Demonstration of regulatory cross-talk between P fimbriae and type 1 fimbriae in uropathogenic Escherichia coli

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The majority of Escherichia coli strains isolated from urinary tract infections have the potential to express multiple fimbriae. Two of the most common fimbrial adhesins are type 1 fimbriae and pyelonephritis-associated pilus (Pap). Previous research has shown that induced, plasmid-based expression of a Pap regulator, papB, and its close homologues can prevent inversion of the fim switch controlling the expression of type 1 fimbriae. The aim of the present study was to determine if this cross-regulation occurs when PapB is expressed from its native promoter in the chromosome of E. coli K-12 and clinical isolates. The regulation was examined in three ways: (1) mutated alleles of the pap regulatory region, including papB and papI, that maintain the pap promoter in either the off or the on phase were exchanged into the chromosome of both E. coli K-12 and the clinical isolate E. coli CFT073, and the effect on type 1 fimbrial expression was measured; (2) type 1 fimbrial expression was determined using a novel fimS:gfp reporter system in mutants of the clinical isolate E. coli 536 in which combinations of complete fimbrial clusters had been deleted; (3) type 1 fimbrial expression was determined in a range of clinical isolates and compared with both the number of P clusters and their expression. All three approaches demonstrated that P expression represses type 1 fimbrial expression. Using a number of novel genetic approaches, this work extends the initial finding that PapB inhibits FimB recombination to the impact of this regulation in clinical isolates.

INTRODUCTION

Urinary tract infections (UTIs) are common, affecting a large proportion of the population. It is estimated that 20% of women develop a UTI in their lifetime, and antibiotic treatment results in approximately 110 000 prescriptions per million inhabitants per annum in Europe (Naber, 2000). Escherichia coli strains are the predominant cause of uncomplicated UTIs, responsible for between 60 and 80% of the cases reported in the UK each year (Graham & Galloway, 2001). Many infections are asymptomatic, especially in the elderly (Nicolle, 2001), but others result in cystitis. If the infection ascends to the kidney, then pyelonephritis can occur. Such infections are a significant origin of Gram-negative sepsis.

Fimbrial adhesins are important virulence factors that allow binding of the bacteria to specific receptors on epithelial cells of the urinary tract. The two adhesins most commonly associated with UTI are type 1 fimbriae, and pyelonephritis-associated pilus (Pap) and Pap-related fimbriae (Prf); the last two are collectively termed P fimbriae in this study. Type 1 fimbriae mediate binding to α-D-mannose-containing receptors and extracellular matrix components, whereas P fimbriae bind to glycireceptors containing the αGal(1-4)βGal moiety (Lindberg et al., 1984). Although type 1 fimbriae are common to the majority of E. coli isolates, the FimH adhesin has been shown to be important in a mouse model of UTI, and a degranulation response to the fimbriae is associated with renal scarring (Mizuno et al., 1997). There is good evidence from a number of epidemiological studies that P fimbriae are important in upper UTI (Johnson, 1991). While the mechanism is debatable (Hedlund et al., 1999; Schilling et al., 2003), P-fimbrial expression has been shown to induce inflammation in humans and in a mouse model. A current model provides evidence for P-fimbrial

Abbreviations: GFP, green fluorescent protein; HA, haemagglutination; Lrp, leucine-responsive regulatory protein; MRHA, mannose-resistant HA; MSHA, mannose-sensitive HA; Pap, pyelonephritis-associated pili; Prf, Pap-related fimbriae; RBC, red blood cell; Sfa, S-fimbrial adhesin; UPEC, uropathogenic E. coli; UTI, urinary tract infection.
adherence provoking inflammation in a cluster of differentiation number 14 (CD-14)-independent manner, probably by association with toll-like receptor number 4 (TLR-4) (Frendeus et al., 2001).

To limit immune exposure and inflammation, the expression of type 1 and P fimbriae is phase variable: both are controlled by reversible genetic switches. The mechanisms that control the phase-variable expression are distinct, with type 1 fimbriae regulated by an invertible DNA element (fim switch), and P fimbriae by mutually exclusive protein complexes initiated by alternative DNA methylation patterns (Blomfield, 2001). Phase variation of type 1 fimbrial expression requires the activity of two recombinases; FimB promotes inversion in both directions, whereas FimE mediates predominately on-to-off inversion (Gally et al., 1996; Klemm, 1986). Previous work has demonstrated that a regulator from the P-fimbrial gene cluster, PapB, when expressed from a plasmid, is able to prevent inversion of the fim switch (Holden et al., 2001; Xia et al., 2000). Cross-talk by PapB has been shown to occur by inhibition of FimB-promoted recombination together with increasing fimE by PapB has been shown to occur by inhibition of FimB-cluster can act on the expression (Xia et al., 1996; Klemm, 1986). Previous work has demonstrated that all the isolates contained at least one P-gene cluster and functional P-fimbrial expression was established for each group of isolates. Type 1 fimbrial expression was then examined at the genetic level through to the production of functional fimbriae, and cross-talk with P fimbriae was demonstrated.

