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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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A novel and functional conjugative transfer system identified in O119:H2 enteropathogenic *Escherichia coli* (EPEC) strain MB80 by subtractive hybridization is encoded on a large multidrug resistance plasmid, distinct from the well-described EPEC adherence factor (EAF) plasmid. Variants of the MB80 conjugative resistance plasmid were identified in other EPEC strains, including the prototypical O111:NM strain B171, from which the EAF plasmid has been sequenced. This separate large plasmid and the selective advantage that it confers in the antibiotic era have been overlooked because it comigrates with the virulence plasmid on conventional gels.

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 E2368/69 (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.

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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 ^r ; TA cloning vector	Promega
pED208	Derepressed form of IncFV plasmid F ₀ lac	10, 18
pB171, pE2348 (pMAR7), and pMB80	Wild-type EAF plasmids from EPEC strains B171 (O111:H2), E2348/69 (O127:H6), and MB80 (O119:H2), respectively	5, 7, 23, 36
pMB80-2	Conjugative resistance plasmid from EPEC strain MB80	This study
pB171-2	Conjugative resistance plasmid from EPEC strain B171	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

(2). E2348/69 carries a larger virulence plasmid (98 kb versus 69 kb in B171 [7, 36]) and was therefore selected as the driver over the more closely related B171. Subtracted sequences were cloned into pGEM-T (Promega), sequenced, and compared to the GenBank nucleotide database (www.ncbi.nlm.nih.gov) by a 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 homology to the system from *Salmonella enterica* serovar Typhi plasmid pED208 (10, 18) (Table 2). We obtained single-strand end-sequence(s) from eight PstI 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 supplemental 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 *orf4*. 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 *traI* and the *traC* genes that are conserved between the MB80 *tra* system and pED208, as well as one primer pair whose product straddles *traU* and *trbC* in pED208 in a region not conserved in MB80 (27) (Table 3). None of the EPEC strains produced an amplicon with pED208-specific *traU-trbC*

primers, but three other strains produced the predicted amplicon for the *traC* and *traI* loci (Table 3). A panel of 26 entero-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 *traI* and *traC* PCRs. Strain B171, a prototypic O111:NM outbreak isolate 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 inhibition zone diameters were interpreted according to Clinical and Laboratory Standards Institute (formerly NCCLS) requirements (24), and *E. coli* NCTC 10418 was used as the control. All wild-type EPEC strains were nalidixic acid sensitive, but most were resistant to one or more other agents. Five of seven O111/O119:H2 EPEC strains were resistant to three or more antimicrobials, and three of these were positive for the pMB80-2 *traI* and *traC* genes. We were able to identify at least two of the resistance loci in each of these strains by PCR (Table 3). Only one strain, 1947 Scottish isolate Stoke W (O111:NM), from early in the antibiotic era, possessed the *tra* genes and was sensitive to all tested antimicrobials.

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 2. Blast hits for MB80 plasmid-specific DNA fragments isolated by subtractive hybridization

Subtracted clone	BLAST hit	GenBank accession no. (region)	BLAST identities (%), expect value
pAN1	Insertion sequence IS26 from <i>E. coli</i> plasmid pAPEC-O2-R	AY214164.3	134/137 (97), 5e-63
pAN2	Insertion element IS2 from <i>E. coli</i> K-12 MG1655	U00096 (region 380068..382096)	187/193 (96), 4e-89
pAN3	<i>traI</i> gene from <i>Salmonella</i> serovar Typhi plasmid pED208 IncFV plasmid transfer region	AF411480.1	177/203 (87), 2e-47
pAN4	<i>trbB-traH</i> from <i>Salmonella</i> serovar Typhi plasmid pED208 IncFV plasmid transfer region	AF411480.1	56/61 (91), 1e-12
pAN5	<i>traV-traC</i> from <i>Salmonella</i> serovar Typhi plasmid pED208 IncFV plasmid transfer region	AF411480.1	273/312 (87), 3e-81
pAN6	<i>traI</i> from <i>Salmonella</i> serovar Typhi plasmid pED208 IncFV plasmid transfer region	AF411480.1	389/446 (87), 6e-118

