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A Second Large Plasmid Encodes Conjugative Transfer and Antimicrobial Resistance in O119:H2 and Some Typical O111 Enteropathogenic Escherichia coli Strains

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Enteropathogenic Escherichia coli (EPEC) continues to be a major cause of diarrhea and death, predominantly in developing countries (8, 22). During infection, EPEC strains attach intimately to the intestinal epithelium and efface the absorptive microvilli, initiating a complex signaling cascade that ultimately leads to diarrhea by mechanisms that are only partially understood (8). These pathogenic effects manifest as an "attaching and effacing" histopathological hallmark, which is conferred by a large chromosomal island called the locus of enterocyte effacement (LEE). The LEE encodes a virulence gene regulator and a type III secretion system and effectors, as well as the intimin adhesin and its translocated receptor. The LEE is present in all EPEC strains, as well as in enterohemorrhagic E. coli and some animal pathogens (reviewed in references 8 and 22).

EPEC strains are classified as being either typical or atypical based on their ability to form densely packed three-dimensional clusters, or microcolonies, a phenotype known as localized adherence (LA). LA is conferred by a large EPEC adherence factor (EAF) plasmid, associated with virulence in epidemiological and volunteer challenge studies, which is present in all typical EPEC strains and absent or altered in atypical EPEC strains (3, 16, 25, 35). The specific virulence factor responsible for LA is an EAF plasmid-encoded bundle-forming pilus (Bfp), which also contributes to antigenicity, autoaggregation, and biofilm formation (3, 11, 15, 20). On a separate region of the EAF plasmid are the perABC genes, encoding the plasmid-encoded regulator (a transcriptional activator for the bfp operon and the LEE, as well as its own promoter) and other virulence loci (9, 19). The EAF plasmid and the LEE are believed to have been acquired by different EPEC lineages in separate horizontal events (30).

EAF plasmids from two well-studied EPEC strains have been sequenced. The major difference between the EAF plasmids from isolates E2348/69 (of the EPEC1 lineage [30]) and B171 (representing the EPEC2 lineage [30]), is the presence of conjugative transfer (tra) genes on pMAR7, the E2348/69 EAF plasmid (7). The B171 EAF plasmid, pB171, does not contain tra genes (36), even though at least one earlier paper reported conjugative transfer of pB171 (31). Other than with respect to tra genes, the two sequenced EAF plasmids are highly conserved, and low-resolution mapping suggests that other EAF plasmids are similarly conserved (23). Many atypical EPEC isolates lack the entire EAF plasmid. Other strains harbor a plasmid that shares a conserved backbone, and is in many ways similar to pB171 and pMAR7, but with inactivating mutations in the bfp and per operons that are required for LA (5, 27). Such strains are phenotypically “atypical,” even though they carry probe-detectable EAF plasmids. We therefore hypothesized that the large plasmids of these strains would carry compensatory virulence and/or fitness genes.

An atypical O119:H2 EPEC strain, MB80, with a deletion in the bfp operon and inactivating point mutations in the plasmid-encoded regulator genes, was selected for study (5, 27). Plasmids used, identified, or constructed in the course of the study are listed in Table 1, and standard molecular biology procedures were used throughout (32). Alkaline lysis-extracted (4) EPEC plasmid bands in the range of 60 to 150 kb were gel-purified away from smaller plasmids and contaminating chromosomal DNA by electroelution. A PCR-select subtractive hybridization kit (Clontech/BD Biosciences) was used to subtract the RsaI-digested EPEC strain MB80 large-plasmid DNA (tester) from a similarly digested preparation from prototypical EPEC strain E2348/69 (driver), according to the manufacturer’s instructions, essentially as described by Akopyants et al.
strains produced an amplicon with pED208-specific 
not conserved in MB80 (27) (Table 3). None of the EPEC 
VOL. 189, 2007 NOTES 6075 
whose product straddles 
traU 

pGEM-T Amp r; TA cloning vector Promega 
pBluescript Cloning vector Stratagene 

the six fragments mapped to a conjugative (..ac.uk/cgi-bin/BLAST/submitblast/escherichia_shigella. Four of 
analysis of the in-process E2348/69 genome at http://www.sanger 
confirmed that all were absent from the driver genome by BLAST 
nucleotide-nucleotide BLAST search.