**Methods**

**Strains and plasmids.** The strains and plasmids used for this study are listed in Tables 1, 2 and 3.

**Cloning and DNA manipulation.** For genetic manipulation, *E. coli* strains were grown on LB medium supplemented with antibiotics as required. Single-copy fusions were as follows: egfp was amplified from pEGFP using primers GFP 5′ and GFP 3′ (Roe et al., 2003) and cloned into pAJR25, forming pAJR28, and into pK88, forming pK12. pap DNA from strains DL2121, DL 2496 (Nou et al., 1995) and J96 (Hull et al., 1981; Marklund et al., 1992) which included pap ORF, papB ORF and the start codon of papA was amplified with the primers 177P and 178P (Table 4), cloned initially into pCR4-TOPO-TA, then subcloned with BamHI and KpnI sites into either pAJR28 or pK12, for exchange into *E. coli* K-12 or CFT073 backgrounds, respectively, as described previously (Blomfield et al., 1991; Porter et al., 2004). The plasmids constructed corresponded to the mutated pap regulatory regions as follows: pNJH83 (K-12) and pNJH86 (CFT073) contained PapBA ON from DL 2121, forming ZAP986 and ZAP989; pNJH84 (K-12) and pNJH87 (CFT073) contained PapBA OFF from DL 2496, forming ZAP987 and ZAP990; pNJH85 (K-12) and pNJH88 (CFT073) contained wild-type PapBA (J96), forming ZAP988 and ZAP991. All insertions were confirmed by specific PCR.

The minimal fim.:gfp ′ construct (pNJH97) was cloned by amplifying fim DNA from pMM34 (Blomfield et al., 1991) in the off orientation with primers 151F and 152F (Table 4), and cloned into pAJR145 using XbaI. The orientation was confirmed by restriction analysis. The minimal fim switch unit was cloned into pDG19, using primers 135F and 136F, generating pNJH79 for allelic exchange into strain BGEC144, generating strain ZAP973, as described in Gally et al. (1994). Insertion was confirmed by specific PCR. It should be noted that the plasmid was not successfully transformed into all of the clinical isolates, resulting in a reduced number of samples tested.

Fimbrial cluster deletions in *E. coli* strain 536 were carried out using the method described in Datsenko & Wanner (2000). The template plasmid pKD3 was used to insert the cat cassette in each operon individually using specific primers for fim (fim1 and fim2, Table 4), pap (pap1 and pap2, Table 4) and sfa (sfa1 and sfa2, Table 4). The cat cassette was excised using pCP20, as described in Datsenko & Wanner (2000). Deletion of the clusters was verified by Southern blotting and specific PCR.

**Southern blotting.** Southern hybridization was used to determine the number of P-fimbriae-related clusters carried by each *E. coli* isolate. CFT073, J96 and 536 reference strains, and pPap5 (Table 1), were used as controls for probe specificity. Genomic DNA from each isolate and plasmid DNA were digested overnight with HindIII or BglII restriction endonucleases, resolved on agarose gels and blotted onto nitrocellulose (Hybond-N⁺, Amersham) using the method described in Sambrook et al. (1989). Two probes were used for hybridization to increase the probability of detecting all P-related clusters: the papF probe was amplified with primers 196P and 197P (Table 4), and the papB probe was amplified with papB1 and papB2, described in Holden et al. (2001); both were labelled with fluorescein-dUTP using the ECF Random Prime Labelling kit (Amersham Biosciences). Following hybridization, the membrane was washed with high-stringency buffers and the signal was amplified using an ECF Signal Amplification System (Amersham Biosciences). The signal was detected with a Fuji FLA-2000 scanner with a 580 nm filter.

**Assay of fim switch recombination and orientation.** FimB-promoted recombination was measured using a minimal MOPS X-Gal plating assay, essentially as described previously (Gally et al., 1993; Holden et al., 2001). In brief, for off-to-on recombination frequencies, a white colony was selected, diluted in PBS to a suitable cell number, and plated onto minimal MOPS X-Gal agar. The number of blue and white colonies was counted, and the frequency calculated as a function of the number of generations that the
original colony had been through, and was expressed as a value per cell per generation. Orientation of the fim switch was carried out essentially as described in Leathart & Gally (1998). fim DNA was amplified by PCR using primers 2535 and 3137, incorporating \( 7 \times 10^5 \) Bq [\(^{32}\)P]dATP for radiolabelling when necessary. The PCR-amplified products were digested asymmetrically with HinfI, and the fragments were resolved on 4% acrylamide gels in sodium borate buffer (Brody & Kern, 2004). If radiolabelled, the gels were dried and exposed to X-ray film for at least 12 h. Exposure of more than 24 h was carried out to determine whether there was any fim DNA in the ON orientation for the lysate containing the PpapBA ON construct.

