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The type 1 pili regulator gene *fimX* and pathogenicity island PAI-X as molecular markers of uropathogenic *Escherichia coli*

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Uropathogenic *Escherichia coli* (UPEC) fall within a larger group of isolates producing extraintestinal disease. UPEC express type 1 pili as a critical virulence determinant mediating adherence to and invasion into urinary tract tissues. Type 1 pili expression is under regulation by a family of site-specific recombinases, including FimX, which is encoded from a genomic island called PAI-X for pathogenicity island of FimX. Using a new multiplex PCR, *fimX* and the additional PAI-X genes were found to be highly associated with UPEC (144/173=83.2 %), and more prevalent in UPEC of lower urinary tract origin (105/120=87.5 %) than upper urinary tract origin (39/53=74 %; $P<0.05$) or commensal isolates (28/78=36 %; $P\leqslant 0.0001$). The Fim-like recombinase gene *fimX* is the only family member that has a significant association with UPEC compared to commensal isolates. Our results indicate PAI-X genes, including the type 1 pili regulator gene *fimX*, are highly prevalent among UPEC isolates and have a strong positive correlation with genomic virulence factors, suggesting a potential role for PAI-X in the extraintestinal pathogenic *E. coli* lifestyle.

INTRODUCTION

Escherichia coli is a typical constituent of the enteric tract in many animals, including humans. Pathogenic *E. coli* strains produce a wide variety of intestinal and extra-intestinal diseases, such as diarrhoea, urinary tract infections, septicaemia and meningitis (Orskov & Orskov, 1992). Urinary tract infection (UTI) is a leading infection of children, women and the elderly (Foxman, 2010; Foxman *et al.*, 2000), and uropathogenic *E. coli* (UPEC) is responsible for over 80 % of the approximately 10 million annual cases of community-acquired UTIs in the USA (Russo & Johnson, 2003). While the majority of UTIs involve bladder infection (cystitis), UPEC may also produce ascending infections to the kidneys, causing pyelonephritis or more advanced diseases such as urosepsis and meningitis among certain patient groups (Laupland *et al.*, 2002; Rushton, 1997).

Molecular epidemiology to assign different unique genetic traits by strain group may be powerful. Defining distinguishing genetic features of commensal strains and pathogenic strains associated with different disease outcomes provides an opportunity to use this information for hypothesis generation, namely the identification of potential virulence loci, as well as for potential diagnostics. While specific pathotypes of *E. coli* are difficult to define given a high degree of genomic heterogeneity, certain genetic features can distinguish pathogenic strains from commensal strains (Groisman & Ochman, 1994). Phylogenetic analyses have shown that *E. coli* strains fall into four main phylogenetic groups (A, B1, B2 and D) (Herzer *et al.*, 1990; Lecointre *et al.*, 1998). Virulent extraintestinal pathogenic *E. coli* (ExPEC) belong mainly to group B2 and, to a lesser extent, to group D and encompass the UPEC strains as

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Abbreviations: ASB, asymptomatic bacteriuria; EHEC enterohaemorrhagic *E. coli*; ExPEC, extraintestinal pathogenic *E. coli*; UPEC, uropathogenic *E. coli*; UTI, urinary tract infection; VF, virulence factor.

One supplementary table is available with the online version of this paper.

well (Boyd & Hartl, 1998; Picard *et al.*, 1999). A large proportion of faecal-commensal, but a minority of UPEC strains, belong to group A (Bailey *et al.*, 2010). However, even among the B2 and D groups, UPEC are genetically heterogeneous with an assortment of factors either directly demonstrated or epidemiologically linked to virulence, including adhesive factors like pili (P pili, type 1 pili and S pili), toxins (HlyA and CNF1), and invasion-promoting factors (Hek and IbeA) (Groisman & Ochman, 1994). Many of these factors are clustered together on horizontally acquired genomic islands termed pathogenicity-associated islands (Hacker, 1990).

Animal and human studies suggest that the type 1 pilus is a critical virulence determinant in the pathogenesis of UPEC cystitis, and bacteria lacking type 1 pili are attenuated, secondary to reduced adherence and invasion into superficial bladder epithelial cells (Connell *et al.*, 1996; Mulvey *et al.*, 1998; Wright *et al.*, 2007). Corroborating the molecular pathogenesis studies of type 1 pili in UTI, vaccination with pili subcomponents in animal studies has been shown to reduce the severity and duration of infection (Palaszynski *et al.*, 1998). Type 1 pili are encoded by the *fim* operon (*fimAICDFGH*) and their expression is controlled at multiple regulatory levels (Corcoran & Dorman, 2009; Kelly *et al.*, 2006; McClain *et al.*, 1991). The orientation of the invertible type 1 pili promoter region (*fimS*) (Abraham *et al.*, 1985) is determined by the action of multiple tyrosine recombinases. UPEC strains are known to possess up to five Fim-like tyrosine recombinases (FimB, FimE, FimX, IpuA and IpuB) based on sequence homology, and all but IpuB are have been shown to invert *fimS* (Bryan *et al.*, 2006; Gally *et al.*, 1996; Hannan *et al.*, 2008; Klemm, 1986; McClain *et al.*, 1991; Xie *et al.*, 2006) with differing biochemical activities: FimE inverts the promoter from ON to OFF, FimB catalyses bidirectional inversion, but favours OFF to ON, IpuA also has bidirectional activity, while FimX is able to invert *fimS* from OFF to ON.

While *fimB* and *fimE* are closely linked to the type 1 pili operon, *fimX* is located at an unlinked genetic locus we have termed PAI-X (Fig. 1a) for pathogenicity island of FimX. Among a small collection of *E. coli* isolates, we previously demonstrated that *fimX* was highly associated with UPEC isolates (Hannan *et al.*, 2008). More recently, we showed that the recombinase FimX exclusively regulates the predicted LuxR-like response regulator HyxR that is encoded from the same pathogenicity island and is a negative regulator of the nitrosative stress response and intracellular macrophage survival (Bateman & Seed, 2012). Therefore, a goal of this study was to determine the prevalence of the type 1 pili recombinase regulator genes and PAI-X genes present in an extended, diverse collection of pathogenic and commensal *E. coli* isolates. Furthermore, by establishing the prevalence of the additional PAI-X genes among *E. coli*, we aimed to determine if PAI-X is associated with UPEC isolates from a particular UTI syndrome, indicating a possible role for the factors encoded by these genes in the UPEC pathogenic lifestyle.

