Research Article

Self-Conjugation of the Enteropathogenic *Escherichia coli* Adherence Factor Plasmid of Four Typical EPEC Isolates

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The enteropathogenic *Escherichia coli* (EPEC) adherence factor plasmid (pEAF) encodes the proteins involved in the biogenesis of the bundle-forming pilus (BFP), a key virulence factor that mediates microcolony formation and the localized adherence phenotype on the surface of the host enterocytes. The presence or absence of this plasmid defines typical EPEC (tEPEC) and atypical EPEC (aEPEC), respectively. Although lateral transfer of pEAF has been evidenced by phylogenetic studies, conjugal transfer ability has been experimentally established only for two pEAF plasmids from strains isolated in the late 60s. In the present work, we tested the self-conjugation ability of four pEAF plasmids from tEPEC strains isolated between 2007 and 2008 from children in Peru and the potential of aEPEC to receive them. A kanamycin resistance cassette was inserted into donor pEAF plasmids in order to provide a selectable marker in the conjugation experiments. Two aEPEC isolated from the same geographic region were used as recipient strains along with the laboratory *E. coli* DH5α strain. Here we show that the four pEAF plasmids tested are self-conjugative, with transfer frequencies in the range of $10^{-6}$ to $10^{-9}$. Moreover, the generation of aEPEC strains harboring pEAF plasmids provides valuable specimens to further perform functional studies.

1. Introduction

*Escherichia coli* is gamma Proteobacteria that colonizes the gastrointestinal tract of humans and other animals [1, 2]. Enteropathogenic *E. coli* (EPEC) is a leading cause of infantile diarrhea in developing countries [3]. EPEC infection is accompanied by a distinct intestinal histopathology, called attaching and effacing (A/E) lesion, characterized by the intimate adherence of bacteria to enterocytes, the formation of actin-rich pedestals underneath the sites of bacterial attachment, and localized destruction of the brush border microvilli [4]. The genes necessary for the establishment of the A/E lesion are located within the pathogenicity island known as the locus of enterocyte effacement (LEE) [5].

EPEC is classified into typical (tEPEC) and atypical (aEPEC) strains based on the presence of a large virulence plasmid known as EPEC adherence factor plasmid (pEAF) [1, 6, 7]. The pEAF encodes the bundle-forming pilus (BFP) that mediates EPEC autoaggregation and microcolony formation on the surface of epithelial cells, a phenotype known as localized adherence [8–11], as well as dispersion of the bacteria through the intestinal mucosa and virulence in adult volunteers [12–14]. BFP biogenesis is specified by the 14-gene *bfp* operon [15, 16].

Although the pEAF is not essential for the formation of the A/E lesion [17], it enhances their efficiency by promoting localized adherence on the host cell and the expression of LEE genes through the *per* regulatory operon, which consists of three genes, *perA*, *perB*, and *perC*. PerA activates the expression of the *bfp* operon and autoactivates its own expression [18–22]. PerC increases the expression of LEE encoded proteins by enhancing the activation of the LEE-encoded regulator (Ler) [20, 23–25].

The pEAF from prototype strains BI71 (O111:NM), isolated in Washington in 1983 [26], and E2348/69 (O127:H6), isolated in Tauton, UK, in 1969 [27], has been fully sequenced. The most conspicuous difference between these two pEAF plasmids is the presence of conjugal transfer (*tra*) genes on E2348/69 pMAR7, a derivative of the wild-type pMAR2 marked with a Tn801 transposon conferring resistance to ...
ampicillin [8, 28], but not in the B171 pEAF, pBI71 [29]. However, the rest of the two plasmids are highly conserved, and they share the genetic organization of bfp and per operons, and three plasmid replication and maintenance regions (repFIIA, repFIB, and stb-par-rsv-cd) [28, 29]. The presence of tra genes in other pEAF has been determined by Southern blot hybridization [28] and genome sequence analysis of tEPEC strains of different phylogenomic lineages [30], but their conjugation ability was not tested experimentally. To our knowledge the E2348/69 pMAR7 and E2347/69 pDEP1, both strains isolated from the same outbreak and belonging to serotype O127:H6, are the only pEAF plasmids for which conjugative transferability has been demonstrated [8, 31, 32]. The aim of this work was to test the self-conjugation ability of four EAF plasmids from tEPEC strains isolated between 2007 and 2008 from children in Peru [33] and the potential of two aEPEC strains isolated from the same geographic region to incorporate them when used as recipient strains in conjugation experiments.

