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Authors

Botts, Ryan

Page, Dawne

Bravo, Joseph

et al.

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Polluted wetlands contain multidrug-resistance plasmids encoding CTX-M-type extended-spectrum β-lactamases

Ryan T. Botts[a], **Dawne M. Page**[b], **Joseph A. Bravo**[b], **Madelaine L. Brown**[b], **Claudia C. Castilleja**[b], **Victoria L. Guzman**[b], **Samantha Hall**[b], **Jacob D. Henderson**[b], **Shelby M. Kenney**[b], **Mariele E. Lensink**[b], **Megan V. Paternoster**[b], **Sarah L. Pyle**[b], **Lucas Ustick**[a,b], **Chara J. Walters-Laird**[b], **Eva M. Top**[c], **David E. Cummings**[b,*]

[a]Department of Mathematics, Information, and Computer Sciences, Point Loma Nazarene University, 3900 Lomaland Dr., San Diego, CA, 92106

[b]Department of Biology, Point Loma Nazarene University, 3900 Lomaland Dr., San Diego, CA, 92106

[c]Department of Biological Sciences and Institute for Interdisciplinary Data Sciences (IIDS), University of Idaho, 875 Perimeter Dr., Moscow, ID, 83844

Abstract

While most detailed analyses of antibiotic resistance plasmids focus on those found in clinical isolates, less is known about the vast environmental reservoir of mobile genetic elements and the resistance and virulence factors they encode. We selectively isolated three strains of cefotaxime-resistant *Escherichia coli* from a wastewater-impacted coastal wetland. The cefotaxime-resistant phenotype was transmissible to a lab strain of *E. coli* after one hour, with frequencies as high as 10^{-3} transconjugants per recipient. Two of the plasmids also transferred cefotaxime resistance to *Pseudomonas putida*, but these were unable to back-transfer this resistance from *P. putida* to *E. coli*. In addition to the cephalosporins, *E. coli* transconjugants inherited resistance to at least seven distinct classes of antibiotics. Complete nucleotide sequences revealed large IncF-type plasmids with globally distributed replicon sequence types F31:A4:B1 and F18:B1:C4 carrying diverse antibiotic resistance and virulence genes. The plasmids encoded extended-spectrum β-lactamases $bla_{CTX\text{-}M\text{-}15}$ or $bla_{CTX\text{-}M\text{-}55}$, each associated with the insertion sequence IS*Ec9*, although in different local arrangements. Despite similar resistance profiles, the plasmids shared only one resistance gene in common, the aminoglycoside acetyltransferase *aac(3)-IIe*. Plasmid accessory cargo also included virulence factors involved in iron acquisition and defense against

*Correspondence: David E. Cummings, PH: 1-619-849-2642, davidcummings@pointloma.edu.

CONFLICT OF INTEREST STATEMENT

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Declaration of interest: None

DEDICATION

We dedicate this manuscript to the memory of our late friend and colleague, Dr. Ryan T. Botts.

host immunity. Despite their sequence similarities, several large-scale recombination events were detected, including rearrangements and inversions. In conclusion, selection with a single antibiotic, cefotaxime, yielded conjugative plasmids conferring multiple resistance and virulence factors. Clearly, efforts to limit the spread of antibiotic resistance and virulence among bacteria must include a greater understanding of mobile elements in the natural and human-impacted environments.

Keywords

antibiotic resistance gene; plasmid; wetland; mobile genetic element; horizontal gene transfer; multidrug resistance; CTX-M; β-lactamase

1. INTRODUCTION

The application of antibiotics to combat bacterial infections is of paramount importance in modern medicine, yet resistance has arisen at an astonishing evolutionary pace, due, at least in part, to horizontal gene transfer mediated by conjugative plasmids (Falagas et al. 2005; Giske et al. 2008; Nordmann et al. 2011; Strahilevitz et al. 2009). Conjugative, or self-transmissible, plasmids are independent DNA elements, generally double-stranded and circular, that replicate separately from the bacterial chromosome. They consist of backbone genes that encode for basic replication, maintenance, transfer, and stability functions as well as accessory genes tied to mobile elements, particularly transposons and integrons. A review of the literature suggests that most acquired antibiotic resistance in pathogenic bacteria stems from specific accessory genes found on conjugative plasmids (*e.g.*, Li et al. 2019; Maslova et al. 2022; Pan et al. 2022; Peterson and Kaur 2018; Wright 2019).

Although largely overlooked, antibiotic resistance genes (ARGs) and the plasmids on which they are found in the natural environment (*i.e.*, outside of the clinic or the community) are of great significance. It has been argued that many, if not all, ARGs originate in microorganisms colonizing natural habitats such as soil and water (Allen et al. 2009; Aminov 2009; Benveniste and Davies 1973; Cantón et al. 2012; D'Costa et al. 2011; Martínez 2009; Poirel et al. 2004; Poirel et al. 2005). How these elements find their way into bacteria causing infections in humans has not yet been established (Forsberg et al. 2012), but there is evidence that animal vectors such as shorebirds may contribute to their redistribution (Luo et al. 2022; Navedo et al. 2021; Zeballos-Gross et al. 2021). Furthermore, the natural environment may provide an ideal milieu in which ARGs and resistance plasmids can evolve through mutation, recombination, and the activity of mobile genetic elements, all in the absence of the strong directional pressures found in the clinical context.

Of particular concern are the extended-spectrum β-lactamases (ESBLs), arguably the most significant obstacles in the clinical battle against infections caused by Gram-negative bacilli (Goldstein 2021). ESBLs inactivate virtually all penicillins and many cephalosporins, leaving few viable treatment options (D'Andrea et al. 2013; Paterson and Bonomo 2005). The presence of ESBLs contributes significantly to increased healthcare costs as well as poor patient outcomes (Centers for Disease Control and Prevention 2013; O'Neill Commission 2014; World Health Organization 2014).

Among the ESBLs, none has had a greater impact than the CTX-M enzymes (Bush and Bradford 2020), named for their ability to inactivate the third-generation cephalosporin cefotaxime (CTX). CTX is a critical drug for combatting drug-resistant Gram-negative bacteria, especially members of the *Enterobacteriaceae*. The first $bla_{CTX\text{-}M}$ gene, reported in Germany in 1990, was found in an *E. coli* strain infecting the ear canal of a newborn (Bauernfeind et al. 1990). Since then, variants have spread to virtually every region of the world (Bevan et al. 2017), becoming the most common ESBL in circulation (Castanheira et al. 2021). This global emergence of highly mobile, plasmid-encoded CTX-M cefotaximases has severely handicapped healthcare providers, requiring the use of less optimal 'last-resort' antibiotics such as carbapenems and polymyxins to combat life-threatening infections and contributing to the rise in carbapenemases and *mcr*-type colistin resistance factors (Dadashi et al. 2021; Halat and Moubareck 2020).

The Tijuana River Estuary is a large coastal wetland along the border of the United States and Mexico, near San Diego, California, USA (Zedler et al. 1992). Surface sediments contain a diverse community of bacteria dominated by photosynthetic and anaerobic taxa during the dry season (Cummings et al. 2010). In the wet season, sewage-laden stormwater flows into the estuary with winter rains where exogenous bacteria intermix with these natural populations. In recent years, we have demonstrated that clinically relevant ARGs and multi-drug resistance plasmids are part of the Tijuana River Estuary metagenome (Borgogna et al. 2016; Botts et al. 2017; Cummings et al. 2011). In one study (Borgogna et al. 2016), we found evidence of a vast reservoir of novel $bla_{CTX\text{-}M}$ alleles, most of which had never been reported in clinical pathogens. More recently (Botts et al. 2017), we described a multi-drug resistance and virulence plasmid harboring $bla_{CTX\text{-}M\text{-}55}$ in an isolate of *E. coli* ST744 in this same wetland. Following up on these two important findings, the aim of the present study was to gain a better understanding of the genetic context of the plasmids on which $bla_{CTX\text{-}M}$ genes are located in this natural, human-impacted environment. Here we describe three new, but related, CTX-M-encoding plasmids isolated from *E. coli* strains in the Tijuana River Estuary. Interestingly, these plasmids share highly similar backbones but harbor different combinations of modules of ARGs and virulence genes. Additionally, three different configurations of the genetic context immediately surrounding $bla_{CTX\text{-}M}$ were seen.

