






Intracellular Transposition and Capture of Mobile Genetic Elements following Intercellular Conjugation of Multidrug Resistance Conjugative Plasmids from Clinical *Enterobacteriaceae* Isolates

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ABSTRACT Mobile genetic elements (MGEs) are often associated with antimicrobial resistance genes (ARGs). They are responsible for intracellular transposition between different replicons and intercellular conjugation and are therefore important agents of ARG dissemination. Detection and characterization of functional MGEs, especially in clinical isolates, would increase our understanding of the underlying pathways of transposition and recombination and allow us to determine interventional strategies to interrupt this process. Entrapment vectors can be used to capture active MGEs, as they contain a positive selection genetic system conferring a selectable phenotype upon the insertion of an MGE within certain regions of that system. Previously, we developed the pBACpAK entrapment vector that results in a tetracycline-resistant phenotype when MGEs translocate and disrupt the *cl* repressor gene. We have previously used pBACpAK to capture MGEs in clinical *Escherichia coli* isolates following transformation with pBACpAK. In this study, we aimed to extend the utilization of pBACpAK to other bacterial taxa. We utilized an MGE-free recipient *E. coli* strain containing pBACpAK to capture MGEs on conjugative, ARG-containing plasmids following conjugation from clinical *Enterobacteriaceae* donors. Following the conjugative transfer of multiple conjugative plasmids and screening for tetracycline resistance in these transconjugants, we captured several insertion sequence (IS) elements and novel transposons (Tn7350 and Tn7351) and detected the *de novo* formation of novel putative composite transposons where the pBACpAK-located *tet(A)* is flanked by *ISKpn25* from the transferred conjugative plasmid, as well as the *ISKpn14*-mediated integration of an entire 119-kb, *bla*_{NDM-1}-containing conjugative plasmid from *Klebsiella pneumoniae*.

IMPORTANCE By analyzing transposition activity within our MGE-free recipient, we can gain insights into the interaction and evolution of multidrug resistance-conferring MGEs following conjugation, including the movement of multiple ISs, the formation of composite transposons, and cointegration and/or recombination between different replicons in the same cell. This combination of recipient and entrapment vector will allow fine-scale experimental studies of factors affecting intracellular transposition and MGE formation in and from ARG-encoding MGEs from multiple species of clinically relevant *Enterobacteriaceae*.

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Antimicrobial resistance (AMR) is a major global public health problem and is likely to get worse without the rapid development of new antibiotics and additional therapeutic options. Every use of antimicrobials provides a selective pressure for the evolution of AMR and associated mobile genetic elements (MGEs).

MGEs, such as conjugative plasmids and integrative and conjugative elements (ICEs), are responsible for the dissemination of antimicrobial resistance genes (ARGs) among bacteria, and they often contain multiple ARGs. ARGs are mobilized onto conjugative MGEs via the activity of smaller MGEs, including insertion sequences (ISs), that are capable of intracellular transposition. Transposons containing ARGs against last-resort antibiotics have been found on different plasmids in different bacterial species. For example, *bla*_{NDM-1}, conferring carbapenem resistance, and *mcr-1*, conferring colistin resistance, were found on IS*Aba125*- and IS*Ap11*-based composite transposons like Tn125 and Tn6330, respectively. Both composite transposons have been found on multiple plasmids in different bacterial species (1–5).

MGEs are usually identified through the phenotypic changes conferred by the accessory genes, including ARGs, or changes caused by insertions of MGEs that result in the activation/inactivation of other genes. For example, insertions of IS26, IS5, IS903, and IS1 into the *ompK36* porin gene and insertion of the IS*Ecp1-bla*_{OXA-181} transposon into the *mgrB* gene were shown to result in carbapenem and colistin resistance, respectively, in *Klebsiella pneumoniae* (6–8). Bioinformatic analysis can also identify MGEs by comparative genomics of whole-genome sequencing (WGS) data and by interrogating WGS data with available databases of MGEs (9, 10); however, this approach can rarely give information on the transposition activity of these MGEs. Contextualization of AMR genes on MGEs from short-read sequencing data is also notoriously difficult (11).

Another approach is to use entrapment vectors to capture MGEs based solely on their transposition activity. Entrapment vectors contain a genetic system that will confer a selective phenotype when MGEs transpose into a defined region of DNA (12–14). Previously, we developed a single-copy-number entrapment vector called pBACpAK and demonstrated that it can detect the insertion of MGEs in both laboratory and clinical *Escherichia coli* isolates (15, 16). pBACpAK contains a *cl-te(A)* gene system in which the λ repressor (encoded by *cl*) constitutively inhibits the expression of the *tet(A)* gene by binding to the P_{RM} promoter, blocking the expression of *tet(A)* (17). When an MGE inserts into the *cl* gene, the expression of the repressor is interrupted, leading to the expression of *tet(A)* and a tetracycline resistance phenotype (Fig. 1). Several IS elements and a novel translocatable unit (TU) carrying a functional trimethoprim resistance gene, *dfrA8*, were captured by using the pBACpAK entrapment vector previously (15).

In this study, we used pBACpAK to identify active MGEs from conjugative plasmids that had transferred via conjugation to a transposon-free, differentially resistant recipient *E. coli* strain from carbapenemase-producing clinical *Enterobacteriaceae* donor strains (Fig. 1). Multiple novel MGEs were detected from the screening of tetracycline-resistant transconjugants, giving insights into the interaction and evolution of MGEs carrying ARGs.

RESULTS

Characterization of donor and recipient strains. Based on bioinformatic analysis of the WGS data of 59 carbapenemase-producing *Enterobacteriaceae* clinical strains, 8 clinical isolates (7 *K. pneumoniae* and 1 *E. coli*) were selected as donors in this study, as summarized in Table 1, with the raw data shown in Table S2 in the supplemental material. Their resistance phenotypes against rifampicin (Rif) and fusidic acid (Fus) were subsequently determined to make sure that both antibiotics could be used for selection of transconjugants following a filter-mating experiment. All 8 clinical strains showed no growth on Luria-Bertani (LB) agar supplemented with rifampicin and fusidic acid (Table 1).