Haemagglutination (HA) and yeast-cell agglutination. Human red blood cells (RBCs) were isolated from 5 ml whole blood and suspended in 10 ml PBS. For HA assays, RBCs were used at \( 1 \times 10^7 \) cells ml\(^{-1}\). Bacteria were harvested from CFA plates [optimal

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**Table 1.** *E. coli* strains used in this study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant genotype</th>
<th>Reference or source</th>
</tr>
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<tbody>
<tr>
<td>MG1655</td>
<td>K-12 F(^-) ( \lambda- )</td>
<td>Guyer et al. (1981)</td>
</tr>
<tr>
<td>AAEC185A</td>
<td>F(^-) ( \lambda- ) supE44 hsdR17 mcrA endA1 thi-1 ( \Delta(fimBEACDFGH) ) ( \Delta recA )</td>
<td>Blomfield et al. (1991)</td>
</tr>
<tr>
<td>AAEC370A</td>
<td>MG1655 ( \Delta lacZYA fimA::lacZYA fimE-am18 )</td>
<td>Blomfield et al. (1993)</td>
</tr>
<tr>
<td>BGEC144</td>
<td>MG1655 ( \Delta lacZYA \Delta fimE-fimA( \Delta sacB-Kan' ) ) fimA::lacZYA fimE-am18</td>
<td>Gally et al. (1994)</td>
</tr>
<tr>
<td>DL 2121</td>
<td>Lrp binding site 3 mutation in pap, results in a predominantly ON phenotype</td>
<td>Nou et al. (1995)</td>
</tr>
<tr>
<td>DL 2496</td>
<td>Lrp binding site 4 mutation in pap, results in a predominantly OFF phenotype</td>
<td>Nou et al. (1995)</td>
</tr>
<tr>
<td>J96</td>
<td>pap, prf</td>
<td>Hull et al. (1981); Marklund et al. (1992)</td>
</tr>
<tr>
<td>CFT073</td>
<td>fim, pap1, pap2, sfr</td>
<td>Mobjley et al. (1990, 1993)</td>
</tr>
<tr>
<td>ZAP973</td>
<td>Allelic exchange of the minimal fim switch unit from pNHT79 exchanged into BGEC144</td>
<td>This study</td>
</tr>
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<td>ZAP986</td>
<td>Allelic exchange of pap regulatory DNA from DL2121 (phase on) into the lac locus of AAEC370A</td>
<td>This study</td>
</tr>
<tr>
<td>ZAP987</td>
<td>Allelic exchange of pap regulatory DNA from DL2496 (phase off) into the lac locus of AAEC370A</td>
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<td>ZAP988</td>
<td>Allelic exchange of pap regulatory DNA from J96 into the lac locus of AAEC370A</td>
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<td>Allelic exchange of pap regulatory DNA from DL2121 (phase on) into the lac locus of CFT073</td>
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<td>ZAP990</td>
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<td>ZAP991</td>
<td>Allelic exchange of pap regulatory DNA from J96 into the lac locus of CFT073</td>
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<td>536</td>
<td>fim, prf, sfa</td>
<td>Knapp et al. (1986)</td>
</tr>
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<td>536 prf</td>
<td>Strain 536 with prf deleted</td>
<td>This study</td>
</tr>
<tr>
<td>536 sfa</td>
<td>Strain 536 with sfa deleted</td>
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</tr>
<tr>
<td>536 fim</td>
<td>Strain 536 with fim deleted</td>
<td>This study</td>
</tr>
<tr>
<td>536 prf sfa</td>
<td>Strain 536 with prf and sfa deleted</td>
<td>This study</td>
</tr>
<tr>
<td>536 prf fim</td>
<td>Strain 536 with prf and fim deleted</td>
<td>This study</td>
</tr>
<tr>
<td>536 prf sfa fim</td>
<td>Strain 536 with prf, sfa and fim deleted</td>
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**Table 2.** *E. coli* clinical isolates used in this study

<table>
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<tr>
<th>Associated symptoms</th>
<th>Isolate names</th>
<th>Reference</th>
</tr>
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<tbody>
<tr>
<td>Cystitis</td>
<td>AUTI 4, AUTI 7, AUTI 12, AUTI 19, AUTI 31, AUTI 36, AUTI 43, AUTI 47, AUTI 48, AUTI 62, AUTI 64, AUTI 66, AUTI 72</td>
<td>Keegan et al. (2003)</td>
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<tr>
<td>Pyelonephritis</td>
<td>AUTI 8, AUTI 11, AUTI 20, AUTI 67, AUTI 68, AUTI 69, AUTI 70, AUTI 71, AP4, AP7, AP18, IHE 1041, IHE 1049, IHE 1086, IHE 1106, IHE 1152, IHE 1190, IHE 1268, IHE 1402, IHE 1431, KS 71</td>
<td>Keegan et al. (2003), Pere et al. (1987), Pouttu et al. (2001)</td>
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<tr>
<td></td>
<td></td>
<td>Nowicki et al. (1984)</td>
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### Table 3. Plasmids used in this study