2. MATERIALS AND METHODS

2.1. Antibiotic abbreviations

AN, amikacin; AM, ampicillin; ATM, aztreonam; AZM, azithromycin; C, chloramphenicol; CAZ, ceftazidime; CF, cefalothin; CIP, ciprofloxacin; CL, colistin; CTX, cefotaxime; CXM, cefuroxime; D, doxycycline; ETP, ertapenem; FEP, cefepime; FOX, cefoxitin; GAT, gatifloxacin; IPM, imipenem; K, kanamycin; LOM, lomefloxacin; LVX, levofloxacin; MEM, meropenem; NA, nalidixic acid; NOR, norfloxacin; OFX, ofloxacin; PIP, piperacillin; RIF, rifampicin; S, streptomycin; SAM, ampicillin/sulbactam; SXT, sulfamethoxazole/trimethoprim; TE, tetracycline; TIC, ticarcillin; TIM, ticarcillin/clavulanic acid; TZP, piperacillin/tazobactam; ZEO, Zeocin.

2.2. Selective isolation and identification of cefotaxime-resistant wetland bacteria

Surface sediments (50 g from top 5 mm) from the Tijuana River Estuary were collected from the main river channel and a nearby tidal channel with sterile spatulas three days after rainfall and transported in sterile centrifuge tubes on ice. In the laboratory, bacteria were gently removed from 5 g homogenized sediments by incubation in 25 mL sterile $Na_4P_2O_7$ (0.1%) for 1 h with stirring. The suspension was allowed to settle for 1 h after which 10 mL of the liquid phase was decanted. Cells were collected from the liquid phase by centrifugation at 10,000 *g* for 10 min. and the resulting pellet was re-suspended in 3 mL sterile saline (0.85% NaCl). One hundred μL of this suspension were spread onto plates of MacConkey agar amended with CTX (5 μg mL^{-1}) and incubated at 37°C overnight. Thirteen red colonies of lactose-fermenting Gram-negative bacteria were formed. Gram-negative, lactose-fermenting isolates were identified with standard growth and biochemical tests: growth on MacConkey, triple sugar iron, and blood agar; spot indole, oxidase, and MUG tests) (Mahon and Lehman 2016) and API20E test strips (bioMérieux, Marcy-l'Étoile, FR).

2.3. Mating experiments

One-half mL of plasmid donor was combined with 0.5 mL recipient bacteria (either *Escherichia coli* HY842 (RIF^R, S^R, ZEO^R) (Brown et al. 2013), *E. coli* JM109 (NA^R) (Promega, Madison, USA), or *Pseudomonas putida* KT2440 (C^R) (Nelson et al. 2002)) (16-h cultures in LB broth) and immobilized onto a sterile 0.22-μm nitrocellulose membrane filter. Control filters comprised either donor or recipient bacteria alone. All filters were incubated on the surface of an LB agar plate (no antibiotics) at 30°C for 24 hours. Biomass was removed with a sterile cotton swab and resuspended in 1 mL sterile saline. LB agar plates with (1) no antibiotics, (2) recipient-selective antibiotics, (3) CTX (5 μg mL^{-1}), or (4) recipient-selective antibiotics and CTX were inoculated with 50 μL of resuspended bacteria, and incubated at 37°C (*E. coli* HY842, *E. coli* JM109) or 30°C (*P. putida* KT2440) for 18–24 h. Recipient-selective antibiotics included RIF (100 μg mL^{-1}), S (100 μg mL^{-1}), and ZEO (100 μg mL^{-1}) for *E. coli* HY842, NA (5 μg mL^{-1}) for *E. coli* JM109, or C (50 μg mL^{-1}) for *P. putida* KT2440. Putative transconjugants were first confirmed to be the intended recipient strain using randomly amplified polymorphic DNA (RAPD) PCR using primers 208 and 272 (Mahenthiralingam et al. 1996) followed by plasmid extraction and gel electrophoresis to confirm the presence of a plasmid.

2.4. Total DNA extraction, plasmid DNA extraction, and polymerase chain reaction

Total DNA was extracted using the GenElute Bacterial Genomic DNA Kit (Sigma-Aldrich, St. Louis, USA) according to the manufacturer's instructions. Plasmid DNA was extracted with Qiagen Plasmid Mini, Midi, or Maxi Kits (Hilden, DE) according to the manufacturer's instructions. Conventional (non-quantitative) PCR and gel electrophoresis were carried out according to standard methods (Ausubel et al. 1993, Green and Sambrook 2012).

2.5. Antimicrobial susceptibility testing

Plasmid-bearing strains were repeatedly (n = 5) subjected to antimicrobial susceptibility testing against a broad range of antibiotics by the disc diffusion method according to CLSI protocols and standards (Clinical and Laboratory Standards Institute 2015, 2017) with

antibiotics obtained from BD (Becton, Dickinson, and Co., Franklin Lakes, USA). Minimum inhibitory concentrations were estimated with ETESTs (BioMérieux, Marcy-l'Étoile, FR). *E. coli* ATCC 25922 was employed for quality control (American Type Culture Collection, Manassas, USA). 'Clinical resistance' is defined as a zone of inhibition smaller than the Resistant breakpoint set for the *Enterobacteriaceae* by the CLSI for a given antibiotic, while the term 'decreased susceptibility' is used to describe a statistically significant decrease of ≥ 3 mm in zone of inhibition relative to *E. coli* HY842 without plasmid (n = 5, two-tailed t-test assuming unequal variance, $p < 0.01$).

2.6. Determination of $bla_{CTX\text{-}M}$ copy number per cell by qPCR

The copy number of the plasmid-encoded $bla_{CTX\text{-}M}$ gene per cell was estimated in *E. coli* HY842 according to the quantitative polymerase chain reaction (qPCR) method of Loftie-Eaton et al. (2014). Total DNA was extracted from 50, 250, and 500 μL of early stationary phase cells. Chromosomal and plasmid DNA (0.04 ng/μL) was quantified in triplicate from each extraction by qPCR with primers (750 nM each) targeting either the *atpB* gene of *E. coli* (Yano et al. 2012), which exists on the chromosome in single copy, or the $bla_{CTX\text{-}M}$ gene on the plasmids (Ellem et al. 2010). Amplification efficiency ($E = 10^{(-1/m)}$) for all primer pairs was greater than 1.59 based on 10-fold serial dilutions of total DNA ($R^2 > 0.98$). Melting curve analysis of the qPCR products confirmed amplification specificity.

2.7. Estimating plasmid transfer frequency

Plasmid transfer frequency was estimated as the fraction of potential recipient cells that received the plasmid and expressed the CTX-resistant phenotype after 1 h of mating (T/R, where T represents the population density of transconjugants and R is the density of possible recipient cells). Mating experiments were set up as described above using *E. coli* HY842 containing plasmid as donor and *E. coli* JM109 as recipient; cell densities were assumed equal. Mating on filters proceeded for 1 h at 37°C after which biomass was removed, resuspended, and aliquots spread onto selective agar plates (37°C, 24 h). Transconjugants were selected with NA (5 μg mL^{-1}) and either C (25 μg mL^{-1}) for plasmids pTREC8 and pTREC9 or CTX (2 μg mL^{-1}) for plasmid pTREC4.