METHODS

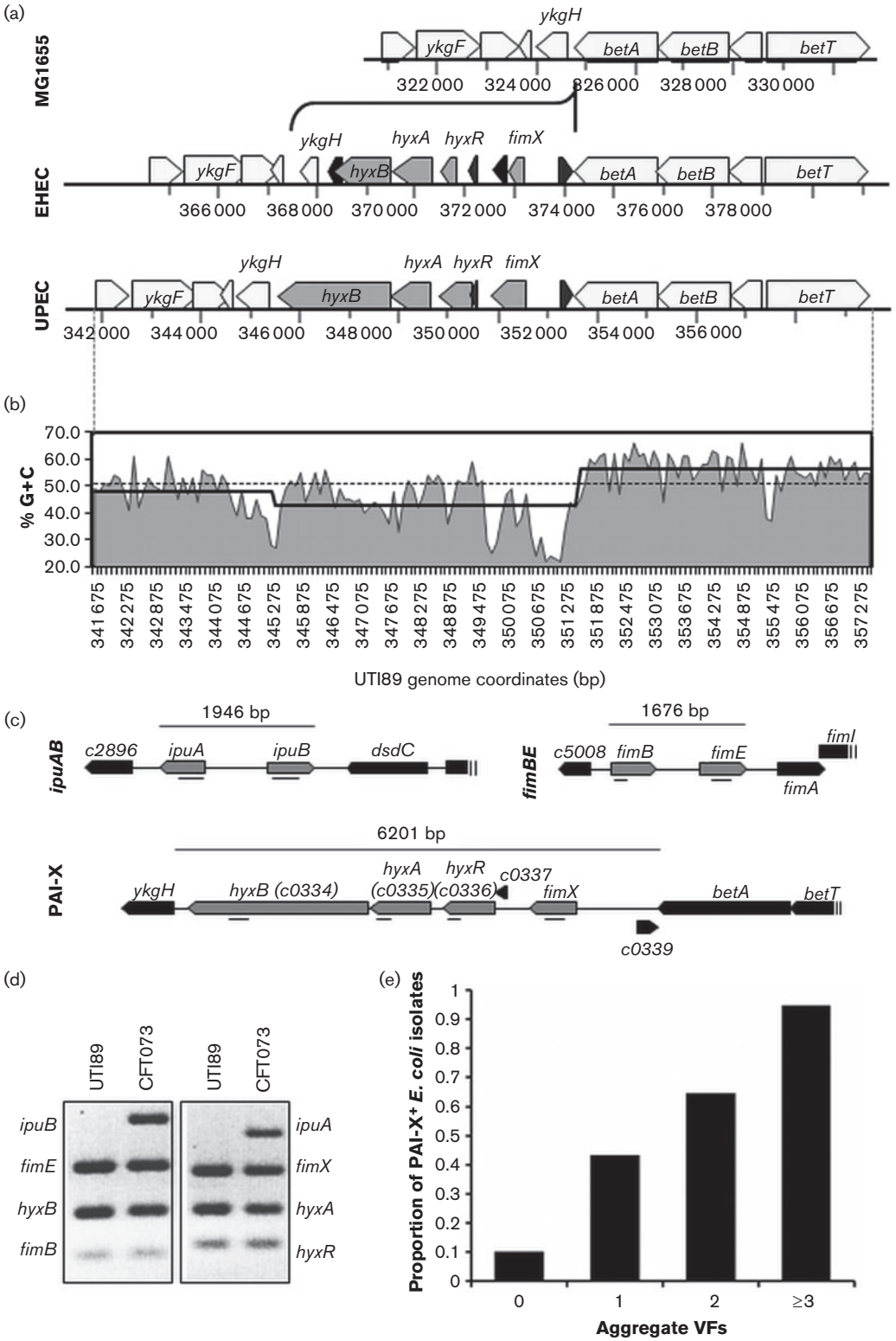
Bacterial strains and cultivation. All strains used in this study are listed in Table 1. A list of all individual clinical *E. coli* isolates with descriptive information and multiplex PCR results are listed in Table S1 (available in *Microbiology* online). Bacterial strains were routinely cultured in Luria–Bertani broth without antibiotics. Isolates were derived from four sources: (1) well-characterized, published representative pathogenic and commensal strains (n=9); (2) isolates collected in previously published clinical studies in Seattle, representing various clinical syndromes of urinary tract infection (n=155) (Czaja *et al.*, 2009; Garofalo *et al.*, 2007; Hooton *et al.*, 1996; Johnson *et al.*, 1987, 1988, 1991) as well as control faecal-commensal isolates (n=20) (Stapleton *et al.*, 1991); (3) strains from the *E. coli* reference (ECOR) collection (n=68) (Ochman & Selander, 1984) and (4) clinical enterohaemorrhagic *E. coli* (EHEC) isolates (n=7).

The characterized representative strains included K-12 strain MG1655 (Blattner *et al.*, 1997); cystitis isolates UTI89 (Mulvey *et al.*, 1998) and NU14 (Hultgren *et al.*, 1986); pyelonephritis isolates 536, DS17, GR12 and J96 (Berger *et al.*, 1982; Hull *et al.*, 1981; Svanborg Edén *et al.*, 1983; Tullus *et al.*, 1984); blood isolate CFT073 from a patient with concurrent pyelonephritis (Mobley *et al.*, 1990) and EHEC isolates EDL933 (Riley *et al.*, 1983).

For clinical isolates collected through studies carried out in Seattle, strains were archived in a specimen repository at the University of Washington UTI Research Laboratory and provided de-identified and anonymously through an Institutional Review Board-approved protocol. The study population for all studies was non-pregnant, outpatient women ages 18 to 49 seen in the Hall Health Primary Care Center (student health clinic) at the University of Washington (Czaja *et al.*, 2009; Garofalo *et al.*, 2007; Hooton *et al.*, 1996) or Group Health Cooperative of Puget Sound (Garofalo *et al.*, 2007; Hooton *et al.*, 1996), except for isolates collected from in-patients hospitalized with pyelonephritis and/or urosepsis. Pyelonephritis isolates were collected from 20 women ages 18 to 45 hospitalized in one of four hospitals in Seattle (Johnson *et al.*, 1991) and 2 were out-patients (Garofalo *et al.*, 2007). Urosepsis isolates were collected from women ages 18 to 45 with bacteraemia arising from a urinary tract source, hospitalized in one of four hospitals in Seattle, WA (Johnson *et al.*, 1987, 1988, 1991). Exclusion criteria for all studies of out-patients included known anatomical or functional abnormalities of the urinary tract, chronic illness requiring medical supervision, pregnancy or planned pregnancy during the next three months and, except for studies of pyelonephritis, symptoms or signs of acute pyelonephritis.