<table>
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<th>Reference</th>
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<td>pCR4 TOPO-TA</td>
<td>Commercial vector</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>pACYC184</td>
<td>Commercial vector</td>
<td>NEB</td>
</tr>
<tr>
<td>pPap5</td>
<td>Complete pap gene cluster from J96</td>
<td>Lindberg et al. (1984)</td>
</tr>
<tr>
<td>pIB307</td>
<td>pMAK705-based vector for allelic exchange; temperature-sensitive replicon</td>
<td>Blomfield et al. (1991)</td>
</tr>
<tr>
<td>pDG19</td>
<td>pMAK705-based vector for allelic exchange into fim; temperature-sensitive replicon</td>
<td>Gally et al. (1994)</td>
</tr>
<tr>
<td>pMM36</td>
<td>pACYC with fim switch in the ON orientation</td>
<td>Mcclain et al. (1991)</td>
</tr>
<tr>
<td>pMM34</td>
<td>pACYC with fim switch in the OFF orientation</td>
<td>Mcclain et al. (1991)</td>
</tr>
<tr>
<td>pEGFP</td>
<td>Commercial vector</td>
<td>Clontech</td>
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<td>pAJR32</td>
<td>pAJR25 with sacB-kan cassette inserted between lacIA</td>
<td>Porter et al. (2004)</td>
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<td>pAJR28</td>
<td>pAJR25 with promoterless egfp inserted between lacIA</td>
<td>This study</td>
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<td>pAJR145</td>
<td>pACYC rpsM::egfp transcriptional fusion</td>
<td>Roe et al. (2004)</td>
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<td>pKC8</td>
<td>CFT073 lacI and lacA regions in pIB307</td>
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<td>pKC11</td>
<td>pCK8 with sacB kan cassette inserted between lacIA</td>
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<td>pKC12</td>
<td>pKC8 with promoterless egfp inserted between lacIA</td>
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<td>pNJH20</td>
<td>pBAD18 containing papB ORF from J96</td>
<td>Holden et al. (2001)</td>
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<td>pNJH79</td>
<td>pDG19 containing the minimal fim switch in the OFF orientation</td>
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<td>pNJH83</td>
<td>pAJR28 with pap regulatory DNA from DL 2121</td>
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<td>pNJH86</td>
<td>pKC12 with pap regulatory DNA from DL 2121</td>
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<td>pNJH84</td>
<td>pAJR28 with pap regulatory DNA from DL 2496</td>
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<td>pNJH87</td>
<td>pKC12 with pap regulatory DNA from DL 2496</td>
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<td>pNJH85</td>
<td>pAJR28 with pap regulatory DNA from J96</td>
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<td>pNJH88</td>
<td>pKC12 with pap regulatory DNA from J96</td>
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<td>pNJH97</td>
<td>pAJR145, minimal fim switch fused to gfp+, replacing rpsM</td>
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### Table 4. Primers used in the study

<table>
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<th>Primer</th>
<th>Sequence (5’–3’)</th>
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<td>CGC GGA TCC GCA TGC CCA CAG ATT TGA GAG TTA</td>
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<tr>
<td>178P</td>
<td>CGG GTA CCC ATA AAT AAC AAT GCT TTT TTC ATT AC</td>
</tr>
<tr>
<td>196P</td>
<td>GTG CAG ATT AAC ATC AGG GG</td>
</tr>
<tr>
<td>197P</td>
<td>ATG CTC ATA CTG GCC GTG GT</td>
</tr>
<tr>
<td>135F</td>
<td>CCG GCG CAT GCT AAA TAC AAG ACA ATT GGG GCC AAA CTG TCC</td>
</tr>
<tr>
<td>136F</td>
<td>CCG GAT CCC CAA AAG ATG AAA CAT TTG GGG CC</td>
</tr>
<tr>
<td>151F</td>
<td>CTA GTC TAG ATG CAT GCT AAA TAC AAG ACA ATT GGG GCC AAA CTG TCC</td>
</tr>
<tr>
<td>152F</td>
<td>CTA GTC TAG ACC AAA AGA TGA AAC ATT TGG GCC C</td>
</tr>
<tr>
<td>2535</td>
<td>GCC GGA TTA TGG GAA AGA</td>
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<td>3137</td>
<td>GCC GCT GTA GAA CTG AGG</td>
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<td>prf1</td>
<td>GCA AGA ATC ATT ATT CTT TTG CCT GAA GCT ATC CGG CAT ACT CAG GCA TTT CAC GCT TTA GTG TAG GCT GGA GCT GT T</td>
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<td>prf2</td>
<td>CTG ATG TAA CTT TTA TCT GTT TCA GTG AAG CAT GTC CAC AAG TTA AGT TAT TAA CAT ATG AAT ATC CTC CTG AGT TGC TA</td>
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<td>sfa1</td>
<td>AAT CTG CAC TCT GAT GTA ACT TTT ATC TGT TTC ATG GAA GTA TGC CCA CAG ATT GAG TTA GTG TAG GCT GGA GCT GT T</td>
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<td>sfa2</td>
<td>TCA CTA GGT CTT TCT GCA ACA CTA CTG CTT TCA ACA AGT CAG GCA TTT CAC ACT CAT ATG AAT ATC CTC CTG AGT TGC TA</td>
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<td>fim1</td>
<td>TAT TGC TAA CCC AGC ACA GCT AGT GCG CGT CTG TAA TTA TAA GGG AAA AAC GAT GGT GTA GGC TGG AGC TGC TT</td>
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<td>fim2</td>
<td>TTT AGC TTC AGG TAA TAT TGC GTA CCA GCA TTA GTA ATG TCC TGT CAT TTC TTT ACA TAT GAA TAT CCT CCT</td>
</tr>
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for expression of colonization factor antigen (Evans et al., 1977),
the OD_{600} adjusted to 1·0 (measured in a Cecil Aurius CE2021 spec-
trophotometer, path-length 10 mm), and diluted twofold in PBS.
Equal volumes of RBCs were mixed with bacterial dilutions in a 96-
well plate and allowed to agglutinate at 4°C for at least 16 h. The agglutinating titre was determined as the lowest dilution that pre-
vented formation of a defined pellet of RBC. Yeast-cell agglutination
was carried out with baker’s yeast (Saccharomyces cerevisiae).
Clinical isolates were plated onto LB agar. Five single colonies of
each strain were pooled, inoculated into 3 ml LB and incubated sta-
tically for 24 h. The cultures were reincubated into a further 3 ml
LB and incubated statically for another 24 h. Agglutination was car-
rried out by mixing bacterial and yeast suspensions on glass slides
and the degree of clumping was assessed. For all agglutinations, 1%
(w/v) mannose was added as required.