2.8. Plasmid genome sequencing, annotation, and analyses

Plasmid DNA was sequenced using a combination of Illumina and Oxford Nanopore sequencing technologies. Illumina sequencing was performed at the Institute for Interdisciplinary Data Sciences at the University of Idaho using a MiSeq sequencer and 250-bp Paired-End Sample Preparation Kits (Illumina, San Diego, USA). Prior to analysis, duplicate-read pairs were removed using a custom Python script. Sequencing adapters and low-quality bases were removed using the software package Seqyclean (https://github.com/ibest/seqyclean).

To close the plasmid sequences, plasmid DNA was subjected to sequencing using MinION technology and the Rapid Sequencing Kit (Oxford Nanopore Technologies, Oxford, UK). A hybrid *de novo* assembly was performed using both the Illumina and Oxford Nanopore sequence data via plasmidSPAdes (Antipov et al. 2016). ORFs from plasmids pTREC4, pTREC8, and pTREC9 were compared to those annotated in the genome of the highly

similar plasmid pTRE-2011 (acc. no. ON943059) (Botts et al. 2017); any ORFs sharing over 95% identity with those in the pTRE-2011 genome were given the same annotation. Regions of the genomes which were different from pTRE-2011 were annotated using the following method: open reading frames were identified using GeneMarkS (Besemer et al. 2001) and then the NCBI protein database was queried using blastp (Gish and States 1993). Any open reading frames shorter than 100 amino acids in length with no alignments to previously annotated sequences were not included in the final annotations.

Plasmid sequences were compared for genomic similarity to fully sequenced plasmids published in GenBank (Clark et al. 2016). Contiguous regions of plasmid backbone genes (*i.e.,* those involved in plasmid replication, conjugation, mating pair formation, partitioning, and stability) were used to identify other similar reference plasmids in the database using NCBI BLAST based on total BLAST alignment scores (Altschul et al. 1990).

Plasmid replicons/Inc groups were identified using PlasmidFinder 2.1 (Camacho et al. 2009; Carattoli et al. 2014) and FABC formulae were determined with pMLST 2.0 (Camacho et al. 2009; Carattoli et al. 2014; Clausen et al. 2018). Antibiotic resistance genes were identified with ResFinder 4.1 (Bortolaia et al. 2020; Camacho et al. 2009; Florensa et al. 2022). VirulenceFinder 2.0 (Joensen et al. 2014; Malberg Tetzschner et al. 2020) was used to identify virulence genes. All features determined by these automated programs were double-checked by direct alignments using BLAST and Geneious Prime v. 2021.1.1 (www.geneious.com).

Plasmid maps were generated from the annotated sequences with SnapGene Viewer v. 6.2 (www.snapgene.com). Sequence alignments, Mauve alignments (Darling et al. 2004), and phylogenetic trees were created using Geneious Prime. The phylogenetic tree was created using Tree Builder within Geneious Prime (v 2021.1.1) using the following settings: pairwise global alignment with free end gaps, neighbor-joining tree build method, Tamura-Nei genetic distance model, no outgroup.

2.9 Nucleotide accession numbers

The plasmid nucleotide sequences reported in this study have been deposited in the GenBank database (http://www.ncbi.nlm.nih.gov/genbank) under the accession numbers NZ_MN158990 - NZ_MN158992.

3. RESULTS AND DISCUSSION

In earlier studies, we demonstrated that ARGs and multi-drug resistance plasmids (Botts et al. 2017) are introduced into the Tijuana River Estuary wetlands with stormwater where they accumulate over time, creating both a sink as well as a possible source of resistance determinants (Borgogna et al. 2016; Botts et al. 2017; Cummings et al. 2011). This high genetic and taxonomic diversity combined with low antibiotic selective pressure in the wetlands may provide the perfect opportunity for mutation and recombination to take place among ARGs and resistance plasmids.

To better understand the genetic context of multidrug-resistance plasmids among bacteria in this environment, we selectively isolated CTX-resistant Gram-negative bacteria and screened them for plasmids. Three of 13 Gram-negative, lactose-fermenting colonies were chosen randomly for further study, each of which was identified as a strain of *Escherichia coli*: aerobic, Gram-negative, lactose-fermenting bacilli; oxidase-negative; indole-positive; sorbitol-positive (indicating non-O157). Identification as *E. coli* was confirmed with API20E test strips (not shown). Strains were named for their source (TRE, Tijuana River Estuary) and the antibiotic used to select them (C, cefotaxime), resulting in *E. coli* strains TREC4, TREC8, and TREC9.

3.1. Plasmid transmissibility, transfer frequency, and $bla_{CTX\text{-}M}$ gene copy number

To determine if the CTX-resistant phenotype was transmissible by conjugation, the native *E. coli* hosts were mated with *E. coli* HY842. All three wild-type strains successfully transferred CTX resistance to the lab strain. Plasmid extraction and gel electrophoresis revealed a single, high-molecular weight plasmid in each recipient, named pTREC4, pTREC8, and pTREC9 (Table 1). Next, plasmid-positive *E. coli* HY842 was mated with *E. coli* JM109, again successfully transferring all plasmids and expressing the CTX-resistant phenotype. Plasmids pTREC8 and pTREC9, but not pTREC4, also transferred CTX resistance to *P. putida* KT2440 via conjugation. Attempts to transfer plasmid pTREC4 from *E. coli* HY842 to *P. putida* KT2440 were unsuccessful. Curiously, neither pTREC8 nor pTREC9 could back-transfer from *P. putida* into *E. coli* by conjugation.

The proficiency with which a self-transmissible plasmid transfers to new host cells is an important factor in its epidemic spread. However, no consensus exists in the literature as to the optimal way to quantitatively describe this property (*e.g.*, Huisman et al. 2022; Kosterlitz et al. 2022). As an approximation, plasmid-positive *E. coli* HY842 was filter-mated with *E. coli* JM109 for 1 h and plated to select for transconjugants. One-hour transfer frequencies (T/R) ranged from 1.31×10^{-5} (pTREC9) to 1.01×10^{-3} (pTREC4) (Table 1). Such population density-based frequencies should be interpreted with caution, however, since many factors can influence this property, including the nature of the plasmid recipient and the conjugation media and other conditions used (Sheppard et al. 2020). Interestingly, pTREC9 displayed nucleotide differences in some of the transfer protein genes, relative to backbone genes in the other plasmids in this study and those most similar in the GenBank database, which may account for its lower transfer frequency. For example, the relaxase TraI in pTREC9 is truncated early due to multiple nonsense mutations in the *traI* gene. Additionally, the DNA transport/coupling protein gene *traD* and the F-pilin acetylase gene *traX* have nonsense mutations near the 3’ ends of their genes, but it is not known if these small truncations would decrease their functions.

An important strategy for plasmid persistence within bacterial populations, given the likely fitness cost to their host, is to carefully regulate the plasmid copy number. Generally speaking, smaller plasmids are maintained at higher copy numbers while larger plasmids exist at lower copy numbers (*e.g.*, Zhong et al. 2011). Here, the average number of copies of each plasmid per cell of *E. coli* HY842 was estimated to range from 2 to 3 copies per cell based on the copy numbers of the $bla_{CTX\text{-}M}$ gene (Table 1). This finding is in

agreement with our understanding that IncF plasmids commonly possess toxin-antitoxin and partitioning systems that aid in plasmid persistence in a population (Baxter and Funnell 2014; Wu et al. 2020).