Clinical UTI syndromes were defined using the same criteria in all studies. Syndromes were defined as follows: acute, uncomplicated cystitis (n=68 isolates) was defined as the presence of typical symptoms (dysuria, frequency and/or urgency), and a midstream urine culture containing $\geqslant 10^2$ c.f.u. bacteria ml^{-1} (Czaja *et al.*, 2009; Garofalo *et al.*, 2007; Hooton *et al.*, 1996); asymptomatic bacteriuria (ASB; n=45) was defined as a midstream urine culture containing $>10^5$ c.f.u. bacteria ml^{-1} documented twice at least 24 h apart in a subject with no typical symptoms of UTI (Garofalo *et al.*, 2007; Hooton *et al.*, 1996); acute uncomplicated pyelonephritis (n=22) was defined as costovertebral angle pain and/or tenderness, pyuria and bacteriuria with $\geqslant 10^4$ c.f.u. ml^{-1} (Garofalo *et al.*, 2007; Johnson *et al.*, 1991) and urosepsis (n=20) was defined as bacteraemia arising from a urinary tract source (Johnson *et al.*, 1987, 1988). Commensal isolates (n=20) were defined as faecal isolates (prospectively collected between 1981 and 1987) from healthy, non-pregnant women with no cystitis symptoms, no history of UTI in the prior year and no antibiotic use in the previous month (Stapleton *et al.*, 1991).

Sixty-eight *E. coli* isolates from the ECOR collection (Ochman & Selander, 1984) representing phylogenetic groups A, B_1, B_2, and D human and non-human faecal-commensal isolates (n=61) and human



urinary tract isolates (ASB, n=1; pyelonephritis, n=6; cystitis, n=4) were used as a well-characterized strain references. ASB, cystitis and pyelonephritis isolates from the ECOR collection were included as UPEC isolates for analysis.

An additional seven previously unpublished, clinical isolates of EHEC were also included for analysis. These isolates were provided de-identified and anonymously through an Institutional Review Board-approved protocol, and represent Shiga-toxin positive, diarrhoeagenic *E. coli* from a diverse patient population at Washington University Medical Center in St. Louis.

Multiplex PCR analysis. One microlitre of bacterial culture suspension was used as template for multiplex PCR. All strains were

Fig. 1. Overview of the genomic location, features, and organization of PAI-X among sequenced *E. coli* isolates. (a) Genomic organization and conservation of PAI-X among sequenced isolates from EHEC (EDL933) and UPEC (UTI89) pathotypes. MG1655 is used as a K-12, commensal reference. (b) Percentage G+C trace of PAI-X_{UTI89} and surrounding flanking regions. Percentage G+C is plotted as area under the curve for the entire region (*betT* through *ykgE*) using a 100 bp window. The average G+C across the entire UTI89 genome is plotted as a grey dotted line. The average % G+C content across PAI-X and flanking sequences is plotted as a black stepped line. (c) Grey arrows represent ORFs detected by multiplex PCR. Directionality of the ORFs, representing forward or reverse strand orientation, is shown. Amplicons are indicated as a black line (_). The genomic sequence for *ipuA* and *ipuB* is shown for CFT073 while *fimB*, *fimE* and PAI-X sequences are shown for the prototypic cystitis isolate UTI89. (d) Representative results of the multiplex PCR for the Fim-like recombinases and associated genomic islands using multiplex PCR. UTI89 and CFT073 are shown as prototypic UPEC strains to show visualization of all bands. (e) All *E. coli* isolates (n=259), regardless of clinical syndrome, were scored based on the number of selective VFs present by PCR analysis and evaluated for the correlation between *E. coli* total VFs and the presence of PAI-X. Aggregate VFs refers to the number of total VFs present (out of the five assayed). Sample sizes varied between groups: 0 VFs (n=30), 1 VF (n=44), 2 VFs (n=68), $\geqslant$3 VFs (n=117).

analysed by multiplex PCR to determine the presence of the Fim-like recombinases and PAI-X genes. Primers were designed to conserved regions from gene alignments based on sequence data from available *E. coli* genomes (NCBI Genomes). The primer specificity was predicted by performing a BLAST analysis of the primer sequence to the UTI89 reference genome (Altschul *et al.*, 1997). Up to four primer pairs were used per reaction (for primer sequences see Table 1). Primer groups were as follows: Group 1 (*ipuB*, *fimE*, *hyxB*, *fimB*), 94 °C (5 min), then 30 cycles of 94 °C (30 s), 51 °C (30 s), 72 °C (30 s), followed by 72 °C (7 min) and 4 °C hold; Group 2 (*ipuA*, *fimX*, *hyxA*, *hyxR*), 94 °C (5 min), then 30 cycles of 94 °C (30 s), 52 °C (30 s), 72 °C (30 s), followed by 72 °C (7 min) and 4 °C hold.

Since UPEC also colonize the enteric tract, we assayed the ECOR reference strains (n=68) and faecal isolates (n=20) for the presence of additional virulence factors (VFs), using the multiplex primers listed in Table 2, to determine if any of the faecal isolates represent potential extraintestinal pathogens. The ECOR strain set has been previously analysed by multiplex PCR for various VFs (Johnson *et al.*, 2001). Primers and programs were grouped as follows: Group 3 (*kpsMT II*, *kpsMT III*, *ibeA*), 94 °C (5 min), then 30 cycles of 94 °C (30 s), 55 °C (30 s), 72 °C (35 s), followed by 72 °C (7 min) and 4 °C hold; Group 4 (*sfa/foc*, *traT*, *papA*), 94 °C (5 min), then 30 cycles of 94 °C (30 s), 55 °C (30 s), 72 °C (50 s), followed by 72 °C (7 min) and 4 °C hold; Group 5 (*cnf1*, *hlyA*, *fyuA*), 94 °C (5 min), then 30 cycles of 94 °C (30 s), 55 °C (30 s), 72 °C (70 s), followed by 72 °C (7 min) and 4 °C hold; and Clonal Groups (*chuA*, *yjaA*, *TSPE4.C2*), 94 °C (5 min), then 30 cycles of 94 °C (30 s), 55 °C (30 s), 72 °C (30 s), followed by 72 °C (7 min) and 4 °C hold.