2. Materials and Methods

2.1. EPEC Strains. The EPEC strains used in this work were isolated in 2007-2008, as part of an epidemiological surveillance program in suburban areas of Lima, Peru [33]. Based on the presence of EAF plasmid markers (see below) four tEPEC strains were selected as pEAF plasmid donors, and two aEPEC strains as recipients. E. coli DH5α was also included as a recipient strain (Table 1).

2.2. PCR Detection of pEAF Genes. Based on available pEAF sequences, primers specific for the amplification of the repFIIA replication region and of two distant regions within the tra operon (traI and traC genes, encoding for the relaxase, and an ATPase required for pilus production of the type IV secretion system, respectively), were designed using the primers4clades web server [34] (Table 2). For the detection of the bfp operon, the bfpA sequence was amplified using the primers described by Lacher et al. [35]. DNA was extracted from liquid cultures by a modification of the salt extraction method described by Miller et al. (1988) [36]. Amplifications were performed in 50 μl reactions using a commercial Taq polymerase kit (Thermoscientific) and 1.5 U Taq polymerase per tube, with a final concentration of 1.5 mM MgCl₂, 0.2 mM dNTPs, and 0.5 μM each primer. Two μl of extracted total DNA was used as a template (roughly 50 ng). The cycling program was as follows: 5 min 95°C followed by 30 cycles of 45 s at 94°C, 30 s at 57°C, and 45 s at 72°C and completed by a final extension for 5 minutes at 72°C.

2.3. Insertion of a Kanamycin Resistance Cassette into pEAF. We used the Lambda Red Recombinase system [38] to insert the kanamycin resistance cassette (KmR) carried in pKD4 into the pEAF ISSfl1 locus of the four selected strains (Table 1) using the recombinase function carried on pKD78. The ISSfl1 locus was selected since this was the target for the insertion of the ampicillin resistance Tn801 transposon in EPEC E2348/69 pMAR7 [8]. The K1 and K2 primers in combination with the IS-F and IS-R primers were used to confirm the correct insertion of the kanamycin resistance cassette into ISSfl1 (Table 2).

2.4. Conjugation Experiments. The strains carrying the pEAF-derivatives marked with the kanamycin resistance cassette were used as donors in mating experiments (Table 1).
Table 2: Primers used in this study.

| Primer name | Region | Sequence 5' to 3' | Reference |
|-------------|--------|------------------|-----------|
| pEAF        |        |                  |           |
| bfpA_1H4F   | bfpA   | GTCTGCGTGTTGATTCCAATA | [35]      |
| bfpA_521R   |        | TCAGCAGGAGTAATGCG | [35]      |
| FIIA-F      | repFIIA| CACTTCAACGACGGTCACC | This study|
| FIIA-R      |        | CAGCAACGGAACCCCGAA | This study|
| tral-F      | tral   | GAGCTGGGAAGAGCAGGC | This study|
| tral-R      |        | CAGTTCCTGTTCCTGCAATTT | This study|
| traC-F      | traC   | GACGGGAGTAATAGC | This study|
| traC-R      |        | ACCCCTTCAATAGGAAATA | This study|
| ISSf1l-F    | ISSf1l | TCGCCTCATGGTGAATGGA | This study|
| ISSf1l-R    |        | AGCCACGAGTCGAAGAAC | This study|
| Mutagenesis |        |                  |           |
| ISSf1l-H1   | ISSf1l | CATGTCCTTCGATGGCAACG | This study|
|             |        | GGGTCGTTGAGCGGTTCCAT | This study|
| ISSf1l-H2   |        | ACCGAGTAACCACATTAGATGCTACG | This study|
|             |        | TCGGCAAGTGGATCCAGCG | This study|
| K1          | kan    | CAGTTCATGGCAATGCACT | This study|
| K2          |        | CGGCCACAGTCGAAGGATCC | This study|