We reproducibly subtracted six MB80-specific sequences and 
confirmed that all were absent from the driver genome by BLAST 
analysis of the in-process E2348/69 genome at http://www.sanger .ac.uk/cgi-bin/BLAST/submitblast/escherichia_shigella. Four of 
the six fragments mapped to a conjugative (tra) system with ho-

tology to the system from Salmonella enterica serovar Typhi 
plasmid pED208 (10, 18) (Table 2). We obtained single-strand end-sequence(s) from eight Pst1 and EcoRI fragments located 
outside the resistance region of plasmid pMB80, and seven of 
these mapped to the tra region (see Table S2 in the supplemen-
tary material). With the subtracted clones, this approach yielded clone 
coverage of about 12 kb of the predicted 32-kb region, at least one 
sequence hit for 15 of the 22 open reading frames between traB 
and traC. From BLAST searches and restriction mapping, we are 
able to infer that this part of the MB80 transfer region shows 
similar gene organization and approximately 80% identity at the 
DNA level to that of Salmonella serovar Typhi plasmid pED208 
(18) (Table 2; see Table S2 in the supplemental material).

We designed primers complementary to internal regions of the 
tra genes and traC genes that are conserved between the 
MB80 tra system and pED208, as well as one primer pair 
whose product straddles traU and traC in pED208 in a region 
not conserved in MB80 (27) (Table 3). None of the EPEC 
strains produced an amplicon with pED208-specific traU-traC 
primers, but three other strains produced the predicted ampli-
con for the traC and traI loci (Table 3). A panel of 26 enter-
aggregative E. coli strains, which belong to a pathotype for 
which plasmid-borne, transferable resistance is common (26, 
28), were also screened, and all were negative for the tra and 
traC PCRs. Strain B171, a prototypic O111:NM outbreak iso-
late from the United States, was one of the EPEC strains that 
screened positive for traI and traC, even though there are no 
sequences homologous to pMB80 tra genes in its EAF plasmid 
(pB171) (36).

Antimicrobial susceptibility testing was performed by the 
disk diffusion method (24), using disks containing ampicillin 
(10 μg), tetracycline (30 μg), trimethoprim (5 μg), nalidixic 
acid (30 μg), chloramphenicol (30 μg), sulfonamide (300 μg), 
streptomycin (10 μg), and ciprofloxacin (5 μg) (Remel, KS). The 

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acid (30 μg), chloramphenicol (30 μg), sulfonamide (300 μg), 
streptomycin (10 μg), and ciprofloxacin (5 μg) (Remel, KS). The 

In a first step towards localizing tra genes in the MB80 and 
B171 genomes, mating reactions were set up with traI- and 
traC-positive EPEC strains as donors and a nalidixic acid-

| TABLE 1. Plasmids and constructs used in this study |
|-----------------|-----------------|-----------------|
| Plasmid(s) or construct | Description | Reference or source |
| pBR322 | Cloning vector | 32 |
| pBluescript | Cloning vector | Stratagene |
| pGEM-T | Amp' ; TA cloning vector | Promega |
| pED208 | Derepressed form of IncFV plasmid F,Jac | 10, 18 |
| pB171, pE2348 (pMAR7), and pMB80 | Wild-type EAF plasmids from EPEC strains B171 (O111:H2), E2348/69 | 5, 7, 23, 36 |
| pMB80-2 | | |
| pB171-2 | Conjugative resistance plasmid from EPEC strain MB80 | This study |
| pINKM1t | Miniplasmid generated from pMB80-2 and containing most of the resistance region | This study |
| pINKE15k, pINKE18t | Resistance region subclones of from pMB80-2 | This study |