3.2. Antibiotic resistance

Plasmids pTREC4, pTREC8, and pTREC9 were selected on the third-generation cephalosporin antibiotic CTX. They share in common resistance (or, in some cases, decreased susceptibility) to several different β-lactam drugs, including penicillins with and without β-lactamase inhibitors, and narrow- and broad-spectrum cephalosporins (Table 2). None provided clinical resistance to the carbapenems, although carriage of plasmid pTREC4 resulted in a slight but statistically significant reduction in susceptibility to MEM.

To quantify the minimum inhibitory concentrations (MICs) of select β-lactams for *E. coli* HY842 with each plasmid, ETESTs were carried out (Table 3). Of particular note were CTX and AM with MICs > 256 μg mL^{-1} for all three plasmids. This is remarkable considering that the wild-type strains were originally selected on only 2 μg mL^{-1} CTX, highlighting the often very high levels of resistance conferred by some ARGs, and the relatively low concentrations necessary to select for them.

The plasmids conferred protection from non-β-lactam drugs as well, including fluoroquinolones, aminoglycosides, macrolides, tetracyclines, anti-folates, and chloramphenicol (Table 2). None of the plasmids decreased susceptibility to the polymyxin antibiotic colistin.

These plasmids qualify for multidrug-resistance (MDR) designation based on the 2012 criteria agreed upon by the European Centre for Disease Prevention and Control and the US Centers for Disease Control and Prevention (Magiorakos et al. 2012). MDR designation is based on resistance to one or more antibiotics from three or more specific categories. Accordingly, the three environmental plasmids described here conferred clinical levels of resistance to at least one antibiotic from seven or more of the designated categories when tested in *E. coli* HY842 (Table 2). Their presence in the natural environment underscores the pressing need to better understand the complex microbial connections among the environment, the community, and the clinic.

3.3. Plasmid genome analyses

3.3.1. General genomic characteristics—Genomic characteristics of the three CTX-resistance plasmids are summarized in Table 4, and maps of features discussed in this report can be found in Figure 1. Plasmids pTREC4, pTREC8, and pTREC9 are relatively large: 168,775 bp, 118,200 bp, and 128,316 bp, respectively. pTREC8 and pTREC9 are highly similar to one another, sharing 89.9% nucleotide sequence similarity along their entire lengths, including both backbone and accessory regions (Figures 2 and 3). pTREC4, however, shows greater divergence, sharing only 61.8% and 62.8% with pTREC8 and pTREC9, respectively. The most similar plasmids in the GenBank database (Figure 2) were reported in *E. coli* strains from humans (pCA28, CP009232; FDAARGOS_1250, CP069572), fowl (p1 from strain 154AHL.1, CP059989), and the Tijuana River Estuary

(pTRE-2011, ON943059), ranging from southern USA/northern Mexico to southern Canada (Botts et al. 2017; Hu et al. 2014; Li et al. 2015; Moffat et al. 2020; Sichtig et al. 2019; Sokurenko et al. 2021).

Alignment of multiple large plasmid sequences is most readily accomplished with an algorithm such as Mauve (https://darlinglab.org/mauve/mauve.html) that identifies and aligns orthologous blocks rather than individual nucleotides. Mauve alignment of pTREC4, pTREC8, pTREC9, and pTRE-2011 revealed multiple rearrangements and other organizational differences (Figure 3). Most notable is a 13-kb segment found on the primary coding strand of pTRE-2011 that is inverted in the same location in pTREC9. A small residual fragment of this segment can also be found in the pTREC4 sequence, and, like pTREC9, it is in reverse orientation relative to pTRE-2011. The full-length segment in pTRE-2011 and pTREC9 encodes *floR*, *lysR*, *estX*, *aadA1*, *qacL*, *sul3*, and *hlyF* bound by IS*26* elements (see Figure 4D).

Evolutionary studies suggest that plasmids tend to take on the genomic signature (%GC, codon usage bias, dinucleotide and trinucleotide bias) of their bacterial hosts (Almpanis et al. 2018; Mann and Chen 2010; Sueoka 1962; Suzuki et al. 2010). Plasmids pTREC4, pTREC8, and pTREC9 comprise 51.7%, 50.4%, and 50.1% GC content, respectively (Table 4), very similar to the reported range for *Escherichia* chromosomes of 50.4–50.8 %GC (Mann and Chen 2010), suggesting that *E. coli* may be their primary host.

Large, low-copy plasmids are inherently at risk of loss through incomplete partitioning. Thus, successful plasmids often include one or more toxin-antitoxin systems that presumably aid in plasmid maintenance via post-segregational killing. Plasmids pTREC4, pTREC8, and pTREC9 share *pemIK*, *vagCD*, and *hok-sok* (Figure 1). Additionally, pTREC4 encodes a *ccdAB* operon. In light of their low copy number, these addiction systems likely contribute to their persistence, particularly in the absence of strong antibiotic selective pressures, such as in most natural environments.

Plasmids pTREC8 and pTREC9 also encode three colicin systems: colicin-M, colicin-B, and colicin-V, along with their cognate immunity proteins (Figure 1, Table 5). In both plasmids, however, the colicin-B and colicin-V genes are truncated and appear to be pseudogenes, leaving colicin-M as the only functional system. Adjacent to this colicin module is a site-specific integrase, suggesting a possible viral origin.

3.3.2. Backbone regions—Plasmids pTREC8 and pTREC9 have typical IncF backbones with largely uninterrupted modules for replication, stability, and transfer. The backbone regions from *traM* to *finO* are nearly identical, sharing 99.7% sequence similarity with one another. Two identical replicons are present in the form of *repA* (IncFII) and *repB* (IncFIB), with specific alleles F18:B1 (Villa et al. 2010) (Table 4). Like the prototypical IncF plasmid R1, and unlike the original F plasmid (Koraimann 2018), *finO* is intact in pTREC8 and pTREC9 and thus would be expected to repress conjugation.

Although plasmid pTREC4 is also in the IncF family, with a complete backbone containing replication, stability, and transfer modules, it is distinct from the other two in several

important ways. The pTREC4 backbone from *traM* to *finO* shares only 92.3% and 92.1% DNA sequence similarity with those of pTREC8 and pTREC9, respectively. In addition to *repA* and *repB*, pTREC4 also contains *repE* (IncFIA) and a second, but different, *repA*, resulting in the allelic formula F31:F36:A4:B1 (Table 4). The main difference between the backbones of these plasmids is an additional 9-kb segment, present in pTREC4 but absent in pTREC8 and pTREC9, between *parB* and *ssb* that contains a DNA methylase, an anti-restriction protein, several hypothetical proteins, and others with unknown functions.

Plasmids of the replicon sequence type F18:A-:B1:C4 (pTREC8 and pTREC9) have been identified in numerous studies of *E. coli* from food animals, pets, and humans across Asia (He et al. 2021; Liu et al. 2013; Nadimpalli et al. 2019; Puangseree et al. 2022; Xia et al. 2017; Yang et al. 2015), with relatively fewer reports from Europe (Villa et al. 2010), Australia (Villa et al. 2010), South America (Liakopolous et al. 2016), and the United States (McGann et al. 2016; Villa et al. 2010). As with pTREC8 and pTREC9, many of the F18:A-:B1:C4 plasmids reported elsewhere harbor the ESBL gene $bla_{CTX\text{-}M\text{-}55}$ (Liu et al. 2013; McGann et al. 2016; Nadimpalli et al. 2019; Xia et al. 2017), indicating that this backbone is a common and important vehicle for the spread of $bla_{CTX\text{-}M\text{-}55}$ in diverse environments around the world.