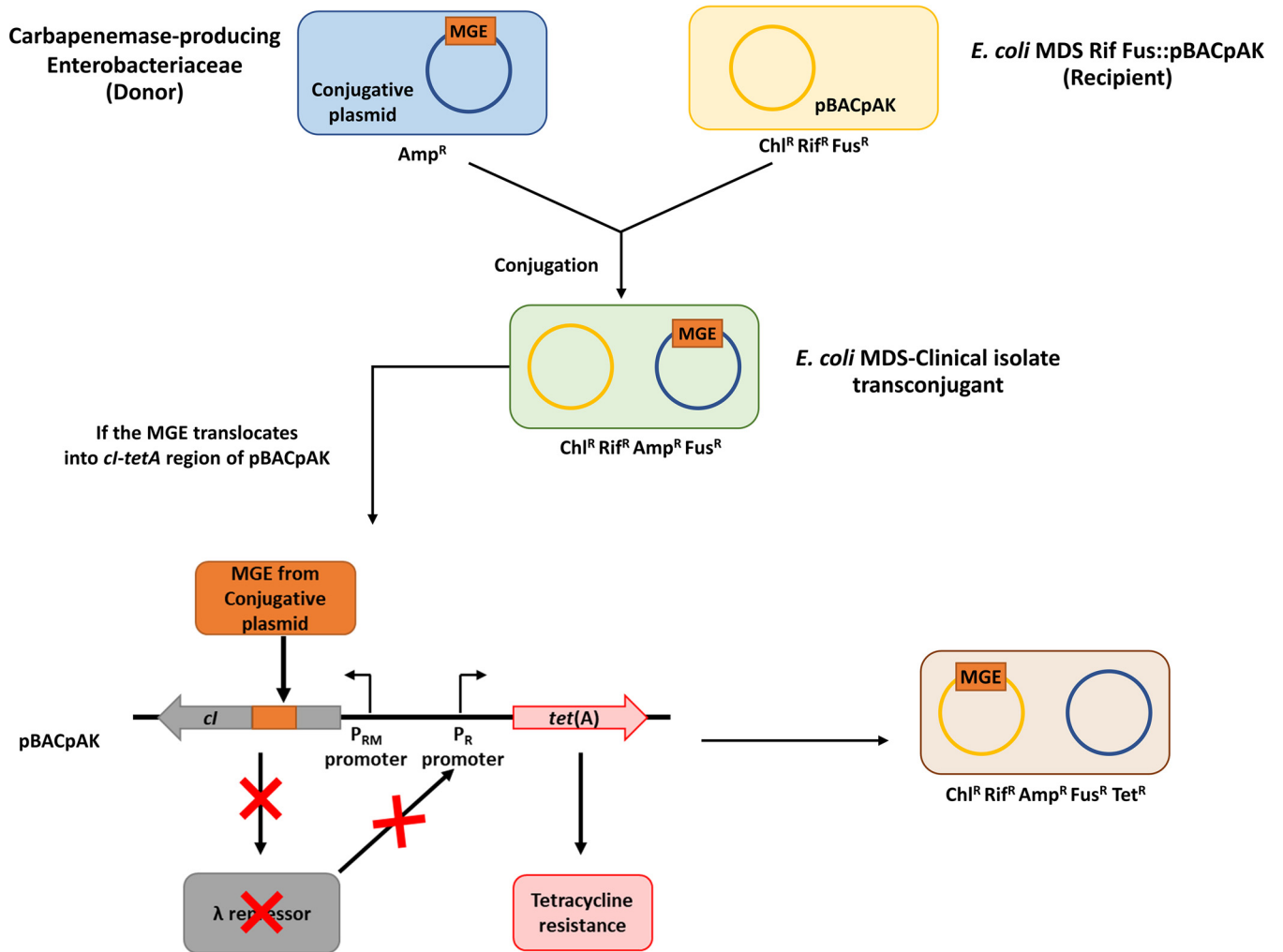


FIG 1 Capture of MGEs from conjugative plasmids by using pBACpAK. The conjugative plasmid from carbapenemase-producing *Enterobacteriaceae* clinical strains were transferred to an *E. coli* MDS Rif Fus::pBACpAK recipient strain through conjugation. Transconjugants were selected on LB agar supplemented with chloramphenicol, rifampicin, ampicillin, and fusidic acid. If the MGE, located on the conjugative plasmid, translocated into the *cl* gene on pBACpAK, it would disrupt the expression of the λ repressor, conferring tetracycline resistance due to the derepression of the P_R promoter. Clones with an insertion of MGEs therefore can be selected on tetracycline-containing agar. The gray and red arrowed boxes represent *cl* and *tet(A)* genes, respectively, which point in the direction of transcription. MGE, λ repressors, and tetracycline resistance protein are shown as orange, gray, and red rectangles. The blue, yellow, green, and brown rectangles represent donor, recipient, transconjugant, and tetracycline-resistant transconjugant cells, respectively. Chl^R, chloramphenicol resistance; Rif^R, rifampicin resistance; Amp^R, ampicillin resistance; Fus^R, fusidic acid resistance; Tet^R, tetracycline resistance.

pBACpAK was electroporated into *E. coli* strain MDS, which was subsequently sequentially evolved to rifampicin and then fusidic acid resistance. The strain was then screened on LB agar containing tetracycline to check for the rate of mutations in the *cl* gene. No tetracycline-resistant colonies were found on any plates from three replicates after 72 h of incubation.

Developing the *E. coli* MDS::pBACpAK strain into rifampicin and fusidic acid resistance was done to use the resistant phenotypes as selective markers for recipient strains, reducing the chance for false-positive transconjugants due to spontaneous mutations in the donor strains. *E. coli* MDS::pBACpAK with rifampicin and fusidic acid resistance was selected and denoted *E. coli* MDS Rif Fus::pBACpAK. *E. coli* MDS Rif Fus::pBACpAK was shown to have point mutations (underlined) in the *rpoB* (D516G [a mutation of D to G at position 516]; GAC→GGC) and *fusA* (L466F; CTC→TTC) genes, which are known to confer resistance to rifampicin and fusidic acid, respectively (18, 19).

Transfer of conjugative plasmids from clinical isolates to *E. coli* MDS Rif Fus::pBACpAK. Filter mating between clinical isolates and *E. coli* MDS Rif Fus::pBACpAK was carried out, and transconjugants were selected on LB agar supplemented with

TABLE 1 The details of carbapenemase-producing *Enterobacteriaceae* clinical strains

Species	Isolate	No. of:		Resistance determinant(s) ^{b,c}		Resistance phenotype ^c			
		Plasmids ^a	MGEs associated with plasmid-derived contigs ^b	β -Lactamase(s) associated with plasmid-derived contigs ^b	Chl	Tet	Chl	Tet	Rif and Fus
<i>K. pneumoniae</i>	K57-33	6	10	<i>bla</i> _{OXA-9f} , <i>bla</i> _{TEM-1A'} , <i>bla</i> _{KPC-2}	No	No	S	S	S
<i>K. pneumoniae</i>	K68-18	6	6	<i>bla</i> _{VIM-27}	No	No	R	S	S
<i>K. pneumoniae</i>	K46-62	2	6	<i>bla</i> _{SHV-12r} , <i>bla</i> _{TEM-1B'} , <i>bla</i> _{VIM-1}	No	No	S	S	S
<i>K. pneumoniae</i>	50825040	4	11	<i>bla</i> _{CTX-M-15r} , <i>bla</i> _{OXA-9f} , <i>bla</i> _{TEM-1B'} , <i>bla</i> _{NDM-1}	No	No	S	S	S
<i>K. pneumoniae</i>	50877064	1	4	<i>bla</i> _{CMY-6r} , <i>bla</i> _{NDM-1}	No	No	S	S	S
<i>K. pneumoniae</i>	50675619	5	8	<i>bla</i> _{NDM-7r} , <i>bla</i> _{OXA-1r} , <i>bla</i> _{CTX-M-15r} , <i>bla</i> _{TEM-1B}	<i>catB3</i> (P)	No	S	S	S
<i>K. pneumoniae</i>	50627996	3	6	<i>bla</i> _{CMY-6r} , <i>bla</i> _{NDM-1r} , <i>bla</i> _{CTX-M-15}	<i>catA1</i> (P), <i>catB3</i> (C)	No	R	R	S
<i>E. coli</i>	50676002	4	5	<i>bla</i> _{CMY-6r} , <i>bla</i> _{OXA-1r} , <i>bla</i> _{NDM-1}	<i>catB3</i> (P)	<i>tet(A)</i> (C)	S	R	S

^aThe number of plasmids was predicted by using PlasmidFinder (49).

^bMGEs and ARGs were analyzed from WGS data by using Mobile Element Finder and ResFinder, respectively (10, 47). mPlasmid was used to predict that the contigs containing each MGE and ARG were likely to be either chromosome-derived (C) or plasmid-derived (P) DNA (48).