All PCRs were performed using APEX *Taq* polymerase and accompanying buffers (Genesse Scientific). Amplicons were visualized on a 2 % TBE gel stained with ethidium bromide and photographed with a UV transilluminator (Bio-Rad). Each reaction was performed a minimum of three times, as biological replicates.

Alignment of PAI-X coding sequences from O157:H7 EHEC strains. The coding sequences for *fimX*, *hyxR*, *hyxA* and *hyxB* were gathered for UTI89 (EMBL coding sequence: ABE05839) and O157:H7 strain ELD933 (EMBL coding sequence: AAG54651) from the EMBL-EBI Integr8 web portal (Kersey *et al.*, 2005). The resulting sequences were aligned using the CLUSTAL W multiple alignment algorithm (Thompson *et al.*, 1994) in the BioEdit Sequence Alignment Editor (Hall, 1999), using the 'Toggle' feature to translate the coding

Table 1. Bacterial strains used in this study

Strains	Relevant features	Reference
UTI89	*E. coli* cystitis isolate	Mulvey *et al.* (1998)
MG1655	K-12 *E. coli* isolates	Blattner *et al.* (1997)
NU14	*E. coli* cystitis isolate	Hultgren *et al.* (1986)
536	*E. coli* pyelonephritis isolate	Berger *et al.* (1982)
DS17	*E. coli* pyelonephritis isolate	Tullus *et al.* (1984)
GR12	*E. coli* pyelonephritis isolate	Svanborg Edén *et al.* (1983)
J96	*E. coli* pyelonephritis isolate	Hull *et al.* (1981)
CFT073	*E. coli* pyelonephritis/blood isolate	Mobley *et al.* (1990)
EDL933	Enterohemhorrhagic *E. coli* isolate	Riley *et al.* (1983)
ECOR	*E. coli* reference set (n=68)	Ochman & Selander (1984)
E. coli cystitis isolates	Single and recurrent clinical *E. coli* cystitis isolates (n=68)	Czaja *et al.* (2009); Garofalo *et al.* (2007); Hooton *et al.* (1996)
E. coli asymptomatic isolates	Single and recurrent clinical *E. coli* ASB isolates (n=45)	Garofalo *et al.* (2007); Hooton *et al.* (1996)
E. coli faecal isolates	Clinical *E. coli* faecal–commensal isolates (n=20)	Stapleton *et al.* (1991)
E. coli pyelonephritis isolates	Clinical *E. coli* pyelonephritis isolates (n=22)	Johnson *et al.* (1991)
E. coli urosepsis isolates	Clinical *E. coli* urosepsis isolates (n=20)	Johnson *et al.* (1987); Johnson *et al.* (1988)

Table 2. Primers used in this study

Multiplex PCR primers and assays for additional VFs: *fyuA* (Johnson & Stell, 2000), *traT* (Johnson & Stell, 2000), *kpsMT II* (Johnson & Stell, 2000), *kpsMT III* (Johnson & Stell, 2000), *papAH* (Johnson & Stell, 2000), *sfa/focDE* (Le Bouguenec *et al.*, 1992), *ibeA* (Huang *et al.*, 1995), *hlyA* (Johnson & Stell, 2000), *cnf1* (Yamamoto *et al.*, 1995), and clonal groups (Clermont *et al.*, 2000) were performed as described. Primer groupings are listed in Methods.

Primers (forward/reverse)	Target	Sequence (5′→3′)	Size	Reference
fimE MPX 1/fimE MPX2	*fimE*	CTAACTGGAAAGGCGCTGAC/GAATATTTCGATGCCCGAGA	225	This study
fimB MPX 1/fimB MPX2	*fimB*	GCCTCATGCTGCACGTAAT/CAATCGACAAATTTCACTCG	79	This study
fimX MPX 1/fimX MPX 2	*fimX*	CCAGAGCATGTCCTTTCCTG/TTCCTCTGCTTAAGCCACAAC	216	This study
ipuA MPX 1/ipuA MPX 2	*ipuA*	GCGATGTTTGCATGATTTTA/TTTTACCCGCAGCAGAAACT	303	This study
ipuB MPX 1/ipuB MPX 2	*ipuB*	TGCGCAAATTTATTACTCATAGTG/TGTCTCGAGATTTTATTTCCTTGA	334	This study
hyxR MPX 1/hyxR MPX 2	*hyxR*	TCGATGAGCGGAATGTTGTC/GGCTGCTCTATACGGGATGC	89	This study
hyxA MPX 1/hyxA MPX 2	*hyxA*	GCATTTCCATCACCGTGAAA/GTGCGCAGTTTCTCAAAACG	142	This study
hyxB MPX 1/hyxB MPX 2	*hyxB*	GGGTATCACCACCCAGCATT/CAGGATGCTGTCCGTCTGAG	139	This study

sequence. Mutations in EHEC, relative to UTI89, that resulted in a premature stop codon were noted.

Sensitivity and specificity calculations. Specificity and sensitivity metrics were calculated as previously described (Altman & Bland, 1994) and were used in this study as a common metric to compare the performance of detecting different *E. coli* genes to classify strains as UPEC. Calculations were based on data derived from Table 3. The formulas used for calculating sensitivity and specificity are shown below:

Sensitivity=number true positive/(number true positive+number false negative)

Specificity=number true negative/(number true negative+number false positive)

Statistical analysis. Statistical analyses using the Fisher's exact test were performed using GraphPad Prism (GraphPad Software) where indicated in figure legends. Two-tails were used in the determination of statistical significance, which was defined by attaining $P \leqslant 0.05$. Statistical determinations were not weighted for epistatic relationships. Logistic regression was performed in R using the MASS, arm, aod and car packages. Model refinement was performed using a forward step-wise approach.