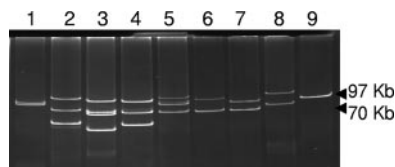


FIG. 1. Resolution of Kado and Liu (14) plasmid preparations by vertical electrophoresis demonstrates multiple plasmids in EPEC2 strains. Loaded (left to right) are plasmid preparations from control strain BA337 with two plasmids (lane 1); O111 strains 009-271082 (lane 2), Stoke W (lane 3), 2309-77 (lane 4), DIF043256 (lane 5), 2966-5 (lane 6), and B171 (lane 7); O119:H2 strain MB80 (lane 8); and control O127:H6 strain E2348/69 with one plasmid (lane 9). Molecular mass markers are on the right.

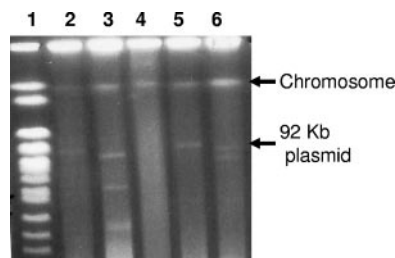


FIG. 2. Pulsed-field gel electrophoresis of undigested DNA from EPEC strain B171 and controls. Lane 1: yeast chromosome markers. Lanes 2 to 6: undigested DNA from *E. coli* strains (with plasmid size in kb, if confirmed by genome sequence) EDL933 (lane 2; single large plasmid, 92 kb); H30 (lane 3); MG1655 (lane 4; no plasmid); E2348/69 (lane 5; single large plasmid, 98 kb); and B171 (lane 6).

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 2×10^{-5} and 7×10^{-5} , respectively, by liquid mating and 6×10^{-5} and 7×10^{-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 smaller EAF plasmid of 69 to 90 kb and a larger conjugative resistance plasmid of 100 to 120 kb for MB80 and B171 (Fig. 1). EAF-negative transconjugants carried only the larger plasmid, which we designate pB171-2. Other O111 strains showed 2 to 4 large-plasmid bands.

Pulsed-field gel electrophoresis has been successfully used to resolve large circular replicons (37). We performed pulsed-field gel electrophoresis of DNA extracted from agarose-embedded B171 and compared the profile obtained with that of genome-sequenced *E. coli* strains. Again the data demonstrate that B171 has two large plasmids, the previously sequenced EAF plasmid, pB171, and a second replicon, pB171-2 (Fig. 2). This finding was supported by MluI restriction analysis of large-plasmid DNA. Thus, the EPEC conjugative system identified in this study, unlike that of strain E2348/69 (7), is present on a plasmid separate from the virulence plasmid, and this

second plasmid also bears multiple antimicrobial resistance genes.

We searched the GenBank database for genetic loci that are present in EPEC but are not present on sequenced EAF plasmids or the LEE. We identified one locus, predicted to be a *csi* (calcium sequestration inhibitor) gene for the well-studied strain B171 (GenBank accession no. Y08258). PCR and sequencing demonstrated that the *csi* gene was unique to strain B171 and encoded on the conjugative resistance plasmid. We did not find epithelial cell adherence, autoagglutination, or biofilm formation associated with the pMB80-2 or pB171-2 plasmids, although it is possible that virulence functions conferred by the plasmid could be identified in the future. The genome of EPEC strain B171 is currently being sequenced (http://msc.tigr.org/e_coli_and_shigella/index.shtml), and any other genes of interest carried by pB171-2 should soon be in the public domain.

Through restriction analysis, end sequencing of MluI miniplasmid pINKM1t 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 *sulIII* 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 Tn1721 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 experimentally or anecdotally assigned to O111 and related EPEC strains but which 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 *incH* 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 *F₀Jac*)-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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