^cChl, chloramphenicol; Tet, tetracycline; Rif, rifampicin; Fus, fusidic acid; S, susceptible; R, resistance.

chloramphenicol, rifampicin, ampicillin, and fusidic acid (LB CRAF agar). Several transconjugant colonies were found from all mating pairs. The donor-only group showed no growth on LB agar supplemented with chloramphenicol, rifampicin, and fusidic acid, while the recipient-only group showed no growth on LB ampicillin plates. Both control groups also showed no growth on any LB CRAF agar plates. The *cl-tet(A)* fragments were successfully amplified from all mating pairs except the *K. pneumoniae* strain K68-18 and *E. coli* MDS Rif Fus::pBACpAK pair, which verified that transconjugants from 7 donors contained conjugative plasmids (conferring an ampicillin resistance phenotype) and the pBACpAK entrapment vector.

Screening for tetracycline resistance transconjugants. All 7 transconjugants from 7 donors were grown on agar plates containing LB CRAF agar plus tetracycline (LB CRAFT agar) to select for colonies with a tetracycline resistance phenotype. Characterizing each tetracycline-resistant clone identified 11 clones with insertion of MGEs on pBACpAK (Table 2). Four of them (*E. coli* MDS-K46-62-TC-Tet-11, *E. coli* MDS-K46-62-TC-Tet-21, *E. coli* MDS-50675619-TC-Tet-4, and *E. coli* MDS-50825040-TC-Tet-2-1) were characterized by sequencing their *cl-tet(A)* amplicons, while the rest failed to be amplified by PCR, so they were characterized by WGS. The results showed that pBACpAK captured 4 different IS elements (*IS26*, *ISSbo1*, *ISKpn14*, and *ISKpn25*) and 2 novel transposons (designated Tn7350 and Tn7351) (Fig. 2). We also detected a recombinant pBACpAK::p50825040 plasmid molecule. Plasmid p50825040 is a previously unnamed plasmid (20) that we transferred from the *K. pneumoniae* 50825040 donor.

Most clones showed an insertion in the *cl* repressor genes; however, *E. coli* MDS-50825040-TC-Tet-2-3 and *E. coli* MDS-50627996-TC-Tet-2 showed an *ISKpn25* insertion between *tet(A)* and *oriV* and an *IS26* insertion in the *sopA* gene on pBACpAK, respectively. The tetracycline resistance phenotype in both clones was a result of a deletion in the *cl* repressor gene (6-bp and 786-bp deletions). *E. coli* MDS-50825040-TC-Tet-3-7 and *E. coli* MDS-50825040-TC-Tet-4-38 each had an insertion of *ISKpn25*, one in the *cl* repressor gene and the other one between *tet(A)* and *oriV*. For clones that were analyzed by WGS, translocation of MGEs into chromosomal DNA was determined by using breseq, which showed no additional insertion within the chromosome of the recipient in any tetracycline-resistant transconjugants.

Tn7350 and Tn7351 were identified from *E. coli* MDS-K46-62-TC-Tet-11 and *E. coli* MDS-K46-62-TC-Tet-21, respectively (Fig. 3). They were similar transposons with 99% identity to a part of plasmid pK45-67VIM found in *K. pneumoniae* (accession number HF955507) (Fig. S1A). Tn7351 was 1,016 bp shorter than Tn7350, missing a recombinase (resolvase) gene. Both transposons contained an *ISSbo1* insertion sequence and genes

TABLE 2 The details of MGEs captured by the pBACpAK entrapment vector

Strain (accession no.)	MGE/mutation	Location on pBACpAK (position)	Size (bp)	% similarity (ISFinder/NCBI)			Direct repeat
				BLASTN	BLASTX	Accession no.	
<i>E. coli</i> MDS-K46-62-TC strains Tet-11	Tn7350	<i>cl</i> repressor (2874)	5,812	99.12		HF955507	
	Recombinase family protein		570		99	WP_195767670	
	Hypothetical protein		849		91	SAP93263	
	RepB family plasmid replication initiator protein		720		99	WP_087871684	
	Transcriptional regulator <i>ardK</i>		342		100	ACI63129	
	Zinc metalloproteinase Mpr		465		99	CDF32051	
	ISS <i>bo1</i>		1,709	96		CP001062	
Tet-21	Tn7351	<i>cl</i> repressor (2963)	4,796	99.25		HF955507	GAAC
	Hypothetical protein		849		95	SXT51837	
	RepB family plasmid replication initiator protein		720		99	WP_087871684	
	Transcriptional regulator <i>ardK</i>		342		100	ACI63129	
	Zinc metalloproteinase Mpr		465		99	CDF32051	
	ISS <i>bo1</i>		1,709		94	CP001062	
	ISS <i>bo1</i>		1,709	96		CP001062	GAAC
<i>E. coli</i> MDS-50675619-TC-Tet-4		<i>cl</i> repressor (2976)					
<i>E. coli</i> MDS-50825040-TC strains Tet-2-1 Tet-2-3 (SAMN21542910)	ISKpn14	<i>cl</i> repressor (3127)	768	100		CP000649	CGGCGTTA
	ISKpn25	Between <i>tet(A)</i> and <i>oriV</i> (4976)	8,154	100		NC_009650	TATTTATC
	Deletion	<i>cl</i> repressor (3174-3179)	6				
	<i>K. pneumoniae</i> bla _{NDM-1} plasmid p2	<i>cl</i> repressor (3126)	118,959	99.99		CP009115	TAACGCCG
	ISKpn25_1	<i>cl</i> repressor (3224)	8,154	100		NC_009650	CATTTTTC
	ISKpn25_2	Between <i>tet(A)</i> and <i>oriV</i> (4980)	8,154	100		NC_009650	TTTTTATT
	ISKpn25	<i>cl</i> repressor (3294)	8,154	100		NC_009650	CTTTTTTG
	Tet-4-13 (SAMN21542913)	<i>cl</i> repressor (3230)	8,154	100		NC_009650	AAAAAAG
	Tet-4-38 (SAMN21542914)	<i>cl</i> repressor (3230)	8,154	100		NC_009650	AATCTTCT
	ISKpn25_1	Between <i>tet(A)</i> and <i>oriV</i> (4941)	8,154	100		NC_009650	AAAGAAAA
	ISKpn25_2	<i>cl</i> repressor (3227)	8,154	100		NC_009650	
	ISKpn25	<i>sopA</i> (8066)	820	100		X00011	CAGATCTT
	<i>E. coli</i> MDS-50627996-TC-Tet-2 (SAMN21542916)						
	Deletion	<i>cl</i> repressor (2305-3090)	786				