RESULTS

Organization of the *fimX*-associated island, PAI-X and association with VFs

Three recombinases FimB, FimE and FimX regulate the phase variation of type 1 pili in the prototypic cystitis strain UTI89 (Hannan *et al.*, 2008). *fimB* and *fimE* are located immediately upstream of the type 1 pili operon, while *fimX* is located at an unlinked genetic locus we have termed PAI-X for Pathogenicity-associated Island of FimX (located at approximately 4.2 min on the UTI89 chromosome), adjacent to the *betABIT* choline-glycine betaine locus (Fig. 1a). The ~6.2 kb region contains four ORFs (>100 aa): *fimX*, *hyxR*, *hyxA* and *hyxB* for Hypotheticals of PAI-X (Fig. 1a). The *fimX* genomic locus (PAI-X) meets most, if not all, criteria for a pathogenicity island as defined by Hacker & Kaper (2000). For instance, PAI-X has disparate G+C content compared to the rest of the UTI89 genome: PAI-X has 42.9 % G+C content relative to 50.6 % G+C content for UTI89 generally (Fig. 1b) (Chen *et al.*, 2006; EnCor Biotechnology, http://www.encorbio.com/protocols/Nuc-MW.htm). As shown later in the results, PAI-X is more prevalent among pathogenic *E. coli* strains than commensal strains, indicating PAI-X is associated with virulence (Table 3). Although prior work demonstrated that FimX plays a role in the regulation of type 1 pili during acute cystitis (Hannan *et al.*, 2008), the role of the *hyx* genes during infection remains unknown.

To determine the prevalence and complement of Fim-like recombinase genes present in the genomes of a range of *E. coli* isolates as well as the association of PAI-X genes with known and epidemiologically linked VFs, a multiplex PCR approach was developed to detect the Fim-like recombinases (*fimB*, *fimE*, *fimX*, *ipuA*, *ipuB*) and *hyx* genes (*hyxR*, *hyxA*, *hyxB*) (Fig. 1c, d) in a variety of pathogenic strains isolated from women with a variety of clinical syndromes (see Methods for a detailed description of *E. coli* strains and patient populations). For comparison, the assay was performed on non-human and the human commensal isolates from the ECOR reference set (Ochman & Selander, 1984), an independent set of faecal isolates from healthy women and a small group of EHEC isolates. Additionally, isolates were screened by PCR for the presence of five known or epidemiologically associated VF genes including *cnf1*, *hlyA*, *fyuA*, *traT* and *ibeA*. The proportion of *E. coli* isolates carrying PAI-X increased with the total number of virulence factor genes (Fig. 1e), demonstrating that PAI-X acquisition has a strong, positive correlation with known VFs.

The type 1 pili regulator gene *fimX* and associated pathogenicity island PAI-X are highly prevalent among UPEC

As expected, *fimB* and *fimE* are ubiquitous among both pathogenic and non-pathogenic *E. coli* as both *fimB* and *fimE* were present in >98 % of UPEC and commensal strains (Table 3). We found that *ipuA* and *ipuB* were always

Table 3. Molecular epidemiology of the Fim-like recombinases and PAI-X factors in commensal and pathogenic *E. coli* isolates

P values (Fisher's exact) were calculated using two-tailed tests. Symbols indicating significance for group comparisons are as follows: human versus non-human commensals: $^{*}P \leqslant 0.05$; lower UTI versus upper UTI: $^{*}P \leqslant 0.05$; $^{**}P \leqslant 0.01$; $^{***}P \leqslant 0.001$. Commensal total versus UPEC total: $^{***}P \leqslant 0.001$; $^{****}P \leqslant 0.0001$.

Target	Commensal†						UPEC							
	Human		Non-human		Total		Lower UTI‡		Upper UTI‡		Total		EHEC	
	(*n*=48)		(*n*=30)		(*n*=78)		(*n*=120)		(*n*=53)		(*n*=173)		(*n*=8)	
	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
fimB	46	96	30	100	76	97	119	99.2	53	100	172	99.4	8	100
fimE	48	100	30	100	78	100	120	100.0	53	100	173	100.0	8	100
ipuA	9	19	3	10	12	15	28	23.3	13	25	41	23.7	1	13
ipuB	9	19	3	10	12	15	28	23.3	13	25	41	23.7	1	13
fimX	17	35	11	37	28	36	105	87.5	39	74*	144	83.2****	5	63
hyxR	17	35	11	37	28	36	105	87.5	39	74*	144	83.2****	5	63
hyxA	17	35	11	37	28	36	105	87.5	39	74*	144	83.2****	5	63
hyxB	17	35	11	37	28	36	105	87.5	39	74*	144	83.2****	5	63
ibeA	6	13	1	3	7	9	56	46.7	11	21**	67	38.7****	2	25
traT	18	38	9	30	27	35	94	78.3	43	81	137	79.2****	5	63
fyuA	27	56	9	30‖	36	46	109	90.8	48	91	157	90.8****	5	63
hlyA	10	21	1	3‖	11	14	45	37.5	20	38	61	37.6****	2	25
cnf1	7	15	1	3	8	10	69	57.5	16	30***	85	49.1****	4	50

†Commensal strains represent the ECOR reference set as well as faecal isolates from healthy women. Only clonal groups A, B1, B2 and D were included. ECOR isolates that were human ASB, CY or PY isolates were included in the UPEC group for analysis.
‡Lower UTI isolates represent single and recurrent ASB and CY isolates. Upper UTI isolates represent PY and urosepsis isolates.

present together, consistent with previous findings (Bryan *et al.*, 2006), and that *ipuA* and *ipuB* were present in commensal (15 %) and UPEC (23.7 %) isolates in statistically equal abundance (Table 3). In contrast, *fimX* was highly prevalent among UPEC (83.2 %), but not commensal isolates (36 %; $P<0.0001$), and was more prevalent in lower (87.5 %) than upper UTI isolates (74 %; $P=0.03$) (Table 3). *ipuA* and *ipuB*, on the other hand, were equally distributed among upper (25 %) and lower UTI isolates (23.3 %). There were no significant differences in the prevalence of *fimX* or *ipuAB* among the various individual UTI syndromes (data not shown). The Fim-like recombinase gene *fimX* is the only family member to have a specific, significant association with UPEC isolates.

Like *fimX*, *hyxRAB* was also more significantly associated with pathogenic (83.2 %) versus commensal isolates (36 %; Table 3). In UPEC isolates, the *hyx* genes were always present with *fimX*, and thus, were also more significantly associated with UPEC isolates, demonstrating that, when present, the major genetic features of PAI-X_{UTI89} are highly conserved among other UPEC isolates of urinary tract and blood.

