractions.

A competitive infection model was also used as described in reference 27. Briefly, a murine ascending UTI model with 9 mice in each group was used for coinfection, in which a virulent Δ*lacZYA* derivative of QT598 (QT4567) was coinfected with the Δ*plf* strain (QT4420). Twenty-five microliters (10^9 CFU) of a mixed culture containing equal amounts of each strain were inoculated through a catheter in 6-week-old CBA/J female mice. Mice were euthanized after 48 h, and bladders and kidneys were harvested aseptically, homogenized, diluted, and plated on MacConkey agar plates.

qRT-PCR to measure PL fimbrial gene expression levels. We compared the expression of PL fimbriae by comparing RNA levels of the *plfA* gene under different conditions: LB medium, minimal M63 medium, and during infection in bladders of mice. For *in vitro* analysis, total RNAs from bacterial samples were extracted according to the manufacturer's protocol EZ-10 spin column total RNA Miniprep kit (BioBasic). For *in vivo* analysis, bladder samples were homogenized with TRIzol LS reagent (Thermo Fisher Scientific), incubated with chloroform followed by centrifugation and incubation in ethanol (95 to 100%) to separate the aqueous phase that contains RNA. Then, RNA samples were extracted using a Direct-zol RNA Miniprep kit (Zymo Research, Irvine, CA, USA) according to the manufacturer's recommendations. All RNA samples were treated with Turbo DNase (Ambion), to eliminate any DNA contamination. The Iscript reverse transcription supermix (Bio-Rad Life Science, Mississauga, ON, Canada) was used to synthesize cDNAs from samples according to the manufacturer's protocol. Primers were specific to the *plfA* gene and the RNA polymerase sigma factor *rpoD* (housekeeping control). Quantitative reverse transcription-PCR (qRT-PCR) was performed in the Corbett Rotorgene (Thermo Fisher) instrument using 50 ng of cDNA, 100 nM each primer, and 10 μL of SsoFast Evagreen supermix (Bio-Rad). Data were analyzed using the threshold cycle ($2^{-\Delta\Delta CT}$) method (56).

Statistical analyses. All data were analyzed with the Graph Pad Prism 6 software (GraphPad Software, San Diego, CA, USA). A Mann-Whitney test was used for mouse infection experiments to determine statistical significance. Analysis of variance (ANOVA) was used to compare the means of samples. Differences between groups were considered significant for P values of <0.05.

Ethics. Protocols for mouse urinary tract infection were approved by the animal ethics evaluation committee (Comité Institutionel de Protection des Animaux; CIPA no. 1608-02) of the INRS-Centre Armand-Frappier Santé Biotechnologie.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, XLSX file, 0.1 MB.

SUPPLEMENTAL FILE 2, XLSX file, 0.3 MB.

SUPPLEMENTAL FILE 3, PDF file, 1.3 MB.

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