Comparative analysis of FimB and FimE recombinase activity

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FimB and FimE are site-specific recombinases, part of the λ integrase family, and invert a 314 bp DNA switch that controls the expression of type 1 fimbriae in *Escherichia coli*. FimB and FimE differ in their activity towards the *fim* switch, with FimB catalysing inversion in both directions in comparison to the higher-frequency but unidirectional on-to-off recombination catalysed by FimE. Previous work has demonstrated that FimB, but not FimE, recombination is completely inhibited *in vitro* and *in vivo* by a regulator, PapB, expressed from a distinct fimbrial locus. The aim of this work was to investigate differences between FimB and FimE activity by exploiting the differential inhibition demonstrated by PapB. The research focused on genetic changes to the *fim* switch that alter recombinase binding and its structural context. FimB and FimE still recombined a switch in which the majority of *fimS* DNA was replaced with a larger region of non-*fim* DNA. This demonstrated a minimal requirement for FimB and FimE recombination of the Fim binding sites and associated inverted repeats. With the original leucine-responsive regulatory protein (Lrp) and integration host factor (IHF)-dependent structure removed, PapB was now able to inhibit both recombinases. The relative affinities of FimB and FimE were determined for the four 'half sites'. This analysis, along with the effect of extensive swaps and duplications of the half sites on recombination frequency, demonstrated that FimB recruitment and therefore subsequent activity was dependent on a single half site and its context, whereas FimE recombination was less stringent, being able to interact initially with two half sites with equally high affinity. While increasing FimB recombination frequencies failed to overcome PapB repression, mutations made in recombinase binding sites resulted in inhibition of FimE recombination by PapB. Overall, the data support a model in which the recombinases differ in loading order and co-operative interactions. PapB exploits this difference and FimE becomes susceptible when its normal loading is restricted or changed.

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INTRODUCTION

Site-specific recombination controls the phase-variable expression of the ubiquitous type 1 fimbrial adhesin in *Escherichia coli*. Type 1 fimbriae are important for bacterial adherence to both biotic and abiotic surfaces and have a specific interaction with α -D-mannosides via the FimH

adhesin (Krogfelt *et al.*, 1990). Type 1 fimbriae are considered to be important for the colonization of the human gastrointestinal tract (Adlerberth *et al.*, 1995) and oropharynx (Donaldson *et al.*, 1991; Guerina *et al.*, 1983). They are a virulence factor for urinary tract infections (Connell *et al.*, 1996; Iwahi *et al.*, 1983; Schaeffer *et al.*, 1987) and degranulation triggered by these fimbriae contributes to renal scarring (Mizunoe *et al.*, 1997).

Phase-variable expression of type 1 fimbriae exhibited by single bacterial cells results from the activity of two site-specific recombinases that invert a 314 bp DNA element termed the *fim* switch (or *fimS*) (Abraham *et al.*, 1985;

Abbreviations: EMSA, electrophoretic mobility shift assay; IHF, integration host factor; IR, inverted repeat; IRL, left inverted repeat; IRR, right inverted repeat; Lrp, leucine-responsive regulatory protein.

A supplementary table listing the strains, constructs, plasmids and primers used is available with the online version of this paper.

Klemm, 1986) (see Fig. 1a). A promoter that drives expression of the *fim* genes required for export and structural assembly of type 1 fimbriae is located within *fimS* (Klemm *et al.*, 1985). The *fim* operon is only expressed when the *fim* switch is inverted to the on orientation, allowing transcription of the *fim* genes. FimB and FimE, required to invert *fimS*, are members of the λ integrase family of site-specific recombinases (Eisenstein *et al.*, 1987) (for a review see Hallet & Sherratt, 1997). Recombination of *fimS* is distinct from the related Xer-mediated recombination in that the recombinases act independently to invert *fimS* (Blakely *et al.*, 1993; Blomfield *et al.*, 1991b; Gally *et al.*, 1996; Klemm, 1986; McClain *et al.*, 1991). Each inverted repeat (IR) is flanked by overlapping FimB and FimE binding sites, and following occupancy of

these sites they recombine the switch within the IR sequence. As for λ phage chromosomal integration and excision, *fim* recombination also requires accessory proteins, specifically integration host factor (IHF) and the leucine-responsive regulatory protein (Lrp) (Blomfield *et al.*, 1993, 1997). These proteins are believed to contribute to the overall architecture of the *fim* switch that facilitates synapse of the 9 bp IRs. The genetic organization and associated protein binding sites of *fimS* are represented in Fig. 1(a).

Lrp interaction with the *fim* switch changes depending on the level of intracellular alanine and leucine, and as aliphatic amino acids are enriched in the structural proteins of type 1 fimbriae, fimbriation may be linked to their abundance (Roesch & Blomfield, 1998). However, the level of these amino acids could also act as a signal of inflammation, as aliphatic amino acids are particularly sensitive to oxidation by reactive oxygen species (Stadtman & Levine, 2003). Recently, it has been proposed that Lrp can act as a recombination directionality factor (RDF) that favours recombination in the off-to-on direction under certain conditions (Kelly *et al.*, 2006).

However, it is the activity of the FimB and FimE recombinases that predominantly determines the orientation of the *fim* switch. While FimB and FimE share 52 % amino acid identity and bind to overlapping sites, both their activity and their directionality are different (Gally *et al.*, 1996). FimB catalyses inversion in both directions, although with a slight bias for the off-to-on orientation, while FimE predominantly catalyses on-to-off inversion. Control of FimE expression is important in bringing about its orientation bias (Kulasekara & Blomfield, 1999); as the *fim* switch is located at the end of *fimE*, the orientation of *fimS* determines the length and 3' sequence of the *fimE* transcript (Hinde *et al.*, 2005; Sohanpal *et al.*, 2001). As a consequence, *fimE* mRNA is likely to be subject to more rapid 3' to 5' degradation when the switch is in the off orientation than when it is in the on orientation. In addition, FimE preferentially binds to *fimS* in the on orientation, as has been demonstrated *in vitro* and *in vivo* (Kulasekara & Blomfield, 1999), which adds to the directional bias. A further difference between FimB and FimE is that FimB inversion frequencies are markedly lower than those exhibited by FimE, both *in vitro* and *in vivo* (Blomfield *et al.*, 1991b; Gally *et al.*, 1993).