Flow cytometry. Bacteria transformed with pNJH97 were plated
on CFA for two subsequent days, recovered as described for HA,
and washed once in an equal volume of PBS, pre-warmed to 37
°C. The bacteria were then suspended in 1 ml PBS containing 0·1%
(v/v) formaldehyde. Fluorescence was measured at 488 nm using a
BD FACS Caliber flow cytometer. At least 20,000 events were gated,
and in each case the negative control was set to 0.5%.

Statistical analysis. Statistical analysis of association of the number
of P clusters with isolate class, correlation between number of P clus-
ters and mannose-resistant HA (MRHA), and analysis of variance of
fluorescence levels using pNJH97 between the isolate classes (Table 2)
were carried out using the Minitab computer program. The threshold
for statistical significance was \( P < 0·05 \).

RESULTS

Single-copy papB represses recombination of
the fim switch in E. coli K-12 and CFT073
backgrounds

To examine repression of fim recombination by papB from the
Pap regulatory region on the chromosome, three variants
of the sequenced urinary tract isolate E. coli CFT073 were
constructed. Each of the constructs contained different pap
regulatory alleles placed in single copy at the lac locus. The regulatory alleles included the ORFs of both regulators, papI
and papB, with the intergenic region containing the divergent
promoters for papI and papB. Alteration of two of the leucine-
responsive regulatory protein (Lrp) binding sites within the
intergenic region has previously been shown to result in apparent
constitutive expression or repression of the papBA
promoter (Nou et al., 1995). We used these altered sequences
to derive three variants of the regulatory alleles: PpapBA ON,
PpapBA OFF and wild-type PpapBA (phase variable). In each
case, the regulatory alleles were fused to the enhanced green
fluorescent protein (GFP) reporter (egfp) to verify expression
status by fluorescence microscopy. In strains containing the
locked ON allele, the majority of the bacterial population
were found to be fluorescent, whereas the majority of bacteria
were found to be non-fluorescent in strains containing the
locked OFF allele (data not shown).

The status of type 1 fimbriae expression was then measured
in the presence of these three different alleles using fim
switch (fimS) orientational analysis and agglutination
methods. PCR and restriction digestion analysis of the fim
switch (Leathart & Gally, 1998) from the E. coli CFT073-
derived strains was carried out with \(^{32}\text{P}\)dATP to increase
the sensitivity with which the fim switch in the on orienta-
tion could be detected. The constructs were grown under
conditions considered to optimize Pap expression (Evans
et al., 1980), which consistently resulted in low levels of type
1 fimbrial expression. When papB was locked in phase off in
the majority of cells [ZAP990 (PpapAB OFF, CFT073)], or
was subject to wild-type phase-variable expression [ZAP991
(PpapAB J96, CFT073)], fimS DNA in both on and off
orientations was detected. However, when papB was
expressed in the majority of cells [ZAP989 (PpapBA ON,
CFT073)], it was not possible to detect any fimS DNA in the
on orientation (Fig. 1).

The presence of functional type 1 fimbrial expression was
assessed in the E. coli CFT073-derived constructs by agglu-
tination with yeast cells and with human RBCs. Expression of
papB in ZAP989 (PpapBA ON, CFT073) resulted in an
absence of type 1-dependent yeast agglutination and a
fourfold reduction in both MRHA and mannose-sensitive
HA (MSHA) titres, relative to the wild-type. In contrast,
constructs that contained the PpapAB OFF, variable (ZAP990
and ZAP991) or wild-type CFT073 were all capable of rapid
and complete yeast cell agglutination and a high level of
MSHA and MRHA [titre of 4 (log_2) or greater]. These results
demonstrate the absence of type 1 fimbrial expression in the
presence of a phase on regulatory allele expressing PapB.