Plasmids pTREC8 and pTREC9 were highly similar to two other IncF conjugative plasmids in the GenBank database (Figure 2). Plasmid pTRE-2011 (Botts et al. 2017) is a 144-kb IncF plasmid (F18:A-:B1:C4) with a backbone that is nearly identical to those of pTREC8 and pTREC9 along with many of the same shared modules (Figures 3 and 4). Like pTREC8 and pTREC9, pTRE-2011 is a large self-transmissible plasmid that was isolated from sediments at the same location of the Tijuana River Estuary. Additionally, plasmid p1 from *E. coli* strain 154AHL.1 (Moffat et al. 2020), isolated from the feces of a presumably healthy Canadian turkey, is a 132-kb IncF plasmid (F18:A-:B1:C4) with nearly identical backbone to pTREC8, pTREC9, and pTRE-2011.

Although phenotypically similar in many ways to pTREC8 and pTREC9, pTREC4 consists of a different replicon combination: F31:A4:B1:C-, shared by its closest relative, pCA28 (Li et al. 2015), recovered from a community-acquired *E. coli* infection in the state of Iowa (USA). pTREC4 (F31:A4:B1:C-) is distinct from the previously discussed plasmids in that it encodes a unique FII replicon, an FIA replicon that was absent in pTREC8 and pTREC9, and no FIC replicon. This backbone, which typically carries $bla_{CTX\text{-}M\text{-}15}$, has been reported in Europe, Africa, and the United States in pets, food animals, wastewater, and humans (Dahmen et al. 2013; Dopouy et al. 2016; Hordijk et al. 2013; Li et al. 2015; Rafaï et al. 2015; Villa et al. 2010; Woodford et al. 2009). In humans, various sequence types of *E. coli* carrying F31:A4:B1:C-($bla_{CTX\text{-}M\text{-}15}$) have been responsible for both community-acquired and healthcare-associated infections (Li et al. 2015, Rafaï et al. 2015).

3.3.3. Antibiotic resistance genes—Although these plasmids share remarkable sequence similarity with one another, the only resistance feature identical across all three is a 4-kb element encoding the aminoglycoside acetylase gene *aac(3)-IIe* along with an AAA family ATPase and an interrupted IS*3* insertion sequence, flanked by inverted IS*26* insertion sequences (Figure 4A). Otherwise, these three similar replicons possess highly dissimilar

accessory regions. It is clear that, although these plasmids confer similar antibiotic resistance profiles to their bacterial hosts, this is achieved through entirely different genes, as *aac(3)-IIe* is the only ARG shared by all three plasmids.

In addition to *aac(3)-IIe*, pTREC4 contains four other modules flanked by either IS*1* or IS*26* elements in opposite orientation to one another. These include *aac(6')-Ib-cr5*, bla_{OXA-1}, and a *catB* pseudogene (IS*26*); *intI1*, *dfrA17*, *aadA5*, *qacE*Δ*1*, and *sul1*(IS*26*); *mph(A)-mrx-mphR(A)* (IS*26*); and *tetR(B)*, *tetB*, and *tetC* (IS*1*) (Figures 4A and 4B). Plasmid pTREC8 encodes the shared *aac(3)-IIe* (IS*26*) in addition to a second IS26-bound element containing *tet(A)* and *tetR(A)* along with three Tn*3* pseudogenes and two others of uncertain function (Figure 4C). Plasmid pTREC9 similarly encodes the shared *aac(3)-IIe* (IS*26*) element described above as well as two more modules: *floR*, *lysR*, *estX*, *aadA1*, *qacL*, and *sul3* (IS*26*) (Figure 4D); and *lnu(F)* and an *aadA22* pseudogene (IS*26*) (Figure 4E). Both of these accessory segments in pTREC9 include various interspersed pseudogenes and others of unknown function.

Virtually all of the Group 1 CTX-M genes described to date, which include $bla_{CTX-M-15}$ and $bla_{CTX-M-55}$, are associated with the facile gene capture and dissemination element IS*Ec9* (also known as IS*Ecp1*) (Rossolini et al. 2007) (Figure 5). The genetic context of $bla_{CTX-M-55}$ in pTREC9 is congruent with most others in the literature in that it is directly upstream of its cognate IS*Ec9* on the same strand. However, $bla_{CTX-M-55}$ in pTREC8 is separated from its IS*Ec9* element by another insertion sequence, IS*903B* from the IS*5* family of mobile elements, on the opposite strand. Additionally, pTREC4 harbors $bla_{CTX-M-15}$ associated with an IS*Ec9* that is interrupted by an IS*26* element at its 5' end. Thus, even in this relatively small sampling of bla_{CTX-M}-harboring plasmids from a single habitat, unique evolutionary histories of the bla_{CTX-M} gene are evident.

Most of the observed resistance phenotypes conferred by the wetland plasmids could be explained by the corresponding genotypes (recognized ARGs in the plasmid nucleotide sequences), with rare exceptions (Table 6). Plasmid pTREC8 encoded no known chloramphenicol or fluoroquinolone resistance genes, however, *E. coli* (pTREC8) was clinically resistant to chloramphenicol and showed significantly reduced susceptibility to the fluoroquinolones. Similarly, *E. coli* (pTREC9) was resistant to tetracycline and showed decreased susceptibility to doxycycline, yet no recognizable tetracycline resistance genes were detected in the pTREC9 genome. These findings suggest that pTREC8 and pTREC9 may possess some as-yet-undescribed mechanisms for defending their hosts from these antibiotics. For example, both plasmids encode numerous 'hypothetical proteins', the functions of which have not been determined and cannot be easily predicted by direct comparison with similar genes in the databases. It is conceivable that the product of one or more of these genes may contribute to antibiotic resistance. Additionally, some well understood ARGs may possess cross-class activities that have yet to be described (*e.g.*, an efflux pump with a substrate range that is in fact broader than reported). Identifying and filling in such knowledge gaps is crucial to a complete understanding of the role of plasmids in antibiotic resistance and virulence in pathogenic bacteria.

3.3.4. Virulence factors—The three IncF plasmids described here share an accessory region bounded by inverted IS*1* elements with frameshifted transposases, between which lie two iron acquisition systems: *sitABCD* and *iucABCD*/*iutA* (Figure 1, Table 5). In addition to these two common virulence modules, pTREC4 also contains a 31-kb region encoding two iron transporters and three ABC transporters along with various proteins that appear to be involved in biochemical pathways. It is unclear if this module contributes to the virulence of plasmid pTREC4 in any way. Plasmids pTREC8 and pTREC9, but not pTREC4, also encode an integrase core domain-containing protein situated immediately upstream of *hlyF*, *mig-14*, and two putative outer membrane proteases, each of which have been implicated in defense against host immunity during an infection (Brodsky et al. 2005; Hritonenko and Stathopoulos 2007; Murase et al. 2015) (Figure 1, Table 5).

4. CONCLUSION

A compelling argument for studying entire resistance plasmids rather than simply individual resistance genes is the opportunity to map genetic linkages (Qian et al. 2021) (Table 6). When patients are treated with a single antibiotic, plasmids may be selected that carry resistance factors to many drug classes in addition to the one used. For example, plasmid pTREC4 confers resistance to broad-spectrum penicillin, carboxypenicillin, and ureidopenicillin, both with and without β-lactamase inhibitors. Likewise, many cephalosporins are ineffective against *E. coli* (pTREC4) including first, second, third, and fourth generation drugs. In addition to these β-lactam antibiotic classes, pTREC4 confers resistance to sulfonamides, aminoglycosides, tetracyclines, and macrolides, leaving very few options for treatment of infection save for carbapenems and colistin, both of which pose risks of serious adverse effects.

An often-overlooked consequence of the use of antibiotics is the potential for increasing bacterial virulence. Selection with an antibiotic that is inactivated by a gene product encoded on a plasmid can also enhance the virulence of the population by co-selecting genetically linked virulence factors, such as the iron acquisition systems and host immunity resistance proteins seen here (Figure 1).