Previous work has demonstrated that when pyelonephritis-associated pili (P-pili) are switched on, PapB prevents expression of type 1 fimbriae in the same bacterial cell by inhibition of FimB activity (Holden *et al.*, 2006; Xia *et al.*, 2000). In contrast, FimE recombinational activity, and hence rapid on-to-off phase switching, is not inhibited by PapB *in vivo* or *in vitro*. In fact, FimE expression is increased at the transcriptional level, raising the frequency with which *fimS* in the on orientation is turned off (Xia *et al.*, 2000). PapB regulates P-pili expression by binding to specific sites on *pap* DNA (Blyn *et al.*, 1989; Forsman *et al.*,

Fig. 1. Illustration of the *fim* switch, minimal switch and replacement *fim* constructs used in the study. (a) Genetic organization of the *fim* switch in the on orientation in strain AAEC370A. (b) Comparative map of the minimal *fimS* region in ZAP973 in which the regions flanking the *fim* switch have been deleted. (c) Comparative map of the replacement *fim* switch. The internal region of the *fim* switch was replaced on the *E. coli* chromosome with 1167 bp of λ DNA. This generates a novel switch that still contains the Fim recombinase binding sites and the IRs, but now lacks the original Lrp, IHF and PapB binding sites both within the switch and externally, as the replacement switch was constructed within the minimal switch shown in (b). A *P_{tac}* promoter was also inserted into the replacement switch to allow measurement of switching frequencies in conjunction with a *fimA::lacZYA* fusion. In all cases, the relevant protein binding sites are shown as black boxes (IHF and Lrp), grey boxes (recombinases) and asterisks (putative PapB binding sites; identified previously by EMSA and described in Holden *et al.*, 2001 and Xia *et al.*, 2000). The IR sequences, IRL and IRR, are shown as white arrowheads. The recombinase binding sites are recognized by FimB and FimE (to different extents) and are located adjacent to and partially within the IR sequences. ORFs for *fimB*, *fimE*, *fimA* and the *lacZYA* fusion are indicated by open boxes. The locations of relevant promoters (arrows) and restriction endonuclease sites are also indicated.

1989; Xia *et al.*, 1998). PapB has also been shown to bind to *fim* DNA (Fig. 1a) (Holden *et al.*, 2001; Xia *et al.*, 2000), which raises the possibility that inhibition may be the result of occlusion of FimB, but not FimE, from *fimS*. Inhibition of FimB-promoted recombination by PapB is not directional and occurs *in vitro*, and hence must arise from a direct effect on FimB recombination. The specificity shown by PapB suggests that the mechanism of inhibition is due to differences in how the recombinases bind to and complex with *fimS*, and not the recombination event per se.

In the current study we aimed to understand the basis of the differences between FimB- and FimE-mediated recombination by making use of the differential inhibition exhibited by PapB. The research made use of deletions, duplications, swaps and replacements within and adjacent to the *fim* switch (*fimS*) and also measured the relative affinities of FimB and FimE for the four 'half sites' that flank the 9 bp IRs.

METHODS

Culture conditions. Media used included Luria–Bertani (LB) broth (per litre: 10 g tryptone, 5 g yeast extract and 5 g NaCl; BDH-Merck) and LB agar (LB broth containing 1.5 % agar; BDH-Merck). MOPS medium supplemented with 10 µM thiamine and glycerol (0.2 %, w/v) was as described elsewhere (Neidhardt *et al.*, 1974). Indicator media were minimal MOPS plates supplemented with X-Gal (40 µg ml⁻¹; Melford Laboratories) and the appropriate antibiotic, as described in Gally *et al.* (1993). Protein expression was induced with arabinose or IPTG at the concentrations indicated. Antibiotics used were ampicillin (Amp; 50 µg ml⁻¹) and tetracycline (Tet; 25 µg ml⁻¹).

Cloning techniques and the generation of different *fimS* alleles

DNA was purified with Promega or Hybaid DNA purification mini columns. Restriction enzymes were purchased from either New England Biolabs or Boehringer Mannheim. All the molecular biology techniques were carried out according to standard protocols (Sambrook *et al.*, 1989). All the primers, plasmids and bacterial strains used or generated are listed in Supplementary Table S1 available with the online version of this paper.

Individual half site cloning. The four different half sites were amplified by PCR, with different primer sets depending on the half site to be cloned. The primers used were 2893 and 3139 [right inverted repeat (IRR) outside], 2774 and 3004 (IRR inside), 2476 and 2693 [left inverted repeat (IRL) outside] and 2632 and 2812 (IRL inside). The PCR products were digested with *Hae*III, and treated with the Klenow fragment of DNA polymerase I, and each half site was cloned into *Sma*I-digested pUC21. Successful clones were selected using *E. coli* XL1. Clones were checked by sequencing and the ones used in the study all had the half sites oriented in the same direction within the vector. This was to help ensure that surrounding vector DNA had no impact on the comparison of binding affinities. Four plasmids were created, each containing different half sites inserted into the multi-cloning site of pUC21 (pJL043, IRR outside; pJL044, IRL outside; pJL046, IRL inside; pJL077, IRR inside).

Cloning half site changes. ZAP1164: wild-type *fimS* in *fimB*⁻ *fimE*⁻ background. Plasmid pDG019 was exchanged into BGEC150 to generate ZAP1164.

ZAP1169: duplication of IRL-out to IRR-out (designed to strengthen FimB and weaken FimE affinities and to disrupt a triple A sequence). PCR was used to generate a downstream fragment with the F107 and F90 primers and an upstream fragment with the F108 and F90 primers. The fragments were digested with *Bgl*II, and annealed and amplified with the F90 and F91 primers, and the complete fragment was cloned into the *Sph*I and *Bam*HI sites in pDG019 to generate pNJH66, via pCR4-TOPO T/A. Strain ZAP1169 was then generated by allelic exchange of pNJH66 into BGEC144.