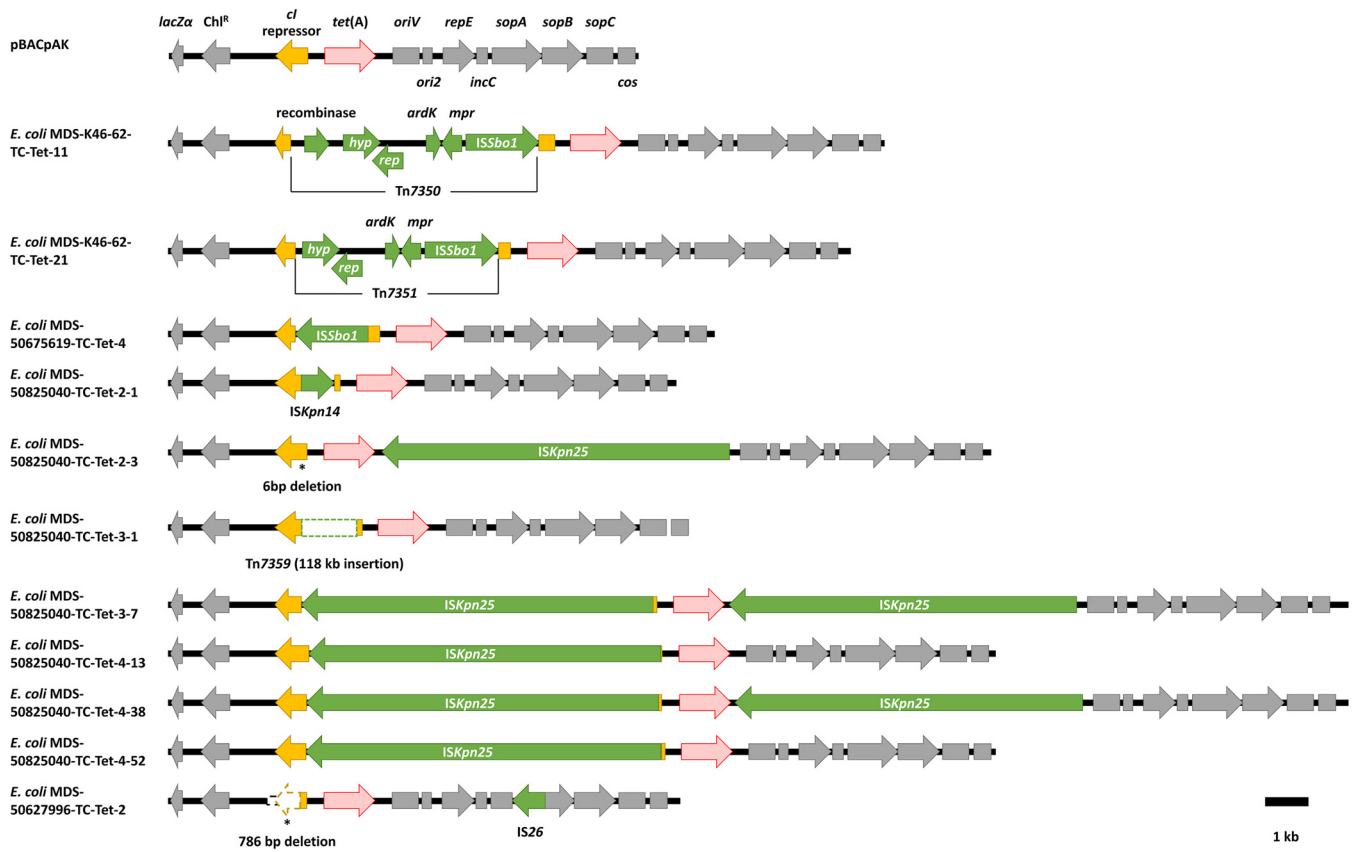


FIG 2 The structures of MGEs captured by pBACpAK in tetracycline-resistant transconjugants. The red, yellow, green, and grey arrowed boxes represent *tet* (A), *cl*, MGEs, and other genes, respectively. The green dashed box represents an insertion of Tn7359, shown in Fig. 4.

encoding a replication initiator protein, an ArdK transcriptional regulator, and an Mpr zinc metalloproteinase. They had different insertion sites on the pBACpAK vector (Fig. 3 and Fig. S2), and only Tn7351 contained a 4-bp direct repeat (GAAC) (Fig. 3). The recombinase gene found only in Tn7350 was similar to the resolvase genes from Tn552, Tn917, and Tn2501 (accession numbers P18358, P06693, and P05823), with percent identities of 42.33%, 35.64%, and 29.10%, respectively. The region of Tn7350 that was not present in Tn7351 was also flanked by the direct repeat GAAC (Fig. 3).

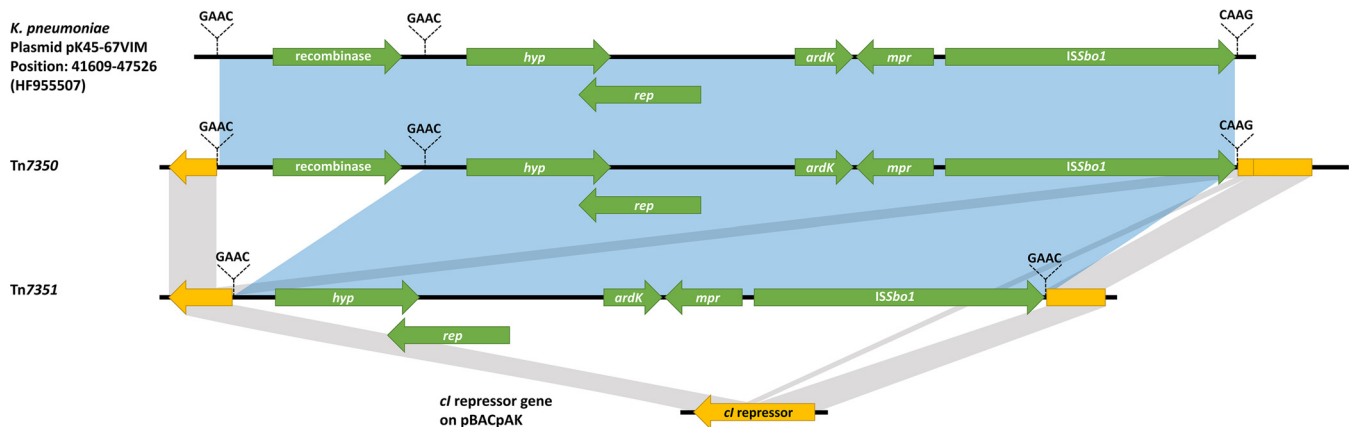


FIG 3 Comparison of Tn7350 and Tn7351 inserted in the *cl* repressor gene on the pBACpAK entrapment vector. Tn7350 and Tn7351 were compared to the *cl* repressor gene on pBACpAK and plasmid pK45-67VIM from *K. pneumoniae* (accession number HF955507). The yellow and green arrowed boxes represent the *cl* repressor gene and genes found on Tn7350 and Tn7351, respectively. The identical DNA regions of the *cl* repressor gene and transposons are shown in gray and blue, respectively. The GAAC direct repeats on Tn7350 and inverted repeats on Tn7351 are shown and indicated with dashed lines.