It has generally been reported that IncF plasmids are restricted to bacteria in the family *Enterobacteriaceae* (*e.g.*, Villa et al. 2010). The observation that pTREC8 and pTREC9 could transfer CTX resistance to *P. putida*, from the family *Pseudomonadaceae*, shows that while the IncF replication range may be limited, these plasmids can still contribute to resistance spread to a broader range of bacteria than commonly believed.

Here, we report the full genome sequences and resistance phenotypes of three novel IncF plasmids captured from the natural environment. These plasmid types, F18:A-:B1:C4 ($bla_{CTX\text{-}M\text{-}55}$) and F31:A4:B1:C1 ($bla_{CTX\text{-}M\text{-}15}$), have been found in both humans and animals from around the world, suggesting facile dissemination between them. In this report, we add urban wetlands to our understanding of their geographic distribution.

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Highlights

- Conjugative IncF-type resistance plasmids were found in polluted wetlands
- Nucleotide sequences revealed globally distributed backbones
- Multiple antibiotic resistance genes were linked to multiple virulence factors
- Whole-genome alignments showed rearrangements and inversions
- *bla*$_{CTX\text{-}M}$ genes were associated with IS*Ec9* in varying genetic contexts

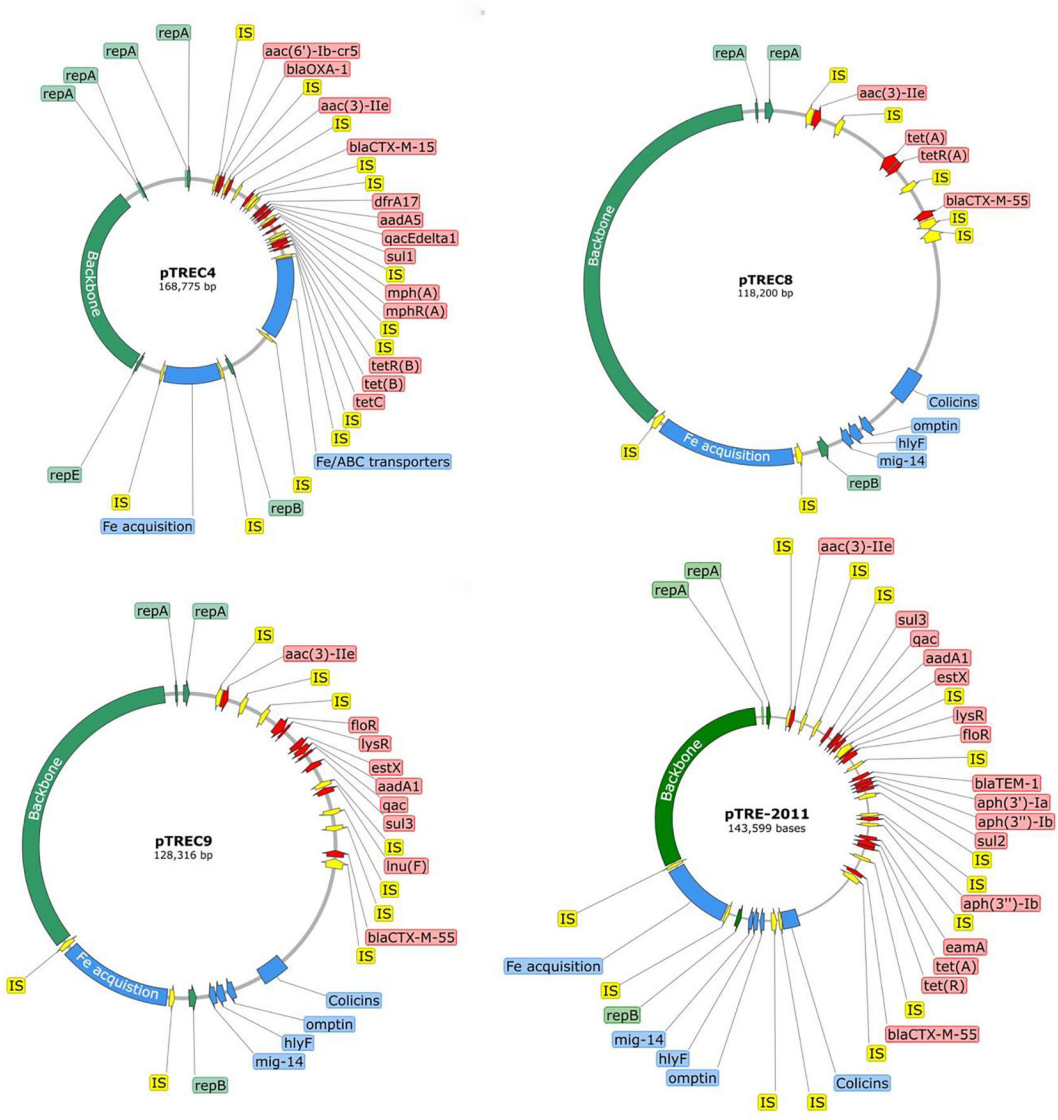

FIGURE 1.

Genetic maps of CTX-resistance plasmids captured from the Tijuana River Estuary. Plasmids pTREC4, pTREC8, and pTREC9 are described here, while the highly similar plasmid pTRE-2011 was described previously (Botts et al. 2017). Only those features discussed in this paper are shown: backbone (green), antibiotic resistance (red), mobile genetic elements (yellow), and virulence factors and bacteriocins (blue). Complete annotated sequences can be found in GenBank (see Table 4 for accession numbers). Note that image sizes are not proportional to plasmid sizes.