Distribution of the Fim-like recombinase and PAI-X genes by *E. coli* phylogenetic group

Phylogenetic group analysis was performed as previously described (Clermont *et al.*, 2000; Gordon *et al.*, 2008). Consistent with previous results (Carlos *et al.*, 2010), commensal *E. coli* of non-human origin contained a larger percentage of group B1 strains (46.2 %) than those of human origin (11.8 %; $P=0.004$) while, not surprisingly, faecal isolates of human origin contained a larger percentage of group B2 strains (26.5 %; $P=0.03$ compared with faecal isolates of non-human origin) (Fig. 2a). Consistent with other published clinical strain sets, the majority of UPEC isolates were group B2 (76.3 %) and to a lesser extent group D (Fig. 2a). Group D strains were at least 2.5-fold more highly associated with pyelonephritis isolates (25.0 %), than isolates from other syndromes (13/141=9.2 %; $P=0.03$). The distribution of the isolates among the phylogenetic groups is consistent with previously published clinical sets.

Analysis of the Fim-like recombinases and PAI-X association with phylogenetic group revealed that *fimB* and *fimE* were equally distributed among all groups (Table 4), consistent with the ubiquitous presence of these genes. One the other hand, the genes *ipuA* and *ipuB* were frequently identified in group B2 (28.6 %) or group D (27.3 %) strains, but were rarely found in group A strains (2.4 %) and were absent in group B1 strains (0 %; Table 4). PAI-X was found in the vast majority of group B2 isolates (95.5 %) but few group A strains (9.5 %; $P<0.0001$), consistent with group A strains making up the largest proportion of commensal isolates (Fig. 2a). PAI-X showed

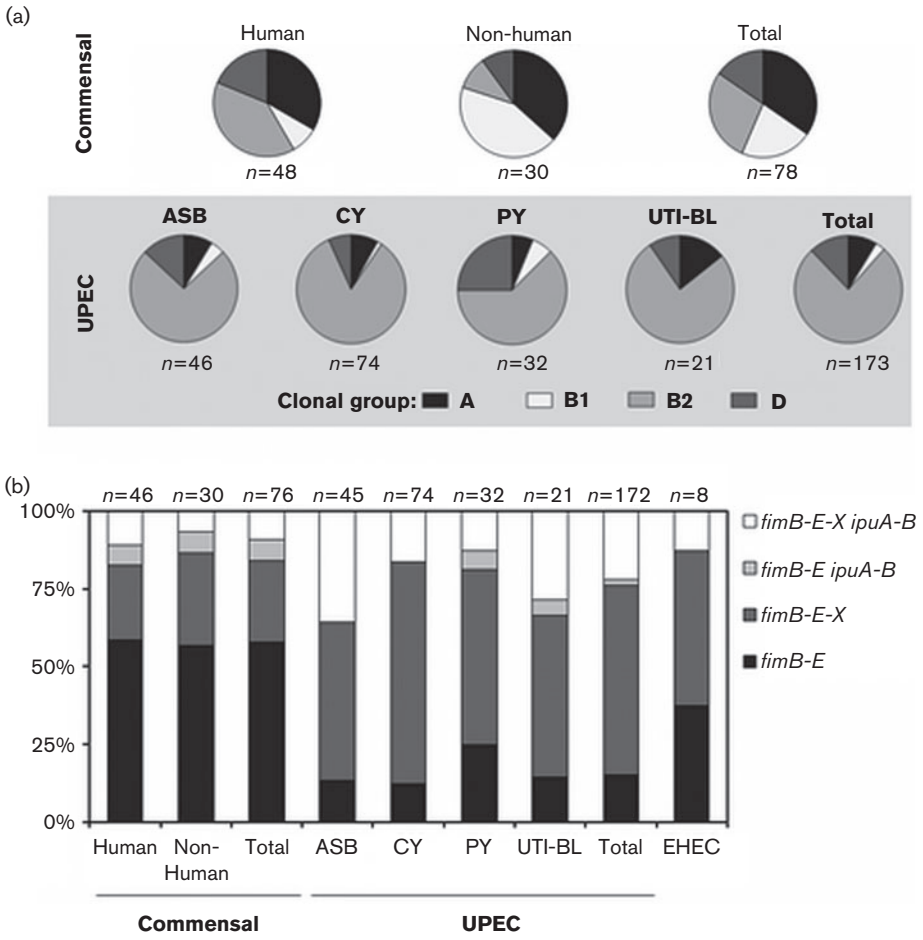


Fig. 2. Clonal group variation and Fim-like recombinase prevalence by isolate source and syndrome. (a) Phylogenetic associations among commensal and pathogenic isolates. The percentage of isolates in each of the four clonal groups is shown. Clonal groups were assigned based on a previously published multiplex PCR for *chuA*, *yjaA*, and genomic fragment TSPE4.C2 (Clermont *et al.*, 2000; Gordon *et al.*, 2008). ECOR strain clonal groups were determined previously (Johnson *et al.*, 2001) and are in accordance with our experimental results. CY, cystitis; PY, pyelonephritis; UTI-BL, urosepsis. (b) The percentage of isolates that encode one of four recombinase profiles observed, grouped as follows: *fimB-E* only (similar to MG1655); *fimB-E-X* only (similar to UTI89); *fimB-E ipuA-B* (rarely observed); and all five recombinases present, *fimB-E-X ipuA-B* (similar to CFT073). Two human commensal isolate and one ASB isolate were excluded from analysis as they did not carry the *fimB* gene by PCR analysis.

a much lower association with group D strains (33.3 %), and all 11 of these isolates were from the human UPEC group (Table 4), which is consistent with PAI-X being a phylotype B2 and D, human UPEC-associated marker. PAI-X was also present in 45.5 % of B1 isolates (Table 4), but the majority (8/10=80.0 %) were from non-human commensals.

UPEC have acquired additional Fim-like recombinase genes

There were four main recombinase profiles observed among isolates: strains that encode *fimB fimE* only, strains that encode *fimB fimE* in addition to *fimX*, strains that encode *ipuA ipuB* in addition to *fimB fimE*, and strains that encode all five recombinase genes, *fimB fimE fimX ipuA ipuB* (Fig. 2b). The majority of commensal strains (both human and animal origin) only carried the genes for *fimB* and *fimE* (58 %; Fig. 2b). In both commensal (7 %) and UPEC isolates (1.7 %), addition of *ipuA ipuB* in the absence of *fimX* was infrequent. Interestingly, UPEC strains generally contained an expanded repertiore of recombinase genes. For instance, 84.8 % of UPEC contained at least one added Fim-recombinase gene in addition to *fimB* and *fimE*, compared with 42 % of commensal isolates ($P<0.0001$) (Fig. 2b). Therefore, UPEC isolates are twofold more likely to have acquired recombinases in addition to 'core genome' family members *fimB* and *fimE*, suggesting that pathogenic *E. coli* have undergone adaptation of the regulatory programme controlling type 1 pili.