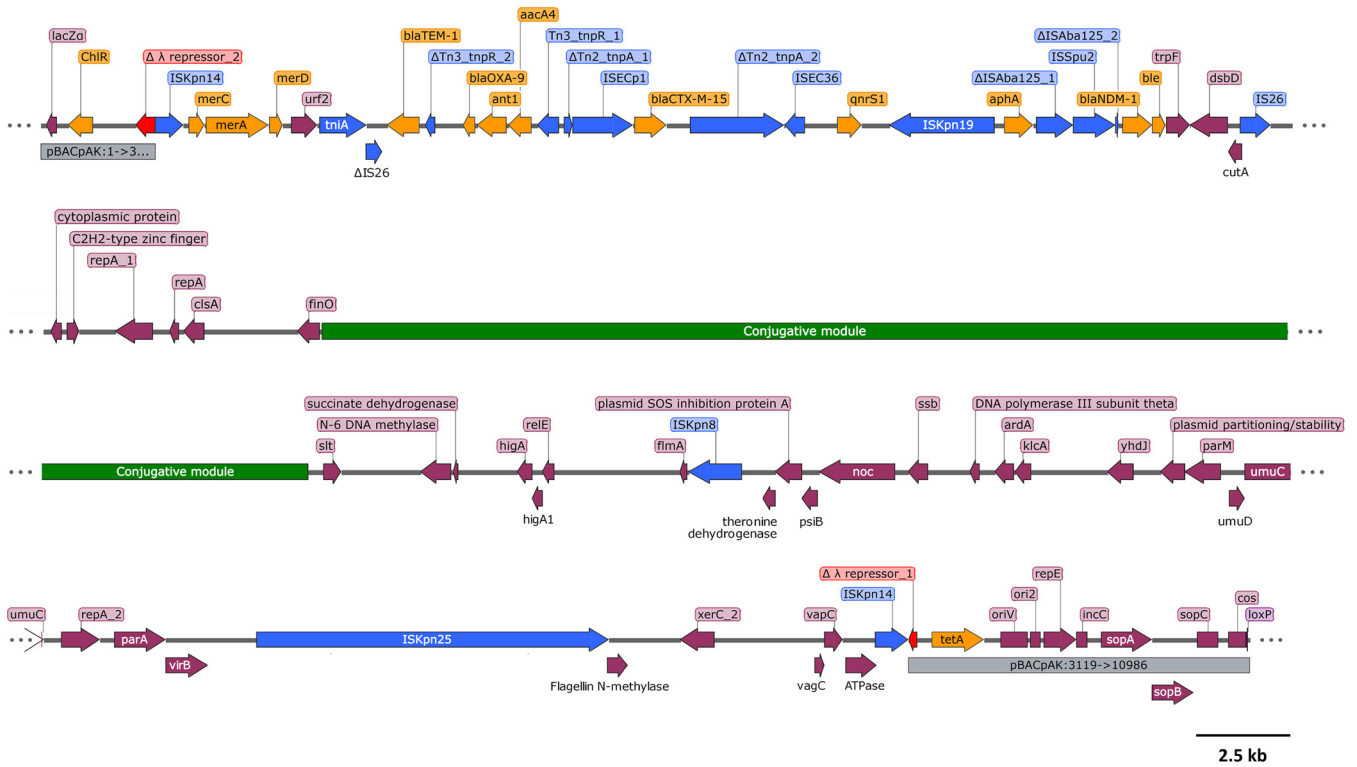


FIG 4 The structure of Tn7359 captured by the pBACpAK entrapment vector from *E. coli* MDS-50825040-TC-Tet-3-1. The blue, yellow, red, green, and purple arrowed boxes represent MGEs, ARGs, the *cl* repressor gene, conjugative genes, and other genes, respectively. The gray boxes represent pBACpAK. Hypothetical genes were omitted from the figure. The figure was constructed using SnapGene software (Insightful Science, USA).

The insertion in *E. coli* MDS-50825040-TC-Tet-3-1 consisted of the p50825040 plasmid originally from the *K. pneumoniae* 50825040 donor. As the inserted plasmid sequence was flanked by ISKpn14 elements in pBACpAK, it fit the definition of a composite transposon (21) and was named Tn7359 (Fig. 4). It was also highly similar to the bla_{NDM-1}-containing plasmid p2 found in *K. pneumoniae* (accession number CP009115) (Fig. S1B) (22). Tn7359 contained a Tn21-like structure (*merCAD* mercury resistance genes, *urf2*, and *tniA* genes) and multiple ARGs within an IS26-based pseudo-compound transposon (PCT)-like structure (23), as one of the IS26 elements was disrupted by the *tniA* gene, a conjugative module, and a plasmid stability/replication module (Fig. 4). The IS26 PCT-like structure contained multiple β-lactamase genes (*bla_{TEM-1}*, *bla_{OXA-9}*, *bla_{CTX-M-15}*, and *bla_{NDM-1}*), aminoglycoside resistance genes (*ant1*, *aacA4*, and *aphA*), the *ble* bleomycin resistance gene, and the *qnrS1* quinolone resistance gene. It also carried several IS elements, such as *ISEcp1*, *ISEc36*, *ISSpu2*, *ISKpn19*, *ISKpn8*, and *ISKpn25*.

DISCUSSION

Entrapment vectors have been used to capture MGEs in multiple bacterial species, both Gram positive and Gram negative, such as *Paracoccus pantotrophus*, *Rhodococcus fascians*, *Agrobacterium tumefaciens*, *Corynebacterium glutamicum*, and *E. coli* (15, 24–26). As this approach relies on the transposition activity of MGEs, it has the potential to identify new MGEs that have not been previously delineated (24, 27, 28). In our study, we have identified 2 novel transposons (Tn7350 and Tn7351) in transconjugants from the conjugations between carbapenemase-producing clinical *Enterobacteriaceae* isolates and *E. coli* MDS Rif Fus::pBACpAK. Both of these transposons, plus the region absent in Tn7351 compared to Tn7350, are flanked by GAAC inverted or direct repeats. GAAC represents the conserved target site of IS91 insertion sequences (29, 30). *ISSbo1*, which is present on both Tn7350 and Tn7351, is a member of the IS91 family of

insertion sequences (31) and is therefore likely to be responsible for the movement of these novel transposons; however, experimental verification is still needed to confirm this.

Prior to the filter-mating experiment, we characterized each carbapenemase-producing *Enterobacteriaceae* strain by using the bioinformatic tools Mobile Element Finder, ResFinder, and PlasmidFinder to analyze their WGS data to use as criteria for a selection of donor strains. Our results showed that we detected novel MGEs with pBACpAK that were not reported by these tools, as they are not present in the databases, but it could also capture other known MGEs that were missed by these tools, such as *ISSbo1* in *K. pneumoniae* 50675619, *ISKpn25* in *K. pneumoniae* 50825040, and *IS26* in *K. pneumoniae* 50627996 (Table S2).

Previously, the pBACpAK entrapment vector was developed and used in laboratory and clinical *E. coli* isolates, as it was designed based on the pCC1BAC vector containing the *E. coli* F factor single-copy origin of replication so that entrapment of larger DNA fragments would be more stable than if we had used a high-copy-number plasmid. In this study, we proposed another approach that could extend the uses of pBACpAK to the detection of MGEs from other bacterial species through a filter-mating experiment between clinical *Enterobacteriaceae* isolates as donors and the differentially marked *E. coli* MDS Rif Fus::pBACpAK as a recipient. This allows the capture of MGEs located on conjugative plasmids/transposons from any bacterial species that can transfer MGEs via conjugation (or transformation) to our MGE-free *E. coli* recipient strain. Conjugative elements, especially from clinical isolates, tend to carry not only multiple ARGs but also smaller MGEs; it has been shown, for example, that bacterial plasmids tend to contain a significantly higher number of IS elements than their chromosomal DNA (32).

This approach also extends the uses of pBACpAK in terms of resistance phenotype, since pBACpAK uses the chloramphenicol resistance gene as a selective marker for the vector and a tetracycline resistance phenotype to screen for clones with MGE insertion. Therefore, it cannot be used directly with *E. coli* strains with either a chloramphenicol or tetracycline resistance phenotype. As only resistance genes associated with conjugative elements will be transferred to the recipient in filter mating, it will reduce the background resistance phenotypes from the clinical isolates. This was shown in our study where *K. pneumoniae* 50627996 and *E. coli* 50676002 had the tetracycline resistance phenotype, but the transconjugants from both clinical isolates were susceptible to tetracycline, allowing us to screen for MGEs on other conjugative elements from both strains.