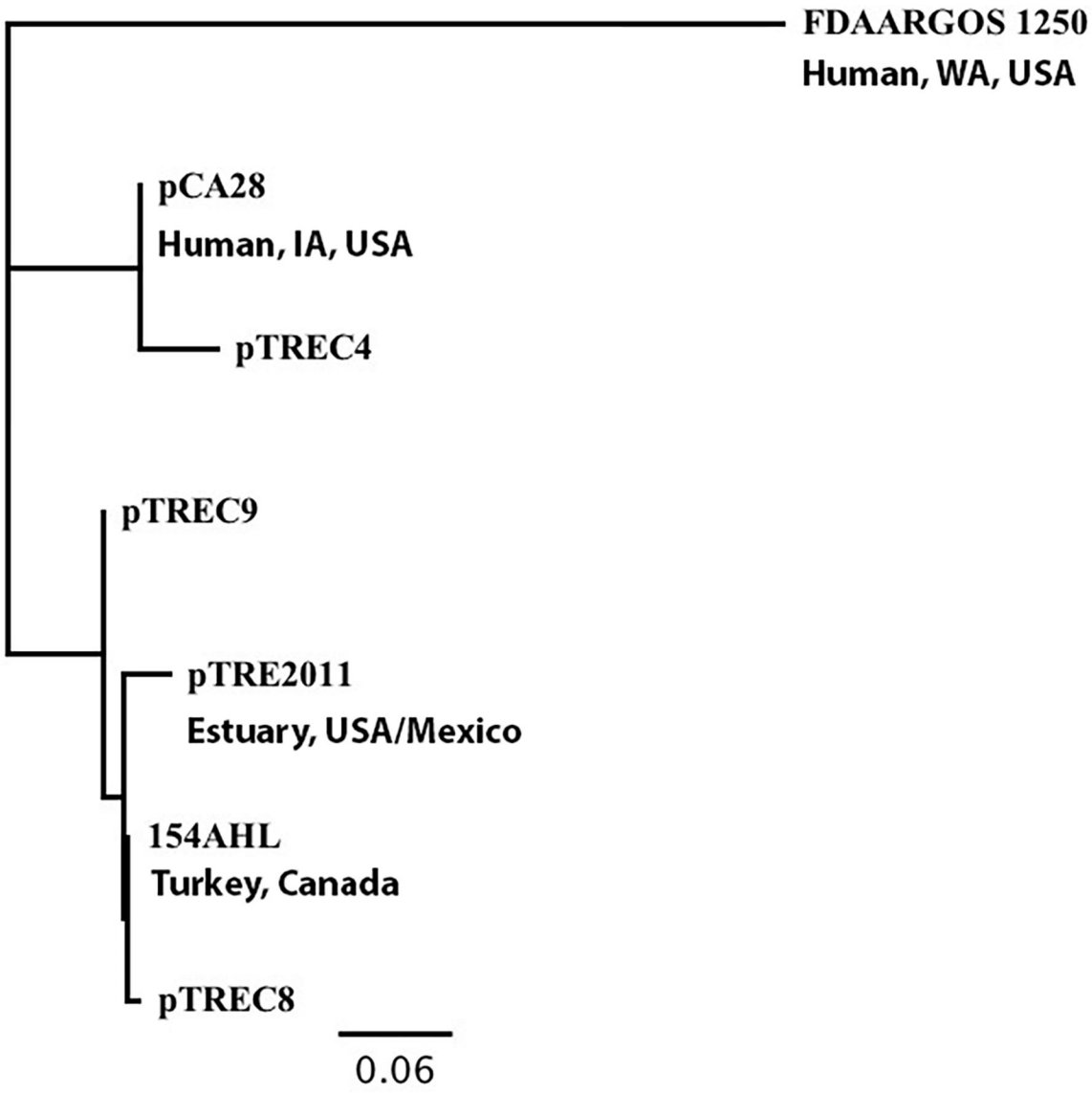

FIGURE 2.

Phylogenetic tree showing relationships among the CTX-resistance plasmids described here and the most closely aligned plasmids in the GenBank database. Full-length plasmid genomes were used. Tree Builder within Geneious Prime (v 2021.1.1) was used (neighbor-joining method, Tamura-Nei genetic distance model, no outgroup). Source is indicated under each plasmid. Bar represents 0.06 nucleotide substitutions per site.

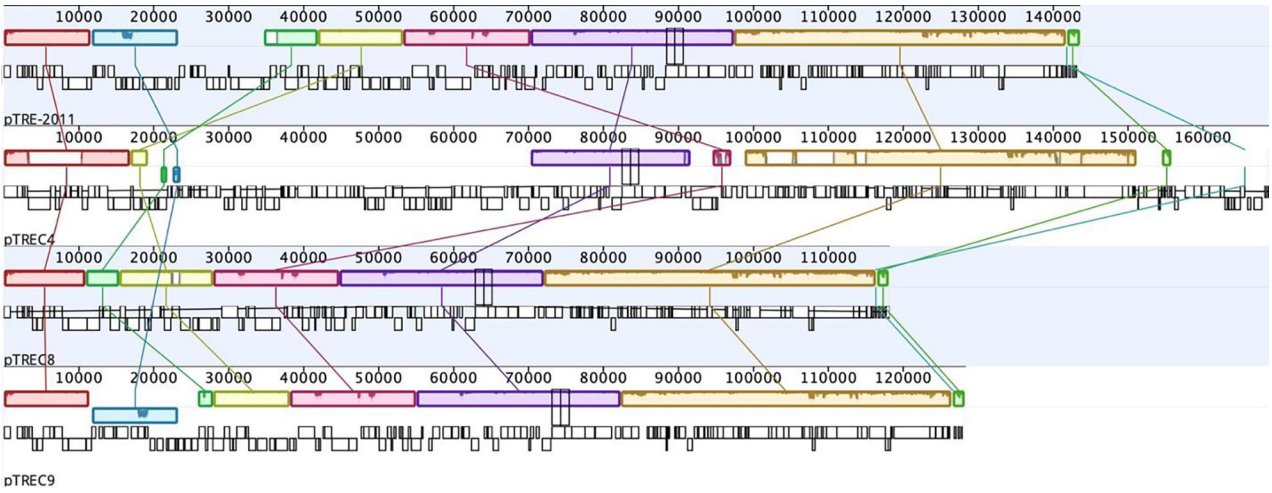

FIGURE 3.

Mauve alignments of the complete nucleotide sequences of CTX-resistance plasmids captured from the Tijuana River Estuary. Mauve alignment plugin in Geneious Prime (v 2021.1.1) was used (match seed weight = 100; all other settings set to default). From top to bottom: pTRE-2011, pTREC4, pTREC8, and pTREC9. Shared modules*: aac(3)-IIe* (orange), tetracycline resistance genes (green), $bla_{CTX\text{-}M}$ (yellow), colicins (red), Fe acquisition systems (purple), backbone (gold), and *floR/sul3* (blue).

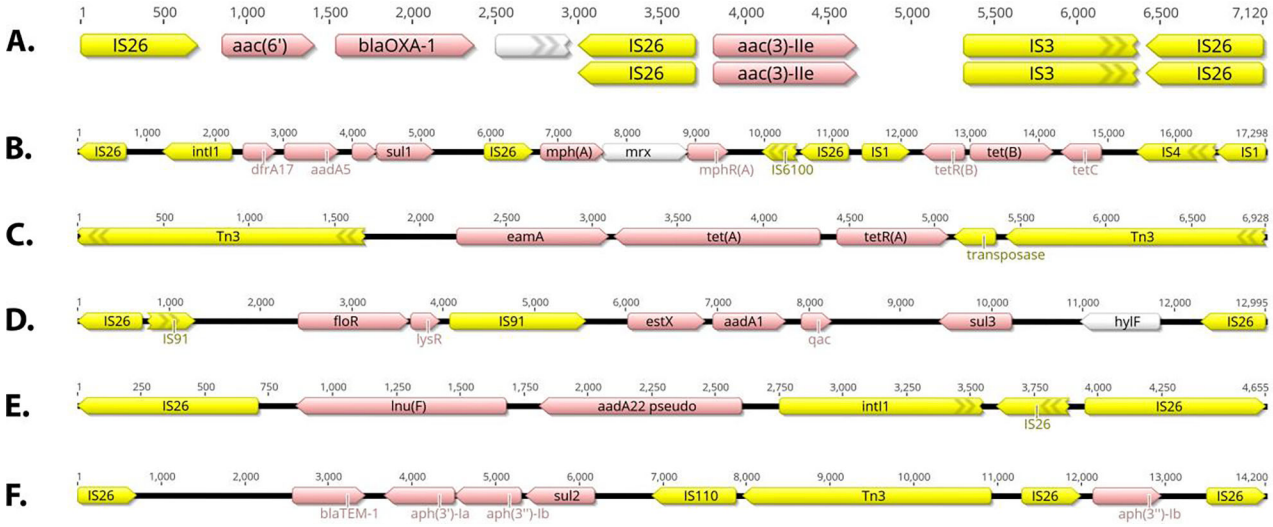

FIGURE 4.

Antibiotic resistance accessory regions of CTX-resistance plasmids captured from the Tijuana River Estuary. (A) *aac(3)-IIe* regions of pTREC4 (top map) relative to pTREC8, pTREC9, and pTRE-2011 (bottom map); (B) 17-kb region unique to pTREC4; (C) 7-kb region unique to pTREC8 and pTRE-2011; (D) 13-kb region unique to pTREC9 and pTRE-2011; (E) 4.5-kb region unique to pTREC9; (F) 14-kb region unique to pTRE-2011.

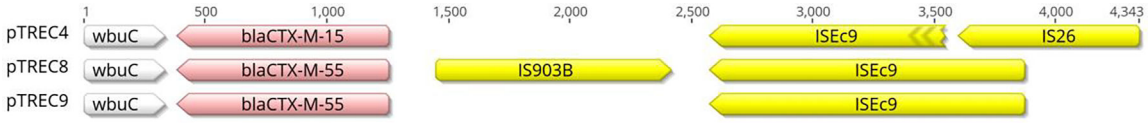

FIGURE 5.