## TABLE 2. Blast hits for MB80 plasmid-specific DNA fragments isolated by subtractive hybridization |
<table>
<thead>
<tr>
<th>Subtracted clone</th>
<th>BLAST hit</th>
<th>GenBank accession no. (region)</th>
<th>BLAST identities (%)</th>
<th>expect value</th>
</tr>
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<tbody>
<tr>
<td>pAN1</td>
<td>Insertion sequence IS26 from E. coli plasmid pAPEC-O2-R</td>
<td>134/137 (97), 5e-63</td>
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<td>pAN2</td>
<td>Insertion element IS2 from E. coli K-12 MG1655</td>
<td>187/193 (96), 4e-89</td>
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<td>pAN3</td>
<td>traI gene from Salmonella enterica serovar Typhi plasmid pED208</td>
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<td></td>
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<td>pAN4</td>
<td>IncFV plasmid transfer region</td>
<td>177/203 (87), 2e-47</td>
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<td></td>
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<tr>
<td>pAN5</td>
<td>trbB-traH from Salmonella enterica serovar Typhi plasmid pED208</td>
<td>AF411480.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pAN6</td>
<td>IncFV plasmid transfer region</td>
<td>56/61 (91), 1e-12</td>
<td></td>
<td></td>
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<tr>
<td>pAN7</td>
<td>trbB-traH from Salmonella enterica serovar Typhi plasmid pED208</td>
<td>AF411480.1</td>
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<td></td>
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<tr>
<td>pAN8</td>
<td>IncFV plasmid transfer region</td>
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<td>pAN9</td>
<td>tral from Salmonella enterica serovar Typhi plasmid pED208 IncFV plasmid transfer region</td>
<td>389/446 (87), 6e-118</td>
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<td></td>
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<tr>
<td>Strain</td>
<td>Serotype</td>
<td>Year</td>
<td>Country</td>
<td>traI (pMB80-2/pED208)</td>
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<td>----------</td>
<td>------</td>
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<td>----------------------</td>
</tr>
<tr>
<td>C54-58 (DEC 1a)</td>
<td>O55:H6</td>
<td>1958</td>
<td>Dutch Guyana</td>
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<td>Beta</td>
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<td>1947</td>
<td>Scotland</td>
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<td>1956</td>
<td>United States</td>
<td>−</td>
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<td>E990</td>
<td>O86:NM</td>
<td>1950</td>
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<td>Stoke W</td>
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<td>Nigeria</td>
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<td>England</td>
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<td>−</td>
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<td>#19</td>
<td>O142:H6</td>
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<td>−</td>
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<td>#15</td>
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<td>−</td>
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<td>pre-1960</td>
<td>Indonesia</td>
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<td>1982</td>
<td>Peru</td>
<td>−</td>
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<td>Canine 4225</td>
<td>O142:H6</td>
<td>1982</td>
<td>−</td>
<td>−</td>
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<tr>
<td>pED208 in K12</td>
<td>O128:NM</td>
<td>1982</td>
<td>−</td>
<td>−</td>
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<tr>
<td>EAEC 042</td>
<td>O44:H18</td>
<td>1993</td>
<td>−</td>
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<td>S. enterica CT18</td>
<td>Typhi</td>
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resistant plasmidless K-12 *E. coli* strain, C600, as the recipient. Transconjugants, selected on plates containing tetracycline and nalidixic acid, harbored an 80- to 120-kb plasmid. In all conjugation reactions, using all *traI* and *traC*-positive EPEC strains as donors, we were able to cotransfer multiple antimicrobial resistance markers along with the *tra* loci. Transfer frequencies from MB80 and B171 were $2 \times 10^{-5}$ and $7 \times 10^{-5}$, respectively, by liquid mating and $6 \times 10^{-5}$ and $7 \times 10^{-6}$ by solid mating (18). In most but notably not all transconjugants, the EAF plasmid-encoded *perA* gene had not been cotransferred. The plasmids could be transferred from C600 transconjugants to a rifampin-resistant derivative of *E. coli* ORN172, verifying that the plasmids were sufficient to mediate their own transfer. Plasmid extraction by alkaline lysis or boiling protocols followed by horizontal gel electrophoresis resulted in a single band or close doublet for large plasmids from wild-type strains MB80 and B171, consistent with profiles presented by earlier investigators (31).

Our own results and other published results for horizontally electrophoresed plasmid preparations suggest that although additional plasmids smaller than 12 kb can be visualized in some EPEC strains, only one large plasmid band is discernible by conventional electrophoresis methods (31). To verify that the second large plasmids recovered from strains MB80 and B171 were present in the wild-type strain (and not, for example, excised from the chromosome during conjugation), we performed plasmid profiling by an alternate method. Vertical electrophoresis of Kado and Liu preparations on E-buffer gels (14) demonstrated in every case that the strains contained a second large plasmid, consistent with profiles presented by earlier investigators (31).