ZAP1170 and ZAP1171: duplication of IRR-in (on) to IRR-out (designed to strengthen FimB affinity marginally and to weaken FimE affinity as well as to disrupt triple A sequence). PCR was used to generate a downstream fragment with primers F107 and F90, and an upstream fragment with primers F109 and F90. The fragments were digested with *Bgl*II, and annealed and amplified with primers F90 and F91, and the complete fragment was cloned into the *Sph*I and *Bam*HI sites in pDG019 to generate pNJH67, via pCR4-TOPO T/A. Strain ZAP1170 was then generated by allelic exchange of pNJH67 into BGEC144. Strain ZAP1171 was generated by allelic exchange of pNJH67 into BGEC132.

ZAP1172 and ZAP1173: deletion of 185 bp downstream of IRR-out. pNJH67 was digested with *Bgl*II and *Bam*HI, the intervening 185 bp were removed, and the plasmid religated to generate pNJH92. Strain ZAP1172 was generated by allelic exchange of pNJH92 into BGEC144. Strain ZAP1173 was generated by allelic exchange of pNJH92 into BGEC132.

ZAP1176: designed to alter IRR-out to more closely resemble IRL-in (on), also to delete flanking DNA upstream of IRL-out and IRR-out. PCR was used to generate the fragment containing the alteration at IRR-out, using primers F135 and F142, and this was cloned into the *Sph*I and *Bam*HI sites of pDG019 via pCR4-TOPO T/A. Strain ZAP1176 was generated by allelic exchange of pNJH137 into BGEC144.

ZAP1177: designed to alter IRR-out to more closely resemble IRL-in (on), with swapped IRs and deletion of flanking DNA upstream of IRL-out and IRR-out. PCR was used to generate the fragment containing the alteration at IRR-out, using primers F143 and F144, and this was cloned into the *Sph*I and *Bam*HI sites of pDG019 via pCR4-TOPO T/A. Strain ZAP1177 was generated by allelic exchange of pNJH138 into BGEC144.

ZAP1178: designed to combine duplication of IRR-in (on) to IRL-in (on) and IRR-out with deletion of flanking DNA upstream of IRL-out and IRR-out. PCR was used to generate the fragment containing the alteration at IRL-in and IRR-out, using primers F145 and F141, and this was cloned into the *Sph*I and *Bam*HI sites of pDG019 via pCR4-TOPO T/A. Strain ZAP1178 was generated by allelic exchange of pNJH139 into BGEC144.

ZAP1179: designed as ZAP1178, but with flanking region upstream of IRL-out restored by PCR to maintain functional *fimE*. PCR was used to generate the fragment to repair *fimE*, using primers F150 and F90, and this was cloned into the *Sph*I site of pNJH139 to produce pNJH140. Strain ZAP1179 was generated by allelic exchange of pNJH140 into BGEC144.

ZAP1174 and ZAP1175: IRL-out swapped with IRR-out. pHK156 was digested with *Sac*I to remove the pUC19-derived origin of replication, creating pNJH91. Strain ZAP1174 was generated by allelic exchange of pNJH91 into BGEC144. Strain ZAP1175 was generated by allelic exchange of pNJH91 into BGEC132.

ZAP1180 and ZAP1181: complete replacement of internal *fimS* with part of λ *cdsH* also containing the *P*_{tac} promoter to drive *lacZ*_{YA} expression. PCR was used to generate the replacement switch of λ *cdsH'* flanked by *fimS* IRL and IRR, using primers F293 and F240; the

resulting fragment was cloned into the *Sph*I and *Bam*HI sites of pDG019, via pCR4-TOPO T/A. The *Ptac* promoter was then inserted by PCR amplification using primers K12 and K14, digested with *Bam*HI and *Xma*I, end-filled with Klenow, and cloned into the *Xma*I site in pNJH127 (located within λ *cdsH'*), generating pNJH130. Strain ZAP1180 was generated by allelic exchange from pNJH130 into BGEC144, and strain ZAP1181 was generated by allelic exchange from pNJH130 into ZAP1164 (see Supplementary Table S1).

ZAP1182: contains wild-type *fimS* in a *fimE*[−] *lrp*[−] background. The *lrp* mutation was introduced by P1 transduction from strain BGEC429A into strain AAEC370A to generate ZAP1182.

ZAP1183: complete replacement of internal *fimS* with part of λ *cdsH* and containing the *Ptac* promoter to drive *lacZYA* expression, in a *fimE*[−] *lrp*[−] background. The *lrp* mutation was introduced by P1 transduction from strain BGEC429A into strain ZAP1180 to generate ZAP1183.

Cloning *fimB*, *fimE* and *papB*. pNJH55 was generated by cloning *fimB*, amplified by PCR using primers F96 and F97, into the *Eco*RI and *Kpn*I sites in pBAD18. pNJH56 was generated by cloning *fimE*, amplified by PCR using primers F100 and F101, into the *Eco*RI and *Kpn*I sites in pBAD18. pNJH20, containing *papB* in pBAD18, was published previously (Holden *et al.*, 2001).

Measurement of recombination frequencies on agar media.

Inversion frequencies of the *fim* switch were determined using *fimA*–*lacZYA* strains cultured on MOPS minimal agar plates, supplemented with ampicillin (50 μ g ml^{−1}) and/or tetracycline (25 μ g ml^{−1}) and X-Gal (40 μ g ml^{−1}). *PapB* was expressed from pNJH20, induced with arabinose (0.2%, w/v), or expressed from pHMG88, induced with IPTG (1 mM). *FimB*, expressed from pNJH55, and *FimE*, expressed from pNJH56, were induced with arabinose (0.0004%, w/v). To assess the effect of *PapB* on *FimB*-promoted recombination, pNJH20 and pBAD18 (control) were transformed into the following strains: AAEC370A, BGEC576, BGEC605, ZAP973, ZAP1169, ZAP1170, ZAP1172, ZAP1174, ZAP1176, ZAP1177, ZAP1178, ZAP1180 and ZAP1183. To assess the effect of *PapB* on *FimE*-promoted recombination, pNJH20 and pBAD18 (control) were transformed into the strains AAEC198A, ZAP1171 and ZAP1173. To assess the inhibitory effect of multi-copy *FimB* or *FimE* on the inhibitory role of *PapB*, pNJH55 or pNJH56 were co-transformed with pHMG88 into ZAP1180 and ZAP1183 (the relevant controls were pBAD18 and pACYC184). The assays were carried out as described in Gally *et al.* (1993) and Xia *et al.* (2000). Briefly, recombination frequencies were determined in strains that contained *fimA*–*lacZYA*, where blue colonies represented *fimS* in the on phase and white colonies, *fimS* in the off phase. Blue (for on-to-off recombination) or white (for off-to-on recombination) colonies were picked from an agar plate containing X-Gal. The colony was suspended in PBS, diluted and replated onto a similar indicator plate, and the resulting proportion of blue and white colonies that had arisen from the original colony, as well as the age of the original colony (in number of generations), were determined. This allowed measurement of the frequency of recombination from one phase to another on a per cell, per generation basis, also described in Gally *et al.* (1993). Each frequency is derived from at least two sets of independent experiments and each set from approximately five different colonies. The mean and SEM for each frequency were determined. Comparison of different recombination frequencies was carried out using a two-sample *t* test, and differences were considered significant at the 95% confidence level.

Measurement of *fimS* on/off proportions. The orientation of non-*fim* switches was determined by PCR amplification with primer combinations of F246 and F128 for on-specific products and F90 and F246 for off-specific products. The orientation of wild-type *fimS* was determined by PCR amplification with primer combinations of F85

and F106 for on-specific products and F90 and F85 for off-specific products. The products were resolved on a 1.5% agarose gel. For proportional determination of non-*fim* switch populations, DNA was amplified by PCR using primers F90 and F128, and then subjected to digestion with *Aat*II. For wild-type *fimS*, DNA was amplified by PCR using primers F1 and F2, and then subjected to digestion with *Hinf*I. The products were then resolved on a 4% polyacrylamide gel.

Preparation of proteins for protein–DNA interactions. Crude cell extracts containing *FimB* or *FimE* were generated from *E. coli* NEC11 transformed with pIB378 and pIB382, respectively, after induction with 0.5 mM IPTG for 2.5 h during mid-exponential growth in rich defined MOPS media, and subsequent sonication. A control extract was obtained in the same way from *E. coli* NEC11 transformed with pET11. Sonicates were cleared by centrifugation at 10 000 g for 30 min at 4 °C. The cleared lysates were stored in 50% (v/v) glycerol at −20 °C.

Electrophoretic mobility shift assay (EMSA). EMSA analysis of *FimB* and *FimE* on the *fimS* half sites was carried out as described in Gally *et al.* (1996) and Holden *et al.* (2001). DNA incorporating each recombinase half site from pJL043, pJL044, pJL046 and pJL077 was amplified by PCR using primers Puc1 and Puc2. *FimB* and *FimE* extracts generated as above were serially diluted (fivefold) in sonication buffer and used in gel retardation assays, as described previously (Gally *et al.*, 1996). The proportion of free DNA was measured using a phosphorimager (Molecular Dynamics) and the relative concentrations of *FimB* or *FimE* extract required to shift 50% of the DNA were calculated. To demonstrate the relative affinities using these figures, an arbitrary value of 1 was assigned to the lowest-affinity half site and the other sites were scaled to this. In the case of *FimB*, even at the highest concentration used it failed to shift 50% of the DNA for its lowest-affinity site, and therefore the neat extract concentration was taken as 1 for calculating the relative affinities of the remaining sites and scored as <1 for this half site. The recombinase affinities shown are therefore only comparable for each recombinase and cannot be used to compare *FimB* and *FimE*.

RESULTS

Minimal sequence requirements for *FimB* and *FimE* recombination

Lrp and *IHF* binding within and adjacent to the *fim* switch has been shown to be necessary for wild-type *FimB* and *FimE* recombination frequencies. We wanted to determine whether recombination could still occur when the complete internal switch region and external flanking regions were replaced, removing *Lrp* and *IHF* sites required for DNA bending by the accessory proteins to align the IRs (Gally *et al.*, 1994; Blomfield *et al.*, 1997). With such structural constraints removed, would this alter recombinase susceptibility to *PapB*? A minimal-switch-region unit has previously been constructed in the chromosome at *fim* by removal of sequences either side of the recombinase external binding sites that flank the IR units; 227 bp upstream of IRL and 200 bp downstream of IRR (strain ZAP973; Fig. 1b). This removes *IHF* site I (Blomfield *et al.*, 1997). Fusion of *fimA* to *lacZYA* allowed *FimB*-promoted recombination to be determined from the proportion of blue and white colonies, as described in Methods. These changes alone only had a minor effect in the off-to-on

direction: 2.63×10^{-3} ($\pm 0.15 \times 10^{-3}$) per cell per generation compared to 2.34×10^{-3} ($\pm 0.18 \times 10^{-3}$) for wild-type *fimS*, and a reduction of 2.7-fold in the on-to-off direction from 1.90×10^{-3} ($\pm 0.46 \times 10^{-3}$) for wild-type *fimS* to 0.73×10^{-3} ($\pm 0.19 \times 10^{-3}$) for the minimal switch. Recombination of this construct in both orientations was still completely inhibited in the presence of PapB. The FimE rate was not measured for this construct as the left-hand flanking region deletion leads to a *fimE* truncation.

The *fim* switch region (270 bp) between the internal recombinase half sites was then replaced within the minimal switch construct, described above, with a larger fragment of non-*fim* DNA (1167 bp λ phage DNA) (Fig. 1c). The novel sequence was screened and did not contain consensus sites for IHF or Lrp binding. While we cannot rule out the possibility that either protein has an interaction with this element, the context is now entirely different and any interaction is unlikely to promote alignment of the IRs. The on and off orientation of this novel switch was defined accordingly to the position of the recombinase half sites in wild-type *fimS*. FimB-promoted recombination of this replacement switch in

the off orientation occurred and could be detected by orientation-specific PCR amplification from individual colonies (Fig. 2a). The lack of a requirement for Lrp for recombination was confirmed by transducing an *lrp* mutation into a strain background containing the replacement switch (strain ZAP1183; Supplementary Table S1). Recombination in the off-to-on direction was still detected in the *lrp*⁻ strain by orientation-specific PCR. In the corresponding wild-type *fimS* strain containing the *lrp* mutation (ZAP1182), the *fim* switch starts in the on orientation and no on-to-off recombination was detected (Fig. 2a). This indicated that *lrp* was required for FimB-mediated recombination of wild-type *fimS* but not of the replacement switch. Despite the lack of putative PapB binding sites in the replacement switch, PapB still inhibited FimB-promoted recombination. FimE was supplied *in trans* (as the chromosomal copy is truncated) and shown to recombine the replacement switch, demonstrated by PCR amplification of the whole switch and subsequent restriction enzyme digestion to generate proportional amounts of on- and off-specific products (Fig. 2b). In addition, the recombination frequency for the replacement switch from on phase to off phase was determined to be 3.31×10^{-4} ($\pm 0.98 \times 10^{-4}$) per cell per generation. However, no

Fig. 2. Recombination of a replacement and wild-type (WT) switch by FimB and FimE. (a) Orientation-specific PCR of the replacement switch was carried out in the absence and presence of Lrp or PapB (encoded by the multi-copy plasmid pHMG88). The first panel shows on- and off-specific products, indicating recombination of the replacement switch by FimB in the presence of *lrp* (WT; ZAP1180), in the absence of *lrp* (Δ Lrp; ZAP1183), and inhibited by PapB (WT+PapB; ZAP1180). The second panel shows on- and off-specific products, indicating recombination of WT *fimS* (on orientation) by FimB in the presence of *lrp* (WT; AAEC370A) but not in the absence of *lrp* (Δ Lrp; ZAP1182). (b) FimE-promoted recombination was determined by PCR amplification of the replacement *fim* switch, which was then digested with *Aat*II to show the relative proportions of both products. The panel shows recombination of the replacement switch in *E. coli* ZAP1181 by multi-copy FimE and inhibition by PapB. Strain ZAP1181 was transformed with pHMG88 alone (PapB), pNH56 (FimE) alone, or pHMG88 (PapB) together with pNH56 (FimE). Digested DNA representative of the on and off subpopulations is indicated. ZAP1181 started in the off orientation. (Only a single representative replicate is shown.)

FimE-promoted recombination of the replacement switch was detected in the presence of PapB, in either the recombination assay or the PCR assay (Fig. 2b). Taken together, these data demonstrate a minimal requirement of just the IRs and the associated recombinase binding sites for recombination by both FimB and FimE. Of note was that FimB recombination of this novel construct was blocked by PapB, indicating that the inhibitory activity is unlikely to be dependent on accessory protein interactions with the *fim* switch. Also, FimE-promoted recombination was shown to be inhibited by PapB, in contrast to its previously reported effect on the wild-type *fimS* (Xia *et al.*, 2000), and this is shown in Fig. 5 for strain AAEC198A.

Relative binding affinities of FimB and FimE for cognate binding sites

The preceding section demonstrated that recombination by FimB/FimE together with PapB inhibition occurs on a DNA switch defined only by the recombinase binding sites and associated IRs. The Fim recombinases bind to two recombinase binding sites, termed half sites, which flank each IR (Gally *et al.*, 1996). To investigate the importance of sequence differences in these sites, relative DNA binding affinities for FimB and FimE were determined. For this, the four half sites were amplified by PCR and cloned into pUC21, as described in Methods. Bacterial extracts containing either FimB or FimE were prepared as described previously, using *fimE* and *fimB* clones in the expression vector pET11 (Gally *et al.*, 1996). EMSA experiments were carried out with serial dilutions of each recombinase preparation. It must be noted that binding affinities would ideally be determined using purified recombinase proteins but, despite considerable efforts from a number of groups, functional proteins have not been purified, so the studies described here are restricted to relative affinities. Loss of free DNA was quantified and the relative binding affinities are shown in Fig. 3(a, b); the nomenclature used for the 'half sites' is that from the on orientation. No discernible protein-DNA complexes were seen using control extracts without FimB or FimE (data not shown). The experiments were repeated with different FimB and FimE preparations and gave similar relative affinity values. The binding sites and relative binding affinities are given in Table 1. It is clear from the binding data that FimE binds with higher affinity to two of the half sites, the IRL internal (on orientation) and IRR external sites. It bound with a lower affinity to the IRL external site, and binding to the internal site at IRR (on-orientation) was only just detectable. In comparison, FimB bound with highest affinity to only one half site, the IRL internal site (on-orientation): eightfold more FimB protein was needed to give 50 % occupancy at the external IRL site, and binding was very weak at both IRR sites (on-orientation). From these results, the key bases required in FimE and FimB recombinase binding sequences have been predicted, but remain to be proven experimentally (Table 1).

Fig. 3. Fim recombinases binding to individual *fimS* half sites. Relative binding affinities of the Fim recombinases for individual *fimS* half sites (on orientation); (a) FimB binding and (b) FimE binding. DNA binding was determined using dilutions of whole-cell extracts containing overexpressed FimB or FimE (described in Methods).

The impact of half site changes on FimB-promoted site-specific recombination and inhibition by PapB

The fact that FimB and FimE have different optimal binding sequences means that the proteins are likely to occupy the half sites in a different order. Differences in their loading order could account for their different activities as well as provide a mechanism to allow PapB to specifically prevent recombination via FimB, but not FimE, on wild-type *fimS*. Based on the relative half site binding affinities shown in Table 1, the recombinase half sites were varied to examine their role in recombinase activity as well as PapB inhibition, by construction of half site duplications, swaps and alterations in an otherwise wild-type *fim* switch, followed by chromosomal integration into the *fim* locus in *E. coli* K-12 (shown diagrammatically in Figs 4 and 5 and listed in Supplementary Table S1). PapB was added to each strain by transformation with an inducible plasmid. We understand that the levels of PapB

Table 1. Position and sequence alignment of Fim recombinase binding sites together with relative binding affinities

It should be noted that the binding constants were not determined (see Methods), and as such the data can only be quantifiably compared for each recombinase, not for each binding site. The conserved CA motif is shown in italic type. Bases predicted to govern the differential binding of FimB and FimE are highlighted as follows: bold type, preferential bases for higher-affinity FimB binding; underlined type, preferential bases for higher-affinity FimE binding.

Position	Sequence	FimB relative affinity	FimE relative affinity
IRL outside	AATACAAGA CA ATT GGG	27	5
IRL inside (on)	TCT ATGAGT CA AAAT TGG	200	29
IRR inside (on)	TGATATGGA CA GTTTGG	4	1
IRR outside	AAGATGAAA CA <u>TTT</u> GGG	<1	44

expression are physiologically relevant, since we have previously demonstrated that single-copy *papB* driven from its native promoter is also sufficient to prevent *fimS* recombination (Holden *et al.*, 2006).

We analysed a first set of mutations with the *fim* switch in the off orientation. The IRL internal (on) site was duplicated at IRR internal (on) so that both half sites internal to the switch were the same (BGEC576) and contained two copies of the highest-affinity site for FimB binding. Attempts were made to measure FimB-promoted recombination in the off-to-on orientation for the duplicated internal half site construct in a *fimB*⁺ *fimE*[−] background (strain BGEC576). However, all colonies were light blue and homogeneous, indicating a high frequency of recombination, too high to determine by the standard plating assay. As an alternative method, the orientation of the *fim* switch in individual colonies was determined by PCR amplification and subsequent restriction digestion with *Sna*BI, which acts on an asymmetric site within the switch. Densitometry analysis of the restriction fragments from both orientations showed that 47 % of the bacteria were in the on orientation and 53 % were in the off orientation, in the colonies examined (data not shown). To achieve these proportions in single colonies, a minimum switching frequency of $>5 \times 10^{-2}$ per cell per generation is required (Gally *et al.*, 1993). The frequency of recombination for the *fim* switch containing the duplicated internal half site is therefore at least 20-fold higher than that of wild-type *fimS* (2×10^{-3} per cell, per generation). Therefore, the lower-affinity IRR internal (on) site must limit FimB recombination. Despite the high level of FimB-promoted recombination in this strain, addition of PapB, induced from a plasmid expression vector (pNJH20), resulted in colonies locked in either the on or the off phase, as determined by a colony-switching assay and orientation-specific PCR amplification reactions (data not shown).

A critical role for the *in situ* location of the highest-affinity FimB binding site (IRL internal, on orientation) was demonstrated with a construct in which the internal half sites were swapped over (strain BGEC605). The prediction was that the half site swap would have little impact on the FimB-promoted recombination frequency, although it might alter the FimB bias to the on-to-off direction, compared to a bias for the off-to-on direction demonstrated on the wild-type *fim* switch sequence (compare data for AAEC370A in Fig. 4a, c). However, FimB recombination was not detected in this construct. Sequence analysis (as with all constructs) confirmed that the modified *fim* switch region was as anticipated. Furthermore, the chromosomal copy of FimB was shown to be functional, and overexpression of FimE but not FimB inverted the altered switch in BGEC605 (data not shown). Therefore, the context of the highest-affinity FimB half site at IRL in (on orientation) is essential for FimB, but not for FimE, recombination.

While the two constructs that affect the internal sites had significant effects on FimB recombination, alterations to the outside half sites had little effect on FimB recombination frequency in the off-to-on orientation (Fig. 4a). For example, swapping of the outside half sites (strain ZAP1174) had no significant effect on the frequency of FimB recombination. Alterations that are predicted to marginally (strain ZAP1170) or substantially (strain ZAP1169) increase the affinity of FimB binding to the outside IRR half site also had little impact on FimB recombination or inhibition by PapB.

The external half site at IRR shows the weakest FimB binding, whereas FimE shows the highest affinity here (Fig. 3). To investigate whether the differential binding to IRR is fundamental to the specificity exhibited by PapB, a series of alterations were made to *fimS* in an attempt first to increase the binding affinity of FimB for IRR-out, and second to reduce any potential binding to IRR-external by PapB. Previous work has shown that PapB binds preferentially to sequences containing triple adenine motifs (Xia *et al.*, 1998), so the alterations disrupted a 5' AAA sequence present at IRR-external. Although two of the changes (strain ZAP1172 and ZAP1176) increased FimB-promoted off-to-on recombination significantly, the level of increase was small (Fig. 4b). As before, PapB activity completely inhibited FimB-promoted recombination. We also investigated FimB activity in the on-to-off orientation, as well as the associated effect of PapB, on a selected number of *fimS* mutants. One of the alterations (strain ZAP1170) increased FimB-promoted recombination 12-fold (Fig. 4c), whereas the others had little or no effect. Furthermore, FimB activity was still inhibited by PapB in every case.

In summary, these data demonstrate that the two internal half sites are critical for FimB recombination. IRR-internal (on) limits FimB recombination frequencies, whereas the context and presence of IRL-internal (on) is essential for

Fig. 4. FimB-promoted recombination of altered *fimS* substrates. Plasmids pBAD18 (control) or pNJH20 (PapB) were transformed into each strain and recombination was assessed as described in Methods. The data shown in each panel are for the relevant strain transformed with pBAD18, as in all cases the level of recombination in the presence of PapB was $<1 \times 10^{-6}$ per cell per generation. (a) Off-to-on recombination frequencies for half site swaps and duplications: AAEC370A [wild-type (WT), on]; BGEC576 [IRL-in (on) duplication]; BGEC605 [IRL-in (on) swap]; ZAP1169 (IRL-out duplicated at IRR-out); ZAP1170 [IRL-in (on) duplicated at IRR-out]; ZAP1174 (external half site swap). (b) Off-to-on recombination for additional IRR-altered *fimS* substrates: AAEC370A (WT, off); ZAP1172 (IRL-in duplicated at IRR-out and deletion of the IRR external sequences); ZAP1176 (like ZAP1172 with a deletion of the IRL external sequences); ZAP1178 [IRR composed of a duplication of IRR-in (on)]; ZAP1177 (like ZAP1176 with a mutation in IRR-out that increases FimB binding and swapped IRs). (c) On-to-off recombination for selected half site mutations: AAEC370A (WT, on); ZAP973 (minimal *fim* switch, on); ZAP1170 (on); ZAP1172 (on); ZAP1176 (IRR mutation); ZAP1177 (on). A diagram of each *fimS* allele is presented beneath its designation. The shaded grey blocks represent FimB/FimE binding sites, with the relative affinities indicated by the degree of shading and values determined shown beneath (Fig. 3, Table 1). The promoter is in either the off or the on orientation, represented by an arrow. The IRs are represented by two white arrowheads, except for strain ZAP1177, where they have been swapped and black arrowheads are used. Crosses represent deleted regions. The frequency for BGEC576 has been estimated from colonies that contained approximately 47% on *fimS* and 53% off *fimS*, as determined by proportional PCR. The frequency of recombination is given per cell per generation; ** $P < 0.001$; * $P < 0.05$; error bars represent the 95% confidence level.

recombination. In contrast, alterations to the external half sites have little effect on FimB recombination, and the sequences external to *fimS* are not key recombination determinants.

Impact of half site changes on FimE-promoted site-specific recombination and PapB activity

Based on the relative half site affinities, it was anticipated that FimE activity would be reduced following alteration of

the sites with highest relative affinity for this recombinase [IRR-external and IRL-internal (on)]. Swapping the external half sites (ZAP1175) resulted in a 4.5-fold reduction in FimE recombination frequency in the on-to-off orientation, from 3.22×10^{-2} ($\pm 0.49 \times 10^{-2}$) for AAEC198A to 0.72×10^{-2} ($\pm 0.11 \times 10^{-2}$) for ZAP1175. Replacing the highest-affinity site (IRR external) with a duplication of the lowest-affinity site [IRL-internal (on), strain ZAP1171] resulted in a non-significant 1.6-fold reduction in recombination frequency (Fig. 5). Deletion of DNA downstream from IRR-external in combination with duplication of IRL-in (on) at IRR-out (ZAP1173) resulted in a 12-fold reduction in FimE activity ($P < 0.001$). FimE recombination is normally increased in the presence of PapB, as PapB upregulates *fimE* expression (AAEC198A) (Xia *et al.*, 2000). However, FimE recombination does not increase in the presence of PapB in either of the related backgrounds (ZAP1173 and ZAP1171), demonstrating that FimE activity can be inhibited, at least to some extent, by PapB when half sites are altered. There remains the possibility that alterations of the half sites have an impact on *fimE* expression. The role of the half sites in the inhibitory activity of PapB was further investigated by using FimE recombination of a previously published *fimS*

Fig. 5. FimE-promoted recombination of altered *fimS* substrates. FimE-promoted recombination in the on-to-off direction was determined in the presence and absence of PapB. In all these strains, *fimE* is present *in cis*, in single copy and under the control of its natural promoter. Plasmids pBAD18 (control) or pNJH20 (PapB) were transformed into AAEC198A (wild-type); ZAP1171 [IRR-out replaced by IRR-in (on)]; ZAP1173 (like ZAP1171 with a deletion of the IRR external sequence); ZAP1175 (external half site swap) and BGEC424 (like wild-type *fimS* with a mutation in IRR-out reducing its Fim binding activity). Each *fimS* allele is presented as described for Fig. 4. P, in the presence of *papB*; C, strain alone without *papB*. The frequency of recombination is given per cell per generation; ** $P < 0.001$; * $P < 0.05$; error bars represent the 95% confidence level.

allele that contains a mutation in the conserved CA nucleotides of the IRR external half site (strain BGEC424). This mutation reduced the FimE switching frequency in rich defined medium by 3.7-fold (compared to previously published data; Gally *et al.*, 1996) to 5.23×10^{-3} per cell, per generation, and this frequency was reduced 30-fold to 1.8×10^{-4} by the presence of PapB. A large degree of variation was seen for recombination of strain BGEC424 (Fig. 5). However, addition of PapB not only reduced the recombination frequency but also reduced the level of variation between single colonies. Since the CA mutation at IRR external has been shown to reduce FimE binding by approximately 40-fold (Gally *et al.*, 1996), the data indicate that the inhibitory activity of PapB is in part reliant on recombinase binding activity.

In summary, differences in the binding affinities of the recombinases for the half sites are largely responsible for the different outcomes in *fimS* recombination promoted by FimB or FimE. This is reflected in the specificity of PapB inhibition of FimB on wild-type *fimS*. However, PapB inhibited FimE when the context of *fimS* was altered by either substantially reducing the affinity of FimE for the

IRR outside half site or removing the switch region between the IRs and therefore the organized structure imposed by Lrp and IHF binding.

DISCUSSION

FimB and FimE are site-specific recombinases that invert a short sequence of DNA containing a promoter required for expression of the structural components of type 1 fimbriae. They are members of the λ integrase, or tyrosine-dependent site-specific recombinase family (for reviews see Chen & Rice, 2003; Hallet & Sherratt, 1997). The Fim recombinases are less-well-characterized members of this family, and much of the current body of knowledge about them stems from detailed work on analogous systems, such as XerC/D, λ Int, P1 phage protein Cre and yeast protein Flp. Interest in these specific recombinases comes from the fact that although they recombine the same substrate they differ in: (i) their recombinational frequencies; (ii) the preferred orientation of their substrate; (iii) their capacity to be inhibited by the PapB regulator. The main aim of this study was to investigate these differences. To do this, each step of the process required for recombination was considered in terms of the above differences.

By analogy with related systems and our preliminary work, the step-wise nature of the inversion of *fimS* by FimB and FimE can be described. Firstly, the Fim recombinases would need to bind to their cognate sites that flank and include the IRs. Each half site is presumably occupied by a monomer. Co-operativity may then aid occupancy of each full site. In the case of the *fim* switch, Lrp and IHF bind within the invertible element to establish a structure competent for recombination, presumably by bringing the IRs into juxtaposition. Co-operative protein–protein interactions may be required to complete the nucleoprotein complex, for example between recombinase dimers. Single-strand cleavage will then occur, and recent work has demonstrated that this may further stabilize the complex (Mumm *et al.*, 2006). Holliday-junction formation and subsequent resolution presumably then occurs by a well-characterized mechanism that is shared by all members of the tyrosine recombinase family.

Given the shared mechanism of recombination, the current research focused on the preceding step of recombinase binding. This was carried out by mutating sequences within and adjacent to the *fim* switch, predominantly at protein binding sites, and the impact was assessed by the effect on recombination frequency. In particular, attention was centred on the specificity exhibited by PapB for inhibition of FimB alone, as this had to reflect a fundamental difference between the two recombinases.

Experiments were carried out to define the minimal requirements for both recombination and PapB inhibition. The research demonstrated that both FimB and FimE were able to act on a novel substrate in which most of the switch was replaced with non-*fim* DNA (derived from λ phage),

leaving only *fim* recombinase binding sites and the two IRs. Therefore, both recombinases can act in the absence of accessory protein, in particular Lrp and IHF, binding. Recombination by site-specific recombinases varies in terms of the complexity of the target sequence and the superhelicity of the substrate. Cre and Flp recognize relatively simple sites and do not require accessory proteins for organization of the substrate. In contrast, higher-order nucleoprotein complex formation is required for alternative systems, dependent on accessory proteins that control the architecture of the complex necessary for recombination (Hallet & Sherratt, 1997; Sadowski, 1986). For example, severe DNA bending is induced by IHF, Xis and factor for inversion stimulation (FIS) to facilitate λ Int-dependent recombination (Kim & Landy, 1992), and a similar role has been demonstrated for ArgR and PepA for Xer-dependent recombination (Colloms *et al.*, 1997; Sherratt *et al.*, 1995). Recombination of *fimS* requires Lrp and IHF to induce sharp bends in the DNA (Blomfield *et al.*, 1993, 1997). To our knowledge, the current study is the first to demonstrate recombination of a DNA sequence from which the structural constraints have been removed by completely substituting the native *fim* DNA. An indication that this would occur comes from earlier research by Dove & Dorman (1996), who have shown that overexpression of FimB promotes *fimS* recombination, even in the absence of Lrp or IHF protein binding to *fim* DNA. Interestingly, after the removal of Lrp- and IHF-dependent intrinsic structure, PapB was able to inhibit both FimB and FimE recombination. Furthermore, this construct no longer contained either of the putative PapB binding sites that we have mapped in the *fimS* region (Fig. 1a), indicating that these also are not required for PapB to inhibit recombination of this novel substrate. We do not know whether the replacement switch DNA contained additional PapB binding sites that may have had an effect. Previous work has demonstrated that PapB can inhibit FimB-, but not FimE-promoted *in vitro* recombination (Xia *et al.*, 2000). Furthermore, there is no evidence that PapB binds directly to any of the four half sites (including IR regions) or directly to FimB or FimE (our unpublished data). The main difference between wild-type *fimS* and the replacement switch would be a lack of structure to facilitate recombinase interactions that lead to synapse formation and recombination. The fact that PapB inhibits FimE recombination under these conditions supports the concept that PapB can act on these later co-operative interactions, i.e. synapse formation, rather than initial recombinase binding.

An alternative way to change the sequence or nature of the co-operative loading is to alter the affinities of the recombinase half sites at *fim*. It was hypothesized that weakening FimE binding at its highest-affinity site would alter the order of interaction and allow inhibition by PapB. The data support this prediction, but are complicated by the demonstration that FimE has two half sites with similarly high affinities, so there may be some flexibility in

the order in which these interactions can occur. In contrast, it was demonstrated that FimB recombination is dependent on a single high-affinity half site. This research has shown that even the position of this site is essential for FimB recombination. Internal duplication of this site did increase recombination significantly, but the reaction was still completely inhibited by PapB. The fact that swapping the internal half sites prevents FimB recombination is indicative of a single pathway leading to synapse formation, and it is likely that this is effectively blocked by PapB. On the other hand, it appears that FimE uses an alternative loading pathway that circumvents PapB inhibition. Only when this pathway is restricted by either structural changes or alteration of binding affinities does FimE recombination become susceptible to PapB inhibition.

The current study shows differences in the Fim recombinase affinities for each half site (Fig. 3), and combining these produces an order of affinities comparable with those determined previously for each whole site/IR (Gally *et al.*, 1996). One clear difference is the magnitude of the differences in binding affinities shown for the half sites in comparison to the whole IRs. This can be explained by the co-operativity of recombinase binding between the half sites. Certain half sites are almost unable to bind recombinase alone and require a stronger half site partner to give occupancy of the whole site. As the previous analysis of binding to the whole sites looked at only the loss of free DNA and did not take into account whether both half sites were occupied, it is likely that the affinity differences described here for the half sites are fundamental in determining the specificity of recombinase activity. From this study, the current working model is that the formation of a recombination-proficient complex requires initial occupancy of certain half sites by the recombinases, and that this binding leads to co-operative binding to the adjacent site. Subsequent interactions that support binding to relatively weak sites occur as a consequence of the IRs being brought into juxtaposition. This allows co-operation across the two IRs. The different affinities of FimB and FimE for the half sites mean that these secondary interactions will also be different. PapB is likely to act at this stage, as current research strongly suggests that it does not prevent initial recombinase binding. To prove this, future work will focus on measuring biophysical interactions between *fimS* complexes involving either FimB or FimE, as well as the impact of PapB. Understanding the mechanism of regulatory cross-talk between these two phase-variable virulence factors will provide insights into how pathogens co-ordinate the expression of cell-surface factors during infection.

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