The same mutated Pap regulatory alleles were exchanged
into E. coli K-12 (AAEC370A, fimE fimA:: lacZYA, Table 1),

enabling measurement of FimB recombination frequencies
using this established lacZ reporter system [described in
Methods and in Gally et al. (1993)]. A 20-fold reduction in
the frequency of FimB recombination in the off-to-on
orientation was observed in strain ZAP986 (Table 1) contain-
ing the phase on allele in which PapB is expressed in the
majority of bacteria. A frequency of \( 3 \times 10^{-5} \pm 1 \times 10^{-5} \)
was measured in the wild-type strain AAEC370A
compared to \( 0 \times 10^{-5} \pm 1 \times 10^{-5} \) in ZAP986 (PpapBA
ON, K-12). In contrast, FimB switching was not affected in
the strains containing either the phase OFF allele or the
wild-type pap regulatory regions (ZAP987 and ZAP988,
respectively, Table 1). The recombination frequency was
shown to be \( 2 \times 10^{-5} \pm 1 \times 10^{-6} \) for ZAP987
(PpapBA OFF, K-12) and \( 3 \times 10^{-5} \pm 3 \times 10^{-5} \) in
ZAP988 (PpapAB J96, K-12). Taken together, the results in
the two different backgrounds prove that expression of papB
from its natural promoter in the chromosome is able to
inhibit FimB recombination and therefore prevent type 1
fimbrial expression.

Deletion of P-related fimbrial clusters in the
clinical isolate E. coli 536 increases fim
expression

A second approach to examine the impact of Pap [and the
related S-fimbrial adhesin (SfA)] clusters on fim was taken
using a novel transcriptional GFP reporter system (GFP^+)
Expression of type 1 fimbriae is inversely related to P-related fimbrial expression in uropathogenic E. coli (UPEC) isolates

We expanded the study to examine whether cross-talk between Pap and type 1 clusters in different clinical isolate backgrounds.

The pyelonephritis isolate E. coli 536 contains a type 1 fimbrial cluster, a Pap-related fimbrial cluster, termed Prf (PapG class III) and an Sfa fimbrial cluster (Berger et al., 1982). There is a high degree of homology between the Prf and Sfa clusters, in particular in the PapB- and PapI-like regulators (Dobrindt et al., 2001; Holden et al., 2001), and SfaB is also capable of repressing FimB-promoted recombination of the fim switch (Holden et al., 2001). All three clusters were deleted to give combinations of single, double and triple knock-outs, using the i-Red allelic exchange system (Table 1). Transformation of the fim readout plasmid (pNJH97, Table 3) into wild-type E. coli 536 resulted in a mean of 2.5% of the bacterial population that expressed the fim::gfp+ fusion. Deletion of the fim cluster (including the recombinase genes) led to only background levels of expression of the fusion, as anticipated (Fig. 2A). In contrast, deletion of the prf cluster resulted in a 1.8-fold increase in the percentage of fluorescent bacteria compared to the wild-type. Deletion of sfa resulted in a similar increase in the level of fim expression, although there appeared to be a larger degree of variation between replicates of the sfa strain. The combined prf and sfa deletions resulted in a 2.3-fold increase in the percentage of bacteria that expressed the fim::gfp+ fusion (Fig. 2A, B). The level of fim expression in the double mutant was consistently higher than that in either of the single-mutant prf or sfa strains. Thus, repression of fim expression, even when present at multi-copy levels on a plasmid, is detectable from chromosomal copies of Pap-related fimbrial clusters in a clinical isolate.
isolates were characterized for Pap-related adhesin gene clusters to determine both genetic carriage and fimbrial expression. The number of Pap-related clusters in each of the clinical isolates was determined by Southern blotting with probes against \textit{papB} and \textit{papF} (Fig. 3, Table 5). Despite selective bias of the presence of at least one P-related cluster (normally asymptomatic isolates tend not to carry P-related clusters), analysis of association using the chi square test revealed that the pyelonephritis isolates were more likely to possess multiple P fimbrial clusters than the asymptomatic isolates \((P=0.009)\). The cystitis isolates also differed from the pyelonephritis isolates, since they were shown to be more likely to carry only one P fimbrial cluster \((P=0.056)\).