Table 4. Phylogenetic distribution of the Fim-like recombinases and PAI-X among all *E. coli* isolates

All *E. coli* isolates (n=251), excluding eight EHEC isolates, were analysed for clonal group by PCR. PAI-X factors were assayed separately but grouped together for analysis. P values (Fisher's exact) were calculated using two-tailed tests. Statistical significance is indicated in the table as: *$P\leqslant0.05$; **$P\leqslant0.01$; ***$P\leqslant0.001$; ****$P\leqslant0.0001$. Significance was calculated for indicated group versus all other strains. Parentheses indicate a negative association.

Target	A		B1		B2		D	
	n=42		n=22		n=154		n=33	
	No.	%	No.	%	No.	%	No.	%
fimB	42	100	22	100	151	98.1	33	100
fimE	42	100	22	100	154	100.0	33	100
ipuA	1	2(***)	0	0(**)	44	28.6***	9	27
ipuB	1	2(***)	0	0(**)	44	28.6***	9	27
PAI-X	4	10(****)	10	45(*)	147	95.5****	11	33(****)

PAI-X is a genetic marker for human UPEC

To elucidate whether specific genetic features of *E. coli* were predictive of isolates being urinary tract-associated UPEC, we evaluated the sensitivity and specificity of VF genes for predicting or excluding an *E. coli* isolate as UPEC (see Methods section for a detailed explanation). These served as common metrics to compare the likelihood that specific *E. coli* genes were predictive of an isolate being part of the UPEC group. Applying this analysis to the data from Table 3, several genes have high sensitivity (equivalent to prevalence) as a positive indicator for an isolate being UPEC. For, instance *fyuA* and PAI-X have the two highest sensitivities at 90.8 and 83.2 %, respectively, indicating that these genomic markers are highly associated with UPEC (Table 5). Conversely, a high specificity would indicate those genes that, when absent, predict an isolate is not UPEC. Genes like *cnf1* and *hlyA*, which are almost never found in commensal isolates, have specificities $\geqslant$97 %, indicating that negative tests for these genomic markers are useful in excluding an isolate from the UPEC group. Although *hlyA* and *cnf1* have high specificities, their low prevalence in the UPEC population yields a low sensitivity value, making their presence less useful in predicting if an isolate is UPEC. Of the VFs tested, PAI-X had the best combination of sensitivity and specificity, at 83.2 and 65 %, respectively (Table 5).

Table 5. Sensitivity and specificity of VFs predictive of human UPEC

Sensitivity and specificity are described in Methods. Calculations are based on data derived from Table 3.

	Sensitivity	Specificity
ibeA	67/173=38.7 %	42/48=88 %
traT	137/173=79.2 %	30/48=63 %
fyuA	157/173=90.8 %	21/48=44 %
hlyA	61/173=37.6 %	38/48=79 %
cnf1	85/173=49.1 %	41/48=85 %
PAI-X	144/173=83.2 %	31/48=65 %

Based on the potential for multiple associations between VFs and sources/syndrome, multivariate logistic regression analysis was used to simultaneously assess bacterial VFs and phylogenetic group as predictors of UTI in general, upper tract disease (pyelonephritis with/without bloodstream involvement) and ASB (Table 6). Logistic regression modelling first included phylogenetic group and the VF genes *ibeA*, *traT*, *fyuA*, *ipuA*, *hlyA*, *cnf1* and *fimX*. Subsequently a forward step approach was used to refine the models. From an analysis of all isolates against candidate predictors of UTI, phylogenentic group B2 and VF genes *ibeA*, *traT*, *fyuA* and *cnf1* emerged as significant predictors of UTI (Wald χ^2, $P=1.6\times10^{-8}$). The models for distinguishing upper tract isolates from lower tract isolates (cystitis and ASB) were not as robust. In the two best models, *ibeA* and *cnf1* had odds ratios (OR) of <1 (Wald χ^2, P=0.025), suggesting they are modest negative predictors of upper urinary tract infections. Significant positive predictors, however, did not emerge, although *ibeA* and *ipuA* had moderate to strong OR (4.1 and 4.5 respectively; Wald χ^2, P=0.0007) as positive predictors of ASB.

DISCUSSION

Pathogenicity-associated islands are common among ExPEC (Blum *et al.*, 1995; Chen *et al.*, 2006; Lloyd *et al.*, 2007) and have some characteristic features, including: (1) disparate G+C content from the core genome; (2) association with pathogenic organisms, but not commensal counterparts; (3) contain virulence-associated genes; (4) insertion sites or flanking direct repeats; (5) a high frequency of insertion at tRNA sites and (6) often mobility (pseudo)genes (Dobrindt *et al.*, 2004; Gal-Mor & Finlay, 2006; Hacker & Kaper, 2000). PAI-X was associated with *E. coli* isolated from patients experiencing a variety of clinical syndromes, including asymptomatic bacteriuria, cystitis,

Table 6. Stepwise multivariate logistic regression analysis for predictors of UTI, upper tract infection, and ASB

Phylogenetic groups (Group) are A, B1, B2 and D. The Group variable was set as B2 versus all other groups. All stepwise models initially included model A values of Group, *ibeA, traT, fyuA, hylA, cnf1, ipuA* and *fimX*. The genes *ipuB* and (*hyxR*, *hyxA* and *hyxB*) were not included because they were found to have 100 % correlation with *ipuA* and *fimX*, respectively. Forward stepwise refinement of model A was performed in each prediction scenario; model B, Group + *traT* + *fyuA* + *cnf1*; model C, *hlyA* + *cnf1*; model D, Group + *ibeA* + *ipuA*. CI, confidence interval; DF, degrees of freedom; OR, odds ratio.