E. coli DH5α and two Peruvian aEPEC strains (D3319 and D3264) were used as recipients. Strains D3264 and DH5α were NalR, and a spontaneous NalR colony was selected for D3319. In order to avoid the selection of spontaneous NalR donor strains instead of transconjugants during the conjugation experiments, spontaneous rifampicin resistant (RifR) colonies of the donor strains were selected to have a second antibiotic resistance selection marker. Therefore, the RifR and NalR recipient strains were named DH5αRN, D3319RN and D3264RN (Table 1). Conjugation experiments were performed as follows: 5 ml LB overnight liquid cultures of donor and recipient strains were pelleted, washed and resuspended in 1 ml of sterile water, mixed and incubated overnight at 37°C. The conjugation mix was removed from the LB plate with 1 ml of sterile water, and transconjugants were selected by plating 100 μl of serial dilutions onto solid LB medium supplemented with kanamycin (60 μg/ml), rifampicin (100 μg/ml), nalidixic acid (30 μg/ml), and kanamycin (60 μg/ml) to quantify the number of transconjugants. Conjugation frequencies were calculated as the ratio of number of transconjugants (KmR, RifR, NalR)/number of donor strains (KmR). Each conjugation experiment was repeated at least twice. The presence of the pEAF plasmid was confirmed by bfpA PCR analysis of ten transconjugant colonies per experiment (Table 2).

To corroborate the self-conjugation ability of the pEAF plasmids, the purified plasmid from a transconjugant colony derived from the first conjugation event was transformed by electroporation into DH5α using kanamycin as selection marker. Transformant colonies were checked by PCR amplification of bfpA. A transformant DH5α strain harboring the pEAF was used as donor in further conjugation experiments using DH5αRN as the recipient strain (Figure 1(a)).

2.5. Plasmid Profiling. To analyze the plasmid content of selected isolates, a modified protocol of the alkaline lysis procedure proposed by Kieser was used [39]. The products were separated in 0.7% agarose gels in 1x TBE buffer at 100 volts for 4 hours, stained with a 1% ethidium bromide solution, and photographed.

3. Results

3.1. tEPEC Strains D0131, D3152, D3048, and D3129 Contain pEAF and Conjugative Transfer Markers. EPEC isolates D0131, D3152, D3048, and D3129, previously reported as tEPEC [33] (Table 1), were selected to analyze the conjugative transfer ability of their pEAF plasmids, which were positive for bfpA, repFIIA, tral, and traC genes based on PCR amplification using specific primers (data not shown). This observation suggested that these plasmids are potentially conjugative. aEPEC strains D3319 and D3264 were selected as recipient strains (Table 1).

3.2. The pEAF Plasmids Were Successfully Marked with a Kanamycin Resistance Cassette. In order to assess the conjugation ability of the pEAF plasmids of tEPEC strains D0131, D3152, D3048, and D3129, these were marked with a kanamycin resistance cassette, since the E. coli virulence plasmids rarely carry antibiotic resistance genes [40, 41]. The region selected for its insertion was the IS element ISSf1l, located downstream of the per operon, because it was the target sequence for the Tn801 transposon inserted in EPEC E2348/69 derivative pMAR7, which was demonstrated not to
For the three recipient strains spontaneous αD3319 and D3264 were chosen, as well as the E. coli pD3129::km Rif marker to counter-select the sensitive donor strains. These transfer frequencies were low in the range between $10^{-6}$ to $10^{-8}$ (Table 3). No consistent differences in transfer frequencies among recipient strains were observed. Our results demonstrate that pEAF plasmids contain the complete molecular machinery for self-conjugation irrespective of the E. coli donor genetic background.