Functional expression of the Pap-related adhesin gene clusters was assessed by MRHA of human RBCs, a measure of P-fimbrial binding capacity (Norgren et al., 1984). Analysis of variance showed that the mean level of MRHA was significantly higher in the cystitis group compared to the asymptomatic isolates \((P=0.037)\). In addition, the median level of MRHA was shown to be higher for both the pyelonephritis and cystitis groups compared to the asymptomatic group (Fig. 4). The asymptomatic group contained the highest number of isolates that did not demonstrate MRHA, 31·25\%, while only 7·70\% of the cystitis and 14·29\% of the pyelonephritis isolates did not show MRHA. There was significant positive correlation between the level of MRHA and the number of P clusters for the isolates, Pearson correlation \((r^2)\) of 0·321, \(P=0.023\), showing that those isolates with more P clusters tended to show an increased level of MRHA, as expected from other studies (Blanco et al., 1997; Hull et al., 1994). It should be noted that although the use of human RBCs alone limits recognition to functional expression of PapG class II, since PapG class III binds preferentially to the Forssman antigen present in other species (Marklund et al., 1992), the majority of isolates were positive for agglutination, so MRHA with human RBCs was judged to be sufficient for the purposes of this study. The fact that the pyelonephritis and cystitis groups were more likely to carry a greater number of P clusters than asymptomatic groups and to express P fimbriae increased the probability of \textit{papB} expression in each cell.

### Table 5. Number of Prf clusters determined by Southern blot

The number of isolates carrying one, two or three Prf adhesin gene clusters is given, together with the value as a percentage in parentheses.

<table>
<thead>
<tr>
<th>Isolate group</th>
<th>1 Cluster</th>
<th>2 Clusters</th>
<th>3 Clusters</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asymptomatic</td>
<td>11 (69)</td>
<td>2 (12)</td>
<td>3 (19)</td>
</tr>
<tr>
<td>Cystitis</td>
<td>8 (62)</td>
<td>4 (30)</td>
<td>1 (8)</td>
</tr>
<tr>
<td>Pyelonephritis</td>
<td>11 (52)</td>
<td>9 (43)</td>
<td>1 (5)</td>
</tr>
</tbody>
</table>
To assess the impact of P-related clusters on fim in the clinical isolates, the fim::gfp plasmid (pNJH97) was transformed into each of the clinical UPEC isolates and expression of the fusions assessed by flow cytometry. The values for each isolate were grouped and analysed for variance between the median values for each isolate class by the Kruskal–Wallis test for nonparametric data (Table 6). Expression of fim::gfp at the population level was clearly lower in the pyelonephritis group, determined from the median values. In contrast, the highest overall level of fim::gfp expression was seen in the asymptomatic group. The rank values for both the asymptomatic and cystitis isolate classes were closest to the overall rank, whereas the corresponding value for the pyelonephritis class was 15.5, indicating that there was a difference from the other two classes, although the P value for the difference is not significant (0.145).

Cross-talk was analysed further in these isolates by HA and orientation analysis of the fim switch (Graham et al., 2001; Lim et al., 1998). Assessment of the potential of each isolate to express fim when grown in static broth, conditions considered to be optimal for type 1 fimbrial expression, resulted in positive mannose-sensitive yeast agglutination (MSYA) for 13 of the 18 pyelonephritis isolates, seven of the 11 cystitis isolates and nine of the 13 asymptomatic isolates (Fig. 5B). Under different culture conditions known to induce P expression (Evans et al., 1980), the HA titres showing the level of type 1 fimbrial expression were reduced (Fig. 5A). Even so, under these conditions, expression of type 1 fimbriae was significantly lower in both the cystitis and pyelonephritis groups compared to the asymptomatic group (P=0.002). This difference was confirmed by PCR and restriction digestion of the fim switch (data not shown). In summary, when P-fimbrial expression is promoted this correlates with a reduction in type 1 fimbrial expression.

**DISCUSSION**

At the single-cell level, expression of adhesins, flagella, capsules and other surface components is likely to be coordinated by bacteria to prevent co-expression of competing factors or occlusion of one factor by another. In turn, such co-ordination should limit immune stimulation. Our previous work has demonstrated the potential for such co-ordination between the P/S and type 1 fimbrial clusters. The work showed that PapB is a repressor of fimA expression, acting at the level of FimB recombinase activity and fimE transcription. The majority of the published work has been carried out in E. coli K-12, and uses multicopy levels of papB from an inducible promoter (Holden et al., 2001; Xia et al., 2000). The aim of the present study was to test whether the regulation is apparent when papB is expressed from its native promoter on the chromosome and whether this regulatory cross-talk can be detected in clinical isolates. The research used three different approaches to investigate cross-talk in UPEC. Two required modification of well-characterized clinical isolates, E. coli CFT073 and E. coli 536, while the third looked at fimbrial expression in a collection of isolates associated with different classes of UTI.

Data generated by all three methods support the conclusion that PapB expressed at wild-type levels does inhibit type 1 fimbrial expression in clinical isolates.

Cross-talk was proven by the use of Lrp-binding-site mutations within the papl–papB intergenic region, originally constructed by David Low’s group (Nou et al., 1995). These mutations limit transition between the phase on and phase off states of P expression, resulting in a bacterial population the majority of which is in either the off or the on expression state. For this study, these mutated regions covering papl–papB were cloned in front of eGFP and then inserted into both E. coli K-12 and CFT073 backgrounds. In the on state, the majority of the bacteria expressed GFP, as determined by fluorescence microscopy, and will contain PapB; in the off state, the converse is true. A twentyfold reduction in FimB inversion of the fim switch from the off to the on orientation was measured in the presence of the ‘on’ P

**Table 6. Kruskal–Wallis test for fim::gfp expression**

<table>
<thead>
<tr>
<th>Isolate group (n)</th>
<th>Median</th>
<th>Mean rank</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asymptomatic (12)</td>
<td>13.87</td>
<td>22.4</td>
</tr>
<tr>
<td>Cystitis (9)</td>
<td>8.92</td>
<td>23.1</td>
</tr>
<tr>
<td>Pyelonephritis (17)</td>
<td>6.91</td>
<td>15.5</td>
</tr>
<tr>
<td>Overall (38)</td>
<td></td>
<td>19.5</td>
</tr>
</tbody>
</table>
promoter region, while no difference was measured in the presence of the ‘off’ P region. As described, these Lrp-binding mutations do not completely lock P phase variation, and so a proportion of the bacterial population does still undergo phase transition. A completely locked on P regulatory region is likely to demonstrate an increased repression of FimB switching, perhaps achieving the complete repression observed for induced levels of the regulator cloned on a plasmid (Holden et al., 2001).

A further demonstration of the repression of type 1 fimbriae by P/S clusters was provided by using the fim::gfp + fusion in different E. coli 536 mutants that had had fim, prf, sfa clusters deleted in various combinations. This work showed that the proportion of bacteria expressing type 1 fimbriae increased by 2-3-fold in a strain that had had the Prf and S clusters deleted, and by 1-8-fold in a strain deleted for Prf. While these differences are subtle at the population level, they were reproducible and support the additive repressive effect of PapB homologues on type 1 fimbrial expression. This regulation was measurable despite the fact that the PapB/SfaB regulators from the chromosome were acting on a plasmid-based fim::gfp + construct.

To examine cross-talk in other E. coli isolates, a set of 50 human clinical isolates that contained P and type 1 fimbrial gene clusters were selected and grouped according to their associated UTI symptoms into the categories asymptomatic, cystitis and pyelonephritis. The isolates chosen for the study all contained at least one P cluster, so that an assessment of cross-talk with type 1 fimbriae could be carried out. Southern blotting revealed that pyelonephritis isolates were more likely to possess two or more P-related clusters when compared to the asymptomatic isolates. The number of P clusters present in the cystitis isolates was also higher than in the asymptomatic group. Functional expression of the P gene clusters was assessed using MRHA, and this correlated significantly with the number of P clusters present in each isolate and therefore with the disease associated with the isolate. While all bacteria assayed contained at least one P cluster, the asymptomatic group had the highest proportion of bacteria that had no clear MRHA, implying either that in these strains the expression of P fimbriae is repressed or that mutations have occurred in the operons that prevent expression. This is consistent with asymptomatic strains not expressing factors that provoke an inflammatory response (Graham et al., 2001; Hull et al., 1999; Lim et al., 1998).

Expression of type 1 fimbriae in the different isolate groups was examined at the level of the DNA (fim switch orientation), promoter activity and functional fimbrial binding. These methods were selected to demonstrate fimbrial expression in the absence of a suitable generic antibody that would recognize fimbriae consistently for a large range of clinical isolates. In each case, the overall trend was the same: type 1 fimbrial expression was repressed in the pyelonephritis group compared to the asymptomatic isolates. This was confirmed using a novel plasmid-based GFP reporter fusion for measurement of fimA expression, supporting the concept that the higher the level of P/S expression, the lower the level of type 1 fimbrial expression.

Taken together with our previous work, the current research demonstrates that P/S expression in clinical isolates leads to type 1 fimbrial repression through the activity of PapB/SfaB. While recent work has uncovered environmental cues in the host that down-regulate type 1 fimbriae, possibly in response to inflammation (El-Labany et al., 2003; Roesch et al., 2003; Schwan et al., 2002), very little is known about the signals that stimulate P expression (Blomfield, 2001). Recently, Snyder et al. (2005) have demonstrated that locking the fim switch in the off orientation in E. coli CFT073 leads to an increase in the expression of one of the P-fimbrial clusters. This provides evidence that cross-talk can work in both directions to prevent multiple fimbrial expression at the level of the single bacterial cell. Other fimbrial and non-fimbrial adhesins, along with flagella and capsule, are also likely to be regulated co-ordinately at the single-cell level to achieve systematic expression that prolongs the infection and maximizes the number of bacteria produced (Barnich et al., 2003; Schwan et al., 2005). Therefore, while phase
variation is considered a stochastic process, it is controlled by other regulatory networks that govern organelle expression and environmental responses. Understanding these regulatory networks will be important in the development of novel strategies to treat UTIs, such as deliberately providing signals that down-regulate or switch off adhesin expression.

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REFERENCES


Cross-talk between type 1 and P/S fimbriae