The conjugation and subsequent detection of MGE movement in transconjugants demonstrate both how MGEs like IS elements and Tns can translocate from one bacterial cell to another with the help of conjugative elements and the consequences of rapid dissemination to other replicons in the recipient cell. The translocation of IS elements can have direct consequences for resistance to their host. *ISKpn26* has recently been shown to insert into *acrR*, leading to inactivation of the AcrAB-TolC multidrug efflux pump and resistance to tigecycline in carbapenemase (KPC-2)-producing ST11 *K. pneumoniae* isolates from Chinese hospitals (33). *ISKpn14* and *ISKpn25* have previously been shown in several studies to be associated with colistin resistance through an insertion that disrupts the expression of the *mgrB* regulator gene, which results in overexpression of PhoPQ, activating the *pmrHFIJKLM* operon and modification of lipopolysaccharide, a drug target of colistin (34–38). While the translocation of ISs in our entrapment vector is also detected by interrupting a gene (*cl*) leading to a tetracycline resistance phenotype, the translocation of MGEs following conjugation can also result in the formation of new composite transposons and variations of known MGEs containing antibiotic resistance accessory genes. In our study, we observed the formation of two independently derived putative novel composite transposons containing the pBACpAK-located *tet(A)* tetracycline resistance gene flanked by copies of *ISKpn25*. The *tet(A)* in these clones may have the potential to be disseminated as a composite transposon. It could also move through an intermediate circular structure containing one copy of *ISKpn25* and the *tet(A)* gene, such as translocatable units (mainly reported in

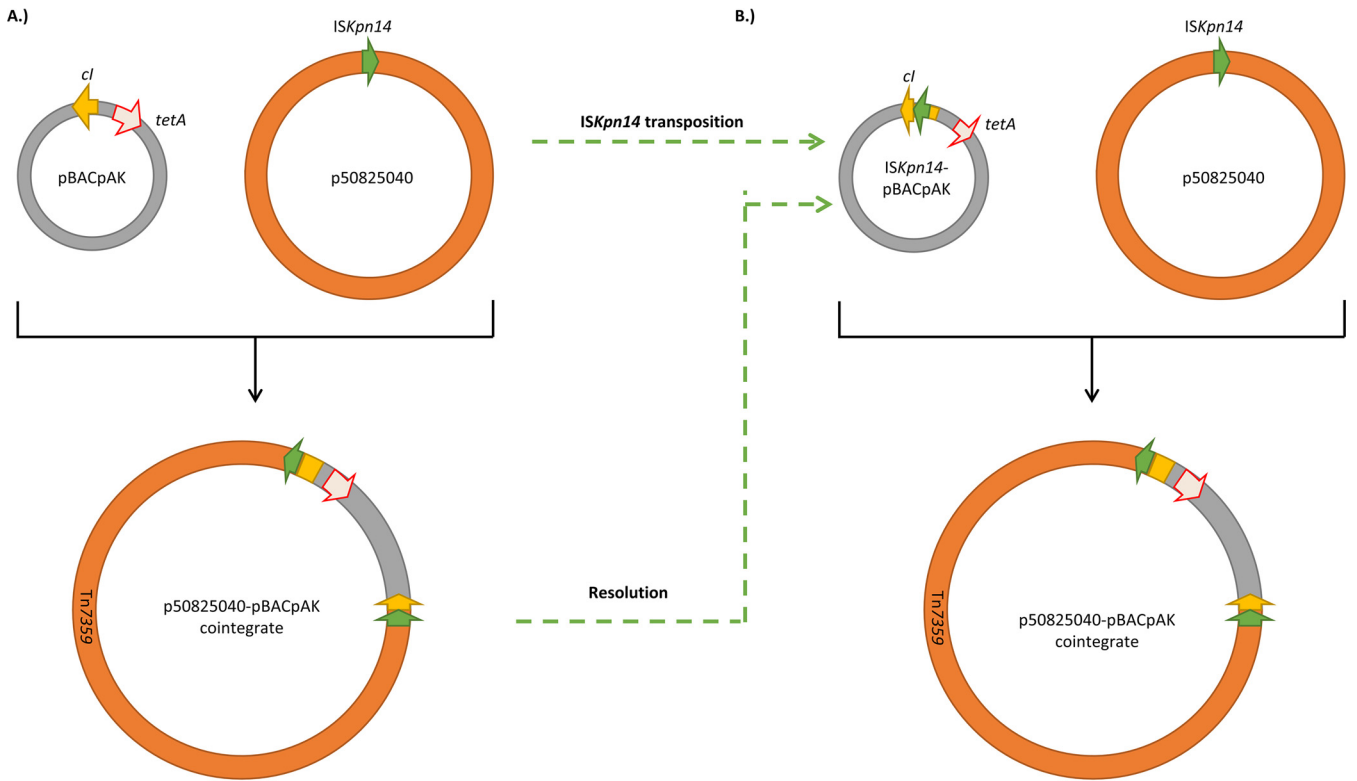


FIG 5 An insertion of Tn7359 into pBACpAK. (A) Tn7359 could form from a replicative transposition (as detailed in Biel and Berg [41]) where the cointegrate did not resolve, so it combined p50825040 with pBACpAK. (B) It could also form through targeted conservative transposition or homologous recombination between the *ISKpn14*-containing pBACpAK and p50825040. The *ISKpn14*-containing pBACpAK could form either through the transposition of *ISKpn14* into the *cl* repressor gene or the resolution of the p50825040-pBACpAK cointegrate, indicated by the green dashed-line arrows. The yellow, green, and red arrowed boxes represent *cl*, *ISKpn14*, and *tet(A)*, respectively. The gray and orange circles represent pBACpAK and p50825042, respectively.

IS26-family PCTs) and unconventional circularized structures (UCSs) (39, 40). However, the estimation of copy numbers of the *tet(A)* resistance gene in *E. coli* MDS-50825040-TC-Tet-3-7 and *E. coli* MDS-50825040-TC-Tet 4-38 (Table S3) showed that they had the same copy number as the chloramphenicol resistance gene on pBACpAK, suggesting that it was unlikely that *tet(A)* was being mobilized, and potentially amplified, from pBACpAK at detectable levels in the bacterial population analyzed; however, planned evolutionary studies will reveal if this gene is able to be acquired by the larger conjugative plasmid.

Comparing the sequences of Tn7359 from *E. coli* MDS-50825040-TC-Tet-3-1 and plasmid p2 from *K. pneumoniae* showed that plasmid p2 contained only one copy of *ISKpn14*. The Tn7359 composite transposon would therefore form via transposition into pBACpAK. *ISKpn14* is an IS1 family IS element, which can transpose through both conservative transposition and replicative transposition (Fig. 5) (41, 42). An insertion of Tn7359 on pBACpAK could occur as a result of a replicative transposition without a resolution of the cointegrate between the conjugative plasmid and the pBACpAK vector. It could also occur as a two-step event by first inserting only *ISKpn14* through replicative transposition, as suggested by an insertion of *ISKpn14* in *E. coli* MDS-50825040-TC-Tet-2-1. The *ISKpn14*-containing pBACpAK and *ISKpn14*-containing conjugative plasmid could subsequently combine through a targeted conservative transposition or homologous recombination at the *ISKpn14* of both replicons (Fig. 5).

The entrapment of Tn7359 by pBACpAK is the first time, to our knowledge, an entrapment vector captured an entire ARG-containing conjugative plasmid. Even though the captured element was not originally a transposon, the structure of the p50825040-pBACpAK cointegrate now fits the definition of a composite transposon, consisting of two IS elements flanking a DNA fragment (21, 43). It also shows how

multidrug resistance conjugative plasmids could extend their genetic complement through fusion with other plasmids, something that has recently been reported in a *K. pneumoniae* isolate containing the IncFIB:IncHI1B hybrid plasmid pEBSI036-1-NDM-VIR from an Egyptian hospital (44). The p50825040 conjugative plasmid from the donor would receive the *tet(A)* tetracycline resistance gene due to a fusion with the pBACpAK vector. Several studies also discovered plasmid fusion mediated by IS elements, such as IS257-mediated generation of multidrug resistance plasmids pSK818 and pSK697 in *Streptococcus epidermidis* and IS*Kpn19*-mediated cointegration between plasmid pBJ114-46 and pBJ114-141 in *E. coli* (45, 46).

