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 \( cI \) 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 conjugal 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.
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, blaNDM-1, conferring carbapenem resistance, and mcr-1, conferring colistin resistance, were found on ISAb125- and ISApl1-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 ISEcpl-blaOXA-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 pBACPvAK and demonstrated that it can detect the insertion of MGEs in both laboratory and clinical Escherichia coli isolates (15, 16). pBACPvAK contains a cl-tet(A) gene system in which the λ repressor (encoded by cl) constitutively inhibits the expression of the tet(A) gene by binding to the 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 pBACPvAK entrapment vector previously (15).

In this study, we used pBACPvAK 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).
pBACpAK was electroporated into *E. coli* strain MDS, which was subsequently sequencially 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 *cI* 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 Rif Fus::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 chloramphenicol, rifampicin, ampicillin, and fusidic acid. If the MