A plasmid-encoded papB paralogue modulates autoaggregation of Escherichia coli transconjugants

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Research note

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Abstract

Objective: Plasmids are key to the transmission of antimicrobial resistance among enteric bacteria. It is becoming increasingly clear that resistance genes alone do not account for the selective advantage of plasmids and bacterial strains that harbor them. Deletion of a 32 Kb fitness-conferring region of pMB2, a conjugative resistance plasmid, produced a hyperautoaggregation phenotype in laboratory Escherichia coli. This study sought to determine the genetic basis for hyper-autoaggregation conferred by the pMB2-derived mini-plasmid.

Results: The 32 Kb fragment deleted from pMB2 included previously characterized nutrient acquisition genes as well as putative transposase and integrase genes, a 272 bp papB/pefB-like gene, and several open-reading frames of unknown function. We cloned the papB/pefB paralogue and found it sufficient to temper the hyper-autoaggregation phenotype. Hyperautoaggregation conferred by the mini-plasmid did not occur in a fim-negative background. This study has identified and characterized a gene capable of down-regulating host adhesins and has shown that trans-acting papB/pefB paralogues can occur outside the context of an adhesin cluster. This plasmid-mediated modification of a bacterial host's colonization program may optimize horizontal transfer of the mobile element bearing the genes.

Introduction

Bacterial autoaggregation is an adherence phenotype that can manifest in macroscopical clumping. Autoaggregation enhances colonization of some niches but because it is not always advantageous, it is typically regulated transcriptionally or post-transcriptionally by antiaggregation proteins or steric hindrance [1-5]. The Escherichia coli pangenome contains hundreds of known autoaggregation/antiaggregation factors, many of which also function in adherence to other cells and surfaces and have been studied in the context of specific virulent E. coli strains or pathotypes [4].

We recently isolated and sequenced a large multi-drug resistance plasmid from a commensal Escherichia strain in Nigeria and mapped a 32 Kb segment that ameliorated the 125 Kb plasmid's carrying cost [6]. Here we report that deletion of this portion of plasmid pMB2 produces the pRMKO miniplasmid that is sufficient to enhance autoaggregation in laboratory E. coli strains in a manner that pMB2 does not. We further demonstrate that the responsible locus was an orphan papB/pefB paralogue, without a cognate fimbral cluster, and propose that this gene may optimize colonization and transmission in conjugation recipients.

The canonical, pathogenicity-island-encoded PapB regulator mediates crosstalk among different fimbrae in extraintestinal pathogenic E. coli, leveraging type I fimbral phase variation to ensure that type I fimbrae and pyelonephritis-associated (P)-fimbrae are not expressed simultaneously [7, 8]. Type I fimbrae or fim genes are present in most E. coli whilst the P-fimbrae-encoding pap genes are typically only found in uropathogenic and other extraintestinal E. coli. ON to OFF phase variation of type I fimbrae is mediated by FimE recombination at the fimS switch locus
within the *fim* cluster. FimB, an analogous site-specific recombinase, predominantly mediates the reverse *fimS* phase OFF to phase ON switching [9]. FimB switching behavior is repressed by PapB and PapB also promotes *fimE* expression, thereby shutting type I fimbriae OFF. Although *papB* lies within the chromosomal pyelonephritis-associated pilus (*pap*) cluster PapB controls *fim* expression even when expressed in *trans* from a plasmid in the laboratory [8, 10].

A number of PapB paralogues (PFAM03333) have been described in enteric bacteria, with varying *fim* regulatory activities. The family includes DaaA of the F1845 fimbrial cluster, SfaB of S-fimbriae, ClpB of CS31 fimbriae, FaeB of K88 fimbriae, FanA and FanB of K99 fimbriae, AfaA of the *afa* adhesin cluster as well as PefB of *Salmonella pef* fimbrial cluster [8, 11]. All these genes occur in the context of an adhesin gene cluster that they regulate and have the potential to crosstalk to other fimbriae.

**Methods**

**Strains and plasmids:** Bacterial strains (Table 1) were cultured in Luria broth containing chloramphenicol (30 μg/mL), ampicillin (100 μg/mL), tetracycline (25 μg/mL), or neomycin (50 μg/mL), where required for selection, and maintained at -70°C in Luria broth:glycerol 1:1. Plasmid pMB2, originally isolated from an *Escherichia* isolate in Nigeria [6], and its derivatives are described in Table 1.

**General microbiology and molecular biology procedures:** Standard molecular biology procedures were used [12]. pMB2, pRMKO and pRMC were extracted using the Qiagen® Large Construct Kit, as previously described [6]. All other plasmid extractions used the Qiagen® MiniPrep Kit. Plasmids were electroporated into *E. coli* strains using a Bio-Rad micropulser. The pMB2 *papB/pefB* analogue was amplified using the oligonucleotides 5’CACCACTCCCTCCCCCTATCCAA-3’ and 5’-CTCAGGTAGAATAATTTAAGGC-3’ and then cloned into pBAD/Thio-TOPO according to manufacturer’s protocols. *In vitro* conjugation was effected by solid mating on LB agar as described previously. The number of transconjugant colonies per donor colony-forming units was computed as the plasmid transfer efficiency [13].

**Sequence analysis:** Sequence was viewed in Artemis [14] and BLAST [15] searches were performed via Artemis on the NCBI platform. Multiple alignments were performed using Clustal W on the EBI server [16] and in MEGA6 [17]. Maximum likelihood phylogenies were computed in MEGA6.

**Autoaggregation:** Hasman et al’s [18] settling assay, with previously detailed slight modifications [19], was used to quantify autoaggregation. Overnight cultures were prepared in test medium containing selective antibiotics and where necessary, gene expression was induced for 90 minutes before the start of the assay. At the assay start, cultures of each strain were adjusted to the same optical density at 600nm (OD600). Eight mL of each adjusted culture was placed into paired tubes. One tube in each pair remained static and the other was lightly vortexed before each optical density measurement. The tubes were incubated without shaking at 37 °C. At designated time points, 0.5
mL was removed from within 2 cm of the surface of the culture, and the OD600 was measured, diluting the sample if required. OD600 supernatant measurements for different strains were compared using a t-test.

Results And Discussion

A PapB/PefB paralogue on pMB2 interferes with autoaggregation. pMB2 was originally extracted from a commensal *Escherichia* isolate from Nigeria [20, 21]. In the course of functional analyses of fitness loci [6], we additionally observed that DH5α carrying the pRMKO miniplasmid autoaggregated significantly, particularly under low nutrient conditions. As shown in Figure 1a, autoaggregation in liquid media was prominent in DH5α (pRMKO) but absent in DH5α (pMB2) cultures grown under the same conditions (p<0.001). Cloning the 32 Kb deleted region into pBluescript also reduced autoaggregation of DH5α compared to an isogenic strain carrying the vector alone (Figure 1a). We examined the sequence of the region deleted from pRMKO for genes with the potential to contribute to autoaggregation. The best candidate is small open reading frame (orf) located from 11,258-11,530 of the plasmid. The predicted amino acid sequence of this gene was 29% identical/ 50% similar to *E. coli* PapB from uropathogenic strain J96 (Genbank Accession ELL39276.1) and was 32% identical/ 48% similar to PefB, a PapB paralogue from *Salmonella enterica* serovariety Typhimurium strain LT2 (Genbank Accession AAL23523). There are no fimbrial genes proximal to the pMB2 *papB*-like gene, however there are mobility genes on either side and the *papB*-like gene region has a noticeably lower G+C content than the surrounding region (Figure 1b).

We cloned the *papB/pefB* paralog under the control of the arabinose promoter and measured the clone's ability to complement pRMKO hyperautoaggregation. As shown in Figure 1c, when the *papB/pefB* parologue is induced by arabinose, autoaggregation was diminished in DH5α (pRMKO, pINKPefB). This phenotype was not seen when the arabinose promoter is glucose-repressed (Figure 1c). Based on this finding, we attribute the hyperautoaggregation phenotype conferred by pRMKO to deletion of the pMB2 *papB/pefB* paralogue.

The pMB2 *papB/pefB* paralogue acts on chromosomally-encoded effectors

PapB binds between *papI* and *papB* in the extraintestinal pathogenic *E. coli* *pap* cluster as well as, if less strongly, upstream of *fimE* [10]. R61A and C65A substitutions, within a hydrophilic region of PapB, produce derivatives unable to bind DNA [11]. As shown in Figure 2, pMB2 PefB paralogue has a cysteine at the position equivalent to 65 in PapB. It does not have an arginine at position 61 but does have a positively-charged lysine residue at the equivalent position. Positions 86 and 91 at the PapB carboxy terminus are also essential for switching activity [8]. Holden et al reported that L82F or F83Q substitutions, which altered these PapB residues to the equivalents in the non-binding DaaA paralogue, reduced switching. When both substitutions were made, activity was obliterated [8]. As shown in Figure 2, the pMB2 PefB paralogue has a leucine at the position equivalent to PapB 82 but has an asparagine
substitution at position 83, and so, even though it is considerably dissimilar to PapB, it could inhibit \textit{fim} switching.

In DH5\textalpha\ (pMB2), PapB/PefB could be repressing either chromosomally-encoded \textit{fim} genes or pMB2 factors not deleted from pRMKO, for example F (conjugative)-pili, which are known to promote autoaggregation [22], although they lack a type I fimbriae-like switch. Indeed any of the several pMB2 orfs of unknown function could contribute to autoaggregation. To determine which possibility could be at play, we transformed pMB2 and pRMKO into ORN172, a fimbrusnegative \textit{E. coli} strain [23]. In this \textit{\textit{fim}} background, there were no \textit{papB}/\textit{pefB}-associated differences in autoaggregation (Figure 1d). Therefore, while it remains to be confirmed in direct experiments, this finding suggests that interactions of \textit{papB}/\textit{pefB} with core chromosomal factors, likely fimbriae, account for the hyperautoaggregation seen in DH5\textalpha\ (pRMKO) and that the hydrophobic residue at position 83 of PapB, may be less critical for FimB regulation than the presence of a leucine at position 82.

PapB is not known to directly regulate conjugative pili, but a \textit{fim} repressor could conceivably enhance F-pilus-mediated conjugation by preventing occlusion by chromosomally-encoded fimbriae. We performed an initial test of this hypothesis by comparing conjugative transfer rates from DH5\textalpha\ (pMB2) and DH5\textalpha\ (pRMKO) to strain EC1502. We recorded a transfer rate of $7.9 \times 10^{-6}$ for DH5\textalpha\ (pMB2) in solid matings, compared to $3 \times 10^{-5}$ of control plasmid pMB80 [24]. In contrast, conjugation from DH5\textalpha\ (pRMKO) could not be detected at the limits of the assay. Due to the low conjugation rate overall and the impossibility of detecting rates below $1 \times 10^{-8}$ we could not perform a robust complementation experiment. However, altogether the available data support the idea that \textit{papB}/\textit{pefB} repressed genes could interfere with pMB2 conjugative transfer.

\textbf{The pMB2 \textit{papB}/\textit{pefB} paralogue is widely distributed and largely overlooked on extraintestinal \textit{E. coli} plasmids}

A BLAST search of the pMB2 nucleotide sequence range 7247-14962, representing the \textit{papB}/\textit{pefB} paralogue, its flanking low G+C region and the adjacent NikM/Hmu genes, reveal that it is a common plasmid-borne feature. Importantly, plasmids of \textit{E. coli} O25b:H4-ST131 strains, commonly implicated in bloodstream, urinary and other extraintestinal infections, bear the entire region depicted in Figure 1b. On the \textit{E. coli} str. UMNO26 plasmid p1ESCUM, often used as an extraintestinal \textit{E. coli} reference sequence (GenBank Accession Number: CU928148.1) [25], the entire region is present, but the \textit{papB}/\textit{pefB} paralog is not annotated as is the case for some well-known resistance plasmids [26], but not for others.

Cystitis and pyelonephritis isolates are more likely to contain one or more \textit{pap} operons than other \textit{E. coli}. Holden et al (2006) [27], who observed this, also showed that cystitis and pyelonephritis isolates are better able to agglutinate red blood cells and attributed this to a higher copy-number and activity of intracellular PapB, which promoted expression of alternate fimbriae whilst shutting type I fimbriae OFF. \textit{papB}/\textit{pefB} paralogues on plasmids could achieve this effect irrespective of the presence of a full fimbrial operon consequently modulating the expression of core adhesins for \textit{in vivo} adherence and/or exposure
to the immune system. Thus, virulence factor profiling of pathogenic *E. coli* needs to take into account the available fimbrial genes as well as fimbrial regulators some of which—like the gene we characterize—could be plasmidborne. As our *papB/pefB* paralogue is borne on a plasmid conferring resistance to no less than eight antimicrobial classes, antibiotic selective pressure could ultimately alter colonization and virulence profiles of strains in the wild.

In conclusion, pMB2 is self-transmissible by an F-type conjugative system that may function more effectively due to repression of chromosomal adhesins by a *papB/pefB* paralogue located on the same plasmid. Plasmids almost identical to pMB2 have been isolated from the USA, Colombia, Italy, the UK and elsewhere. The *papB/pefB* paralogue we describe is highly conserved among them and therefore the autoaggregation it represses is likely common. Our findings add to experimentally validated reports of two other non-resistance loci of pMB2 that promote *E. coli* survival and adaptability [6, 28]. Thus there are multiple genes resistance plasmids that compensate the costs of their carriage or, as in this case, potentially propagate transmission. This study also provides yet another example [29] horizontally acquired DNA that radically affects the expression of recipient cell core genes.

**Limitations**

This study used convincing indirect methods to infer a regulatory link between the *papB/pefB* paralog on pMB2 and chromosomal adhesins and our findings agree with existing evidence on the mechanism of action of genes that belong to this family. We however lack the resources to demonstrate that the new *papB/pefB* paralog directly interacts with *cis* or *trans* fimbrial regulatory elements, or that fimbrial production is altered by the presence or absence of the *papB/pefB* gene.