Previously, all entrapment vector studies used *cl-tet(A)* primers and primer walking to identify MGEs in the tetracycline resistance clones. However, we found that the *cl-tet(A)* region of some samples could not be amplified by both standard and long PCR protocols, so they could not be sequenced by Sanger sequencing. We therefore used WGS sequencing to characterize these clones. We found that *E. coli* MDS-50825040-TC-Tet-3-7 and Tet 4-38 had double insertions of IS*Kpn25* both at the *cl* gene and downstream from *tet(A)*. Insertions of MGEs in other locations, not in the *cl-tet(A)* region, on pBACpAK were found in the WGS analysis of *E. coli* MDS-50627996-TC-Tet-2 as well, including an IS26 insertion in *sopA* and a 786-bp deletion in the *cl* gene, resulting in tetracycline resistance and a failed *cl-tet(A)* PCR, as it lost a *cl-tet(A)*-F1 primer binding site. *E. coli* MDS-50825040-TC-Tet-2-3 showed a wild-type *cl-tet(A)* PCR amplicon in colony PCR screening. This plasmid was then extracted and used to represent the wild-type pBACpAK in XhoI plasmid digestion to compare with other tetracycline-resistant transconjugants. However, XhoI plasmid digestion showed that the 3-kb *cl-tet(A)* band of *E. coli* MDS-50825040-TC-Tet-2-3 shifted up to more than 10 kb (Fig. S3); it was therefore sent for WGS sequencing, which showed a 6-bp deletion in the *cl* gene, conferring tetracycline resistance, and the insertion of IS*Kpn25* at a site downstream from *tet(A)*.

With the advancement of the sequencing technologies and the declining cost, using WGS sequencing to analyze all tetracycline resistance clones is becoming a viable option, as it will give information on translocation of MGEs into other locations, including the recipient chromosomal DNA. In our study, the WGS data did not show MGE insertions in the host chromosomal DNA. Such insertions are likely in a cellular population but in this case were not selected for, as our assay screened for transconjugants with a tetracycline resistance phenotype that would occur only in cells with an inactivating mutation or insertion of an MGE into the *cl* repressor gene on the pBACpAK vector.

In conclusion, we have demonstrated an approach to use the pBACpAK entrapment vector to capture MGEs from conjugative elements through a filter-mating experiment between clinical *Enterobacteriaceae* isolates and the *E. coli* MDS Rif Fus::pBACpAK recipient, which extended the utility of pBACpAK to other bacterial species. We also showed here that pBACpAK had the potential to capture large (120-kb) MGEs, including conjugative plasmids. Our results also demonstrated several aspects of MGE evolution after conjugation, including the rapid movement of IS elements and transposons, the formation of drug-resistance putative composite transposons, and a plasmid fusion likely mediated by IS elements.

MATERIALS AND METHODS

Bacterial strains and culture conditions. All bacterial strains used in the study are listed in Table 3. All bacterial strains were grown at 37°C in Luria-Bertani (LB) medium supplemented with appropriate antibiotics (Sigma-Aldrich, UK) with concentrations as follows: chloramphenicol at 12.5 µg/mL, rifampicin at 20 µg/mL, ampicillin at 100 µg/mL, fusidic acid at 400 µg/mL, and tetracycline at 5 µg/mL.

Clinical isolates in this study were selected from a carbapenemase-producing *Enterobacteriaceae* (CPE) collection at the Norwegian National Advisory Unit on Detection of Antimicrobial Resistance (20). The whole-genome sequencing (WGS) data of these isolates (BioProject accession number PRJNA295003) were used to initially screen for strains that either did not contain tetracycline resistance genes or contained tetracycline resistance genes on chromosome-derived contigs by using ResFinder and mlplasmids (47, 48). The numbers of plasmids, MGEs, and β-lactamase genes associated with plasmid-derived contigs were also determined by using Mobile Element Finder, PlasmidFinder, and

TABLE 3 Bacterial strains used in this study

Strain	Characteristics and MLST ^a	Resistance phenotype ^b	Reference of source
Donor strains			
<i>K. pneumoniae</i> strains			
K57-33	ST461, isolated in 2009	Amp ^r	20
K68-18	ST147, isolated in 2010	Amp ^r Chl ^r	20
K46-62	ST2134, isolated in 2007	Amp ^r	20
50825040	ST17, isolated in 2014	Amp ^r	20
50877064	ST37, isolated in 2014	Amp ^r	20
50675619	ST336, isolated in 2012	Amp ^r	20
50627996	ST11, isolated in 2012	Amp ^r Chl ^r Tet ^r	20
<i>E. coli</i> 50676002	ST131, isolated in 2012	Amp ^r Tet ^r	20
Recipient strains			
<i>E. coli</i> strains			
MDS	Electrocompetent; reduced genome including deletion of mobile DNA		Scarab Genomics, USA
MDS::pBACpAK	<i>E. coli</i> MDS containing pBACpAK	Chl ^r	This study
MDS Rif::pBACpAK	<i>E. coli</i> MDS::pBACpAK with spontaneous rifampicin resistance	Chl ^r Rif ^r	This study
MDS Rif Fus::pBACpAK	<i>E. coli</i> MDS Rif::pBACpAK with spontaneous fusidic acid resistance; recipient for filter-mating expt	Chl ^r Rif ^r Fus ^r	This study
Transconjugant strains			
<i>E. coli</i> strains			
MDS-K57-33-TC	Transconjugant from filter mating between <i>E. coli</i> MDS Rif Fus::pBACpAK and <i>K. pneumoniae</i> K57-33	Chl ^r Rif ^r Fus ^r Amp ^r	This study
MDS-K46-62-TC	Transconjugant from filter mating between <i>E. coli</i> MDS Rif Fus::pBACpAK and <i>K. pneumoniae</i> K46-62	Chl ^r Rif ^r Fus ^r Amp ^r	This study
MDS-50825040-TC	Transconjugant from filter mating between <i>E. coli</i> MDS Rif Fus::pBACpAK and <i>K. pneumoniae</i> 50825040	Chl ^r Rif ^r Fus ^r Amp ^r	This study
MDS-50877064-TC	Transconjugant from filter mating between <i>E. coli</i> MDS Rif Fus::pBACpAK and <i>K. pneumoniae</i> 50877064	Chl ^r Rif ^r Fus ^r Amp ^r	This study
MDS-50675619-TC	Transconjugant from filter mating between <i>E. coli</i> MDS Rif Fus::pBACpAK and <i>K. pneumoniae</i> 50675619	Chl ^r Rif ^r Fus ^r Amp ^r	This study
MDS-50627996-TC	Transconjugant from filter mating between <i>E. coli</i> MDS Rif Fus::pBACpAK and <i>K. pneumoniae</i> 50627996	Chl ^r Rif ^r Fus ^r Amp ^r	This study
MDS-50676002-TC	Transconjugant from filter mating between <i>E. coli</i> MDS Rif Fus::pBACpAK and <i>E. coli</i> 50676002	Chl ^r Rif ^r Fus ^r Amp ^r	This study

^aMultilocus sequence types (MLST) were reported in a previous study (20).

^bChl^r, chloramphenicol resistance; Rif^r, rifampicin resistance; Fus^r, fusidic acid resistance; Amp^r, ampicillin resistance.

mplasmids (10, 48, 49) and were used as criteria to select 8 potential donors for the filter-mating experiments with our *E. coli* MDS Rif Fus::pBACpAK recipient (see below).

Preparation of *E. coli* MDS Rif Fus::pBACpAK recipient strain. For recipient cells, the *E. coli* MDS strain (Scarab Genomics, USA) was used, as it has been genetically modified to remove all mobile DNA and error-prone DNA polymerases (50), reducing the possibility of false positives from the transposition of MGEs from an *E. coli* host and *de novo* mutations within *cl* during screening.