Candidate predictor values	OR (95 % CI)	*P* value	Wald χ^2 (DF, *P* value)	Model
Predictors of UTI				
Group	3.6 (1.4–9.7)	2.7×10^{-15}	42.1 (4, 1.6×10^{-8})	A
ibeA	1.4 (0.52–3.8)	0.004		
traT	6.7 (3.2–15.0)	9.4×10^{-9}		
fyuA	4.3 (1.9–10.2)	0.0003		
Group	4.4 (2.0–10.1)	2.7×10^{-15}	54.3 (4, 4.7×10^{-11})	B
traT	7.7 (3.8–16.5)	5.3×10^{-10}		
fyuA	4.2 (1.9–9.8)	0.0002		
cnf1	2.5 (1.1–6.2)	0.03		
Predictors of upper tract infection				
ibeA	0.4 (0.17–0.94)	0.003	7.4 (2, 0.025)	A
cnf1	0.3 (0.11–0.69)	0.007		
cnf1	0.19 (0.07–0.45)	0.0001	12.5 (2, 0.002)	C
Predictors of ASB				
ibeA	3.8 (1.5–10.4)	0.003	12.1 (2, 0.002)	A
ipuA	4.3 (1.6–12.8)	0.004		
ibeA	4.1 (1.7–10.7)	0.003	14.6 (2, 0.0007)	D
ipuA	4.5 (1.7–12.6)	0.002		

pyelonephritis and urosepsis. Prior studies have also clearly demonstrated that FimX-mediated control of type 1 pili expression is activated *in vivo* and that independent expression of type 1 pili by FimX *in vivo* is sufficient, but not necessary, to produce acute cystitis (Hannan *et al.*, 2008). PAI-X is not associated with a tRNA and direct repeats or insertion elements were not immediately apparent. Many islands are also associated with phage genes and transposases and, while FimX is part of the phage integrase superfamily, it has never been observed to function as an integrase or excisionase, rather mediating site-specific recombination or inversion.

Our molecular epidemiology data provide strong evidence that this locus is widespread among a diverse group of UPEC clinical isolates of urinary tract origin but is present in a minority of commensal *E. coli* isolates. UPEC are true commensal-pathogens, meaning that these potential pathogens cycle through the commensal reservoir of the gastrointestinal tract without causing symptoms. However, phylotype B2 *E. coli*, especially those encoding UTI-associated VFs, likely represent potential pathogens even if they are isolated from a commensal niche. This suggests that the prevalence of PAI-X and other VFs is likely to be overrepresented among *E. coli* strains defined as commensals based solely on host site and not taking into account genomic virulence potential. Our molecular epidemiology data also support the idea that *fimX* is the only Fim-recombinase gene specifically associated with UPEC isolates, suggesting that strains carry *fimB fimE* as part of the 'core genome' then can acquire *ipuA ipuB* subsequent to *fimX* acquisition in pathogenic isolates. Our findings are consistent with the notion that UPEC strains have acquired additional regulatory inputs to control and possibly fine-tune the expression of a major VF, type 1 pili. Alternatively, the high prevalence of *fimX* among UPEC may be conserved due to its role regulating *hyxR* and the subsequent effects on UPEC intracellular survival within macrophages and tolerance to reactive nitrogen intermediates. Future work will be needed to ascertain the evolutionary benefit of FimX coordinated regulation of type 1 pili and *hyxR* during *in vivo* infection.

Among ExPEC in general, *fimX* was always found associated with *hyxR*, *hyxA* and *hyxB*, suggesting that when this locus is present, it is broadly structurally conserved. Although sequence data from model strains suggest these genes reside together at the same locus, hybridization and/or sequencing studies will be necessary to ascertain if *fimX* and *hyxRAB* are always linked. Our molecular epidemiology data also suggest that this locus is less prevalent among pyelonephritis and urosepsis isolates than lower UTI isolates (ASB, cystitis); although, the majority of these strains still carry PAI-X, perhaps accounting for why *fimX* (representing PAI-X) did not emerge as a predictive factor for any particular syndrome in multivariate logistic regression. Future studies will be needed to determine if there is a biological significance to this statistical difference. Many isolates that cause urosepsis are genetically related to strains known to cause cystitis and pyelonephritis, suggesting that the simple

presence or absence of any genetic factors is insufficient to define urosepsis isolates as a group.

As illustrated by several of the sequenced EHEC isolates, the simple presence or absence of PAI-X by PCR analysis is unlikely to predict the functional state of the factors. FimX, HyxR, HyxA and HyxB are highly conserved at the amino acid level with greater than 98 % identity, respectively, among the prototypic UPEC isolates UTI89, 536 and CFT073. However, sequence analysis of PAI-X in EHEC 0157:H7strains EDL933 and EC4115 indicated the presence of a premature nonsense mutation in *fimX* (Tyr128→Stop), *hyxB* (Ser370→Stop) and *hyxR* (Ser56→Stop) (Fig. 1a). In EHEC strains, there is also a potential in-frame secondary translational start site at residue 107 of *hyxR*, which would create a significant N-terminal truncation. Thus, it is likely that *fimX*, *hyxR* and *hyxB* do not yield functional products in these EHEC strains. These data indicate that PAI-X is likely widely distributed among UPEC, although sequencing efforts will reveal if these strains carry polymorphisms producing coding sequence disruptions as found in many EHEC isolates. In contrast, the high identity at the nucleotide level for all of the PAI-X genes between sequenced EHEC isolate EDL933 and prototypic UPEC isolate UTI89 suggests that PAI-X may have a common provenance. PAI-X, including *fimX*, was highly conserved in UPEC isolates but disrupted among sequenced EHEC isolates, suggesting a potential evolutionary benefit of this locus specific to the colonization, transmission or pathogenesis of UPEC isolates generally.

A role for PAI-X in virulence is supported by our molecular epidemiology data and the strong, positive correlation between the number of VFs present in the genome of an isolate and the presence of PAI-X. Previous studies have shown that FimX is capable of regulating virulence during experimental cystitis (Hannan *et al.*, 2008). FimX is sufficient but not necessary to mediate type 1 pili expression during cystitis, thereby promoting UPEC bladder epithelial invasion. Translation and BLAST analysis of *hyxR* suggest it is a putative helix–turn–helix, LuxR-like response regulator. Recently, our laboratory has also shown that FimX exclusively regulates the PAI-X gene *hyxR* through bidirectional promoter inversion, and that HyxR regulates the intracellular survival of *E. coli* during macrophage infection (Bateman & Seed, 2012). The additional two conserved ORFs found in this locus appear to code for a putative outer membrane autotransporter (HyxB) and a conserved hypothetical protein (HyxA), which has homologues only in the genomes of *E. coli* and *S. enterica* (protein BLAST E-score $<5\times10^{-5}$). Further work will be needed to discern the individual contributions of the other PAI-X genes in colonization and extraintestinal disease, but it is clear that PAI-X constituents alter UPEC virulence. The high prevalence of PAI-X genes distributed among UPEC may suggest that PAI-X encoded factors like FimX and HyxR have frequent and conserved roles in host interactions, warranting future studies to assess their full biological functions.

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