4. Discussion

In this study, we report that the pEAF plasmids from four tEPEC strains isolated in 2007-2008 from children in Peru are self-conjugative. To our knowledge, self-conjugation ability has been reported only for the highly similar pMAR7 and pDEPI pEAF plasmids from two EPEC O127:H6 strains DH5α/pEAF::km was used as donor for conjugation with recipient strain DH5α RN (R2), from which transconjugant colonies (Tc2) were derived. (b) Plasmid preparations of donor (D) strain D0131/pD0131::km (1), recipient (R1) strain D3264RN (2), transconjugant (Tc1) strain D3264RN/pD0131::km, obtained from the first-generation conjugation (3), transformant (TfD) DH5α harboring the transconjugant pD0131::km, used as donor in the second-generation conjugation experiment (4), and transconjugant strain D3264RN harboring the pD0131::km (Tc2), obtained in the second-generation conjugation experiment (5).

Table 3: Frequencies of conjugal transfer of the pEAF plasmid of four tEPEC strains.

| Donor tEPEC strain | Recipient strains | Donor tEPEC strain | Recipient strains |
|--------------------|------------------|-------------------|------------------|
| D0131 pD0131::km   | DH5αRN           | 1.1 × 10^{-7} to 7.9 × 10^{-8} |
| D3152 pD3152::km   | 2.8 to 4.2 × 10^{-8} | 2.3 × 10^{-7} |
| D3048 pD3048::km   | 5.2 × 10^{-7} to 9.3 × 10^{-7} | 1.8 × 10^{-8} to 3.6 × 10^{-9} |
| D3129 pD3129::km   | 2.4 × 10^{-6} to 2.0 × 10^{-8} | 1.5 × 10^{-8} to 3.3 × 10^{-7} |

The experiments were repeated at least twice. *Rifampicin and nalidixic acid resistant derivatives.*
isolates [31, 32]. For TEPEC strain B171 (O111:NM), Riley et al. (1987) initially reported that it was able to transfer localized adherence and antibiotic resistance phenotypes [42]; however, sequence studies showed that the B171 pEAF does not carry tra genes [29], and later Nwaneshiudu et al. (2007) demonstrated that the transfer of the adherent and antibiotic resistance phenotypes was due to the mobilization of B171 pEAF by a conjugative antimicrobial resistance plasmid [43]. The pEAF of our strains were tra positive, and subsequent mating experiments supported their self-conjugation (Table 3 and Figure 1).

Our experiments also demonstrate that aEPEC strains can receive the pEAF. These transconjugant strains are suitable candidates for studying the effect of the introduction of the pEAF into aEPEC genetic backgrounds, as well as its stability under laboratory conditions considering that pEAF instability has previously been reported [30, 44, 45]. Future research will also be conducted to analyze the functional responses and gene expression profiles of these aEPEC pEAF-carrying strains, including the expression of BFP and the localized adherence phenotype, as well as the regulatory effect of the per locus over the LEE island. In this regard, recent global transcriptional analyses have demonstrated that pEAF genes influence the expression of a number of chromosomal genes in addition to the LEE [30, 46].

Studies tracing the evolution of EPEC have provided phylogenetic evidence for the multiple gain and loss of the pEAF virulence plasmids [30, 47–49]. Although our mating experiments show low conjugation frequencies ($10^{-6}$ to $10^{-9}$), the present study provides experimental evidence for the self-conjugation of pEAF virulence plasmids between E. coli strains, supporting the role of pEAF transfer in EPEC ecology and evolution.

5. Conclusions

In this study four TEPEC strains were challenged for conjugation of their pEAF virulence plasmids. All the pEAF plasmids were able to self-conjugate into three E. coli recipient strains including a laboratory strain, with transfer frequencies ranging from of $10^{-6}$ to $10^{-9}$. In a second-generation experiment, two of the transconjugant pEAF plasmids were able to conjugate even from a non-EPEC genetic background (DH5α), indicating that these plasmids fully possess the ability for lateral transfer. Moreover, the generation of transconjugant aEPEC strains harboring pEAF plasmids provides valuable specimens to further perform functional response analysis.

Disclosure

The current address of Crispín Zavala-Alvarado is Unit of Biology of Spirochetes, Department of Microbiology, Institute Pasteur, Paris, France.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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