E. coli MDS::pBACpAK was prepared by introducing a pBACpAK entrapment vector into *E. coli* MDS electrocompetent cells (Scarab Genomics, USA) through electroporation. Amounts of 50 μ L of the electrocompetent cells and 10 ng of pBACpAK plasmid were mixed in a prechilled 1.5-mL microcentrifuge tube and transferred to a prechilled, 0.1-cm electroporation cuvette (Bio-Rad, UK). The cells were then electroporated, and 950 μ L of prewarmed SOC medium (New England Biolabs, UK) was added into the cuvette. The cell mixture was transferred to a 50-mL tube and incubated in a 37°C shaker for 1 h. After the incubation, cells were grown on LB agar containing chloramphenicol and incubated overnight. The transformants were screened and checked for the presence of pBACpAK by performing *cl-tet(A)* PCR with *cl-tetA-F1* and ERIS primers (Table S1).

To generate *E. coli* MDS Rif::pBACpAK, *E. coli* MDS::pBACpAK was subcultured in LB broth containing chloramphenicol (for selection of pBACpAK) and incubated overnight. An aliquot of 100 μ L of the overnight culture was plated onto LB agar supplemented with 20 μ g/mL rifampicin and incubated overnight. The colonies grown on the selective plates were subcultured onto another fresh rifampicin

selective plate to confirm their rifampicin resistance phenotype. *E. coli* MDS Rif Fus::pBACpAK was then generated from *E. coli* MDS Rif::pBACpAK with the same process but with LB agar supplemented with 400 $\mu\text{g}/\text{mL}$ fusidic acid. Rifampicin and fusidic acid resistance in *E. coli* MDS Rif Fus::pBACpAK were confirmed by PCR amplification and sequencing of genes previously shown to be responsible for the resistance phenotypes and subsequently confirmed by WGS of *E. coli* MDS Rif Fus::pBACpAK transconjugants.

Filter mating between clinical isolate donors and *E. coli* MDS Rif Fus::pBACpAK recipient. The frequency of spontaneous mutation of *E. coli* MDS Rif Fus::pBACpAK exposed to tetracycline was determined by spreading an overnight culture of *E. coli* MDS Rif Fus::pBACpAK on 3 LB agar plates supplemented with rifampicin, fusidic acid, chloramphenicol, and tetracycline and incubating at 37°C for 3 days.

Filter mating was performed by following the protocol described previously (51). The donors (*Enterobacteriaceae* clinical isolates) (Table 3) and the recipient (*E. coli* MDS Rif Fus::pBACpAK) were grown overnight in 5 mL LB broth supplemented with appropriate antibiotics in separate 50-mL tubes. Each overnight culture was subcultured into 10 mL of fresh LB broth with no antibiotics with an optical density at 600 nm (OD_{600}) of 0.1 and incubated at 37°C until mid-exponential phase (OD_{600} of 0.5 to 0.6). The cells were centrifuged and resuspended in 500 μL of LB broth. The donor and recipient cells were then mixed together and 150 μL spread on a 0.45- μm -pore-size sterilized nitrocellulose filter (Sartorius, UK) on antibiotic-free LB agar plates. Control groups were also included by adding only the donor or recipient strain to filters. After 5 h, the filters were transferred into 50-mL tubes. Cells on the filters were resuspended in 1 mL of prewarmed LB broth by vortexing the tubes at high speed for 1 min. The cell suspension was spread onto plates containing LB agar supplemented with chloramphenicol, rifampicin, ampicillin, and fusidic acid (LB CRAF agar) to select for transconjugants. Ampicillin was used to select for the transfer of the β -lactamase-containing plasmid(s) to the recipient strain. The transconjugants were confirmed by subculturing on fresh selective LB CRAF agar plates and carrying out a *cl-tet(A)* colony PCR (*cl-tetA-F1* and ERIS primers) to confirm that they were recipient cells (Table S1).

Screening for transconjugants with insertion of MGEs within pBACpAK. All transconjugants were subcultured into 5 mL of LB CRAF broth and incubated for 4 h in a 37°C shaker. An aliquot of 500 μL of culture was plated onto two plates of LB agar supplemented with chloramphenicol, rifampicin, ampicillin, fusidic acid, and tetracycline (LB CRAFT agar). One of them was incubated at 37°C, while the other one was incubated at room temperature. The 4-h culture was returned to the 37°C shaker overnight, and then 100 μL of the overnight culture was spread onto another two LB CRAFT agar plates and incubated at 37°C or room temperature separately. The overnight culture was also used to subculture into 5 mL of fresh LB CRAF broth and the same plating and subculture repeated for another 3 days. All plates were checked for colony growth every day for a week, and any resulting colonies were subcultured on fresh LB CRAFT agar to confirm the tetracycline resistance phenotype.

All confirmed tetracycline resistance transconjugants were screened for insertion of MGEs into the *cl-tet(A)* region of pBACpAK by colony PCR with *cl-tetA-F1* and ERIS primers (Table S1) as described previously (15). The colony PCR was first performed with a standard PCR protocol using 2 \times Biomix red (Bioline, UK) with an elongation time of 3 min to amplify up to 6 kb to initially rule out clones with mutations irrelevant in this study (point mutations, deletions, and small insertions). Clones that failed to amplify using the standard PCR were repeated with Q5 high-fidelity 2 \times mastermix (New England Biolabs, UK) with a 10-min elongation time to amplify up to 20 kb. The amplicons with more than a 500-bp increase in the size of the *cl-tet(A)* amplicon compared to a wild-type *cl-tet(A)* amplicon (1.35 kb) were sequenced by the Sanger sequencing service from Genewiz, Germany. BLASTN, BLASTX, and ISFinder were used to compare the sequences to nucleotide, protein, and IS element databases, respectively (52, 53).

Genetic analysis of tetracycline-resistant transconjugants. Clones that failed to amplify a product with *cl-tetA-F1* and ERIS primers with both standard and long colony PCR protocols were initially analyzed by extracting their plasmids and comparing their XhoI plasmid digestion pattern with the wild-type XhoI pBACpAK digestion pattern. WGS was performed by using MiSeq version 3 with 600 cycles (300-bp paired-end reads) at the Norwegian Sequencing Centre (Oslo University Hospital, Ullevål, Oslo, Norway). Genomic DNA was extracted from the bacterial pellet using the QIAcube automated station (Qiagen, Norway) following the QIAamp DNA mini-QIAcube kit protocol. DNA libraries were prepared using Nextera DNA flex tagmentation (Illumina).

The raw reads were processed with AfterQC version 0.9.7 to trim and filter low-quality reads (54), followed by *de novo* genome assembly with SPAdes 3.13.1 (55). The contigs containing pBACpAK were identified by using BLAST to compare the assembled contigs with pBACpAK sequences. Insertion of MGEs in chromosomal DNA of the *E. coli* recipient was checked by using breseq version 0.35.6 to map the filtered reads with the *E. coli* MDS reference genome (accession number [AP012306](#)) (56). The comparison of Tn7350-, Tn7351-, and Tn7359-containing pBACpAK sequences with their best match from BLASTN was performed with BLAST Ring Image Generator (BRIG) version 0.95 (57). The estimation of gene copy number was done by determining the number of filtered reads mapped to each gene with BWA version 0.7.17 and SAMtools version 1.11 (58, 59) and normalized by dividing the read counts by the size of each gene. The copy numbers of each gene were calculated by dividing each normalized read count by the normalized read counts of the reference genes (the chloramphenicol resistance gene and *repE* for pBACpAK and the *bla*_{NDM-1} resistance gene and *repA* for the conjugative plasmid).

Data availability. Novel transposons were assigned the following Tn numbers by The Transposon Registry (43): Tn7350 ([OK245453](#)), Tn7351 ([OK245454](#)), and Tn7359 (accession number [SAMN21542911](#)). The WGS data were deposited at the National Center for Biotechnology Information (NCBI) with accession numbers [SAMN21542910](#) to [SAMN21542916](#).

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.9 MB.

SUPPLEMENTAL FILE 2, XLSX file, 0.02 MB.

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