Through restriction analysis, end sequencing of *MluI* miniplasmid pNKMI1 subcloned from pMB80-2, and PCR using primers for resistance genes on *Salmonella* serovar Typhi plasmid pHCM1 (Table 3; see Table S2 in the supplemental material), we were able to determine the resistance gene content and order within the pMB80-2 resistance region. The pMB80-2 plasmid bears a β-lactamase gene flanked by IS26 elements, a *sulII* gene encoding dihydropterate synthase, which mediates sulfonamide resistance, *strAB* genes encoding streptomycin resistance, a trimethoprim resistance gene, and a mercury resistance (*mer*) operon. These loci are 99% identical to the resistance genes on plasmid pHCM1 (29), and their organization is similar. Downstream of this 30-kb cluster lies a tetracycline resistance operon, bordered by *pem* genes and identical to the Tn721 tetracycline resistance determinant found on the *E. coli* multiresistant plasmid pC15-1a (6).

Initial optimism that resistance would spread slowly among EPEC strains (34) has been overshadowed by reports of multidrug-resistant EPEC strains, particularly O111 strains, from diverse parts of the world (1, 12, 13, 17, 21, 33, 38, 39). We have identified a clandestine plasmid that accounts for this resistance in a subset of O111:H2/NM and O119:H2 EPEC strains, but which is absent from most other EPEC strains. The conjugative multidrug resistance plasmid identified in this study has been previously undetected or ignored due to its similarity in size with the EAF virulence plasmid in B171 and other strains. Its presence explains a number of properties that have been previously undetected or ignored due to their similarity in size with the EAF virulence plasmid in B171 and other strains.
been experimentally or anecdotaly assigned to O111 and related EPEC strains but were not found on the B171 virulence plasmid (36), including antimicrobial resistance, conjugative transfer, and the putative virulence gene csi (13, 31). The revelation that this plasmid is present but has been previously unreported from a well-studied strain caused us to examine the literature on strain B171 and other EPEC strains closely.

Nataro et al. (23) found that resistance was a common feature of EPEC strains but were unable to select plasmid co-transformants that harbored resistance genes and hybridized to a genetic marker on the EAF virulence plasmid in multiple strains, not including strain B171. In contrast, Riley et al. (31) were able to demonstrate conjugative transfer of resistance and LA in strain B171, but they identified only one 54-MDa plasmid in their transconjugants and therefore concluded that these genes were on the same plasmid. However, when the large plasmid from strain B171 was later sequenced, no tra genes were found. The inability of the whole-plasmid sequencing project to detect the second plasmid, pB171-2, is explained by the fact that the sequence was assembled from subclones, rather than by more-commonly employed shotgun protocols (36). A retrospective assessment of the data presented in these papers suggests that resistance and EAF plasmids in strains studied by both groups were, as in this study, separate entities and that the element was not excised from the chromosome during more recent storage or transport. It is likely that the conjugative multiresistance plasmid has been overlooked or ignored because it does not carry key EPEC virulence genes and because, in routine plasmid profiles, it overlaps or runs closely with the EAF plasmid.

Multidrug-resistant O111 EPEC strains have been predominant causes of nursery outbreaks since the 1970s (1, 12, 13, 17, 21, 33, 38, 39). The efficiently transferred conjugative resistance plasmid in MB80, B171, and other O111 strains could have assisted in maintaining this epidemiologic importance, due to the selective advantage conferred by antimicrobial resistance. This opens the question of whether the plasmid, or at least one of its precursors, such as the 1947 Scottish Stoke W plasmid, was acquired vertically, before clonal expansion of this EPEC lineage, or horizontally in relatively recent times. If the latter is the case, the question of how the resistance plasmid spread rapidly and apparently selectively within the O111: H2/NM and O119:H2 EPEC lineages arises. Epidemic spread of resistance plasmids within a pathotype has been documented for successful inH plasmids among Salmonella serovar Typhi isolates in Asia (40). The ability of the resistance plasmid to occasionally mobilize the EAF plasmid means that it can, at least in theory, play some role in pathogen evolution. Finally, the identification of these EPEC conjugation plasmids represents the first example of an IncFV pED208 (or F Iac)-like conjugative system reported from nature since the 1960s. More expansive studies of the epidemiology of this plasmid family in EPEC strains, using markers identified in this study, could address the question of its origin and potential for persistence or further spread.

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