Genetic organization of $bla_{CTX\text{-}M}$-harboring IS*Ec9* elements in CTX-resistance plasmids captured from the Tijuana River Estuary. pTRE-2011 is identical to pTREC9 in this region.

TABLE 1.

Plasmid transmissibility, 1-h transfer frequencies, and copy numbers of CTX-resistance genes captured

Plasmid	Transmissibility[a]	1-h transfer frequency[b]	Copy number[c]
pTREC4	*E. coli*	$1.01 \times 10^{-3} \pm 6.18 \times 10^{-4}$	2.42 ± 0.10
pTREC8	*E. coli, P. putida*	$1.28 \times 10^{-4} \pm 3.00 \times 10^{-5}$	3.32 ± 0.60
pTREC9	*E. coli, P. putida*	$1.31 \times 10^{-5} \pm 4.60 \times 10^{-6}$	2.20 ± 0.89

[a] Recipients that could successfully receive the plasmid by conjugation and express the CTX-resistance phenotype.

[b] 1-h transfer frequency is defined as the fraction of potential recipient cells that have taken up the plasmid by conjugation in the first hour of filter mating with *E. coli* HY842 as plasmid donor and *E. coli* JM109 as plasmid recipient (mean ± SD, n=3). Transfer frequencies were not determined with *P. putida* as recipient.

[c] Copy number of $bla_{CTX\text{-}M}$ per cell (mean ± SD, n=3).

TABLE 2.

Antibiotic susceptibility of *E. coli* HY842 carrying CTX-resistance plasmids captured from the Tijuana River Estuary[a]

Plasmid	Resistance[b]	Decreased susceptibility[c]
pTREC4	AM, PIP, TIC, SAM, TIM, CF, CXM, CTX, CAZ, ATM, SXT, K, TE, D, AZM	TZP, FEP, MEM, AN
pTREC8	AM, PIP, TIC, CF, CXM, CTX, CAZ	SAM, TIM, FEP, CIP, GAT, LOM, LVX, NA, ATM, C, K, TE, NOR, OFX, D
pTREC9	AM, PIP, TIC, CF, CXM, CTX, ATM, SXT, C, K, TE	SAM, TIM, CAZ, FEP, D

[a]Antibiotic abbreviations can be found in the Materials and Methods section.

[b]Clinical resistance is defined as a zone of inhibition smaller than the resistance breakpoints set for the *Enterobacteriaceae* by the CLSI.

[c]Decreased susceptibility is defined here as a statistically significant decrease of ≥ 3 mm in zone of inhibition relative to *E. coli* HY842 without plasmid (n=5, two-tailed t-test assuming unequal variance, $p < 0.01$).

TABLE 3.

Minimum inhibitory concentrations[a] (μg mL^{-1}) of β-lactam antibiotics for *E. coli* HY842 harboring CTX-resistance plasmids captured from the Tijuana River Estuary[b].

						β-lactam antibiotics
Plasmid	**CAZ**	**FOX**	**AM**	**IPM**	**ATM**	**CTX**
pTREC4	16 (R)	4 (S)	>256 (R)	0.19 (S)	192 (R)	>256 (R)
pTREC8	40 (R)	3 (S)	>256 (R)	0.25 (S)	32 (R)	>256 (R)
pTREC9	24 (R)	6 (S)	>256 (R)	0.25 (S)	>256 (R)	>256 (R)

[a]MICs were measured with ETESTS (bioMérieux) and susceptibilities are reported as R (resistant), I (intermediate), or S (susceptible) based on clinical breakpoints.

[b]Antibiotic abbreviations can be found in the Materials and Methods section.

TABLE 4.

Summary of the complete nucleotide sequences of CTX-resistance plasmids captured from the Tijuana River Estuary.

Plasmid	Acc. No.	Size (bp)	Inc Groups[a]	FABC Formula[b]	%GC
pTREC4	NZ_MN158990	168,775	FII, FIA, FIB	F31:A4:B1:C-	51.7
pTREC8	NZ_MN158991	118,200	FII, FIB, FIC	F18:A-:B1:C4	50.4
pTREC9	NZ_MN158992	128,316	FII, FIB, FIC	F18:A-:B1:C4	50.1

[a]Putative incompatibility (Inc) groups were identified using PlasmidFinder v 2.1.

[b]Replicon sequence types (RST/FABC formulae) were determined using pMLST 2.0.

TABLE 5.

Putative antibiotic resistance and virulence genes[a] encoded by CTX-resistance plasmids captured from the Tijuana River Estuary.

Plasmid	Resistance genes	Virulence genes
pTREC4	*aac(6')-Ib-cr5, bla*$_{OXA-1}$*, aac(3)-IIe, bla*$_{CTX-M-15}$*, dfrA17, aadA5, qacEΔ1, sul1, mph(A)-mrx-mphR(A), tetR(B), tetB, tetC*	*sitABCD, iucABCD* + *iutA,* Fe transporters (2), ABC transporters (3)
pTREC8	*aac(3)-IIe, tet(A), tetR(A), bla* $_{CTX-M-55}$	*sitABCD, iucABCD* + *iutA, hlyF, mig-14,* outer membrane proteases (2)
pTREC9	*aac(3)-IIe, floR, lysR, estX, aadA1, qacL, sul3, lnu(F), bla* $_{CTX-M-55}$	*sitABCD, iucABCD* + *iutA, hlyF, mig-14,* outer membrane proteases (2)

[a]Only in-frame resistance and virulence genes that appear to be of full length and not interrupted by mobile elements are included.

TABLE 6.

Proposed correlation between antibiotic resistance phenotype and genotype for CTX-resistance plasmids captured from the Tijuana River Estuary.

Plasmid	Phenotype[a]	Genotype
pTREC4	Penicillins (AM, PIP, TIC, SAM, TIM) Cephalosporins (CF, CXM, CTX, CAZ, FEP) Monobactams (ATM) Carbapenems (MEM) Sulfonamides (SXT) Aminoglycosides (K, AN) Tetracyclines (TE, D) Macrolides (AZM)	*bla_{OXA-1}, $bla_{CTX-M-15}$* *bla_{OXA-1}, $bla_{CTX-M-15}$* *bla_{OXA-1}, $bla_{CTX-M-15}$* *bla_{OXA-1}* *sul1, dfrA17* *aac(3)-IIe, aac(6')-Ib-cr, aadA5* *tetR(B), tetB, tetC* *mphA-mrx-mphR(A)*
pTREC8	Penicillins (AM, PIP, TIC, SAM, TIM) Cephalosporins (CF, CXM, CTX, CAZ, FEP) Monobactams (ATM) Aminoglycosides (K) Tetracyclines (TE, D) Chloramphenicol (C) Fluoroquinolones (CIP, GAT, LOM, LVX, NA, NOR, OFX)	*$bla_{CTX-M-55}$* *$bla_{CTX-M-55}$* *$bla_{CTX-M-55}$* *aac(3)-IIe* *tetA, tetR(A)* *?* *?*
pTREC9	Penicillins (AM, PIP, TIC, SAM, TIM) Cephalosporins (CF, CXM, CTX, CAZ, FEP) Monobactams (ATM) Sulfonamides (SXT) Chloramphenicol (C) Aminoglycosides (K) Tetracyclines (TE, D)	*$bla_{CTX-M-55}$* *$bla_{CTX-M-55}$* *$bla_{CTX-M-55}$* *sul3* *floR* *aac(3)-IIe, aadA1* *?*

[a]Clinically resistant based on CLSI breakpoints or statistically significant decrease in halo diameter.