**Abbreviations**

Not applicable.

**Declarations**

**Ethics approval and consent to participate**

Not applicable

**Consent for publication**

Not applicable

**Availability of data and materials**
The sequence of plasmid pMB2, on which this manuscript is based is available from Genbank, 3 Accession number MK492688. Other data generated or analysed during this study are included in this article.

**Competing interests**

The authors declare that they have no competing interests

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**Authors’ contributions**

The authors co-conceived the project and both performed different aspects of the experimental work and data analyses. INO prepared the first draft of the manuscript and both authors contributed substantially to the writing.

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**Authors’ information**

The laboratory work for this paper was performed at Haverford College at which neither author is now presently affiliated. RM is currently a medical resident at the Rubin Institute for Advanced Orthopaedics, Baltimore, MD, USA. INO is currently a Professor at the University of Ibadan Nigeria, where in silico work was performed and the manuscript was written.

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Tables
### Table 1. Strains and Plasmids used in this study

| Designation | Description                                                                 | Reference or Source |
|-------------|------------------------------------------------------------------------------|---------------------|
| Strains     |                                                                              |                     |
| M63c        | *E. coli* wild type, S<sup>8</sup>, Na<sup>R</sup>, Su<sup>R</sup>, Amp<sup>R</sup>, Cip<sup>R</sup>, Te<sup>R</sup>, W<sup>R</sup>, K<sup>R</sup> | [20]                |
| DH5α        | F<sup>−</sup> Δ80dlacZΔM15 Δ(lacZYA-argF)U169 deoR recA1 endA1                 | Invitrogen          |
|             | hsdR17(k<sup>R</sup> m<sup>R</sup>+) phoA supE44 λ− thi-1 gyrA96 relA1           |                     |
| ORN172      | thr-1 leuB thi-1 A(argF-lac)U169 marL11 xyl-7 ara-13 26 mit-2 gal-6            | [23]                |
|             | rpsL tonA2 supE44 Δ(fimBEACDFGH)                                        |                     |
| EC1502      | Rifampicin-resistant, plasmid free *E. coli* strain                          | University of Bradford |
| Plasmids    |                                                                              |                     |
| pMB2        | 125 Kb Naturally occurring *aac(6')-Ib-cr-* bearing plasmid                   | [6]                 |
| pMB80-2     | Naturally occurring conjugative plasmid from enteropathogenic *E. coli* strain | [24]                |
| pRMKO       | 93 Kb Miniplasmid constructed by deleting a 32,331 bp Nor1 - Xba1 fragment from pMB2 | [6]                 |
| pRMC        | pBluescript II SK containing a 32,331 bp Nor1 - Xba1 fragment from pMB2 cloned | [6]                 |
| pBAD/Thio-TOPO | Arabinose inducible expression vector                                       | Invitrogen          |
| pBluescript II SK + | High copy number cloning vector                                    | Agilent             |
| pINKpefB    | *papB*/*pefB* parologue from pMB2 cloned into pBAD/Thio-TOPO                | This study          |
| pLMJ50      | 90 Kb aggregative adherence plasmid from enteraggregative *E. coli* strain 60A marked with a *cat* cassette | [30]                |

### Figures
Figure 1

Autoaggregation in DMEM measured as absolute OD600 values from the top of a static culture sampled over time. (a) Autoaggregation of E. coli DH5α carrying pBluescript (grey closed circles on broken line), pMB2 (grey squares on broken line), pRMKO (black diamonds on broken line), pRMC (black line without marker) and control aggregative plasmid pLMJ50 (black crosses). (b) The pefB region of pMB2. The pefB-like allele is colored yellow and is flanked by transposase/ integrase genes (green). The nik
transporter system genes are colored orange and the blue open-reading frame is a conserved hypothetical gene of unknown function. The %G+C content plot above the genes used a 120 nucleotide sliding window and the average value for the plasmid is 51.7%. (c) E. coli DH5α carrying miniplasmid pRMKO and pINKpefB1, the pefB gene from pMB2 cloned under the control of the arabinose promoter. Autoaggregation was measured after growth in arabinose (solid grey line) or glucose (broken dark line) (d) E. coli ORN172 carrying pMB2 (solid grey line) and pRKMO (dark broken line)
(a) Alignment of the pMB2-encoded PefB-like protein with a selection of known PapB homologs. PapB residues known to be required for fim-switching activity of PapB are indicated in bold. (b) Unrooted maximum likelihood tree of PapB paralogues including the PefB-like protein in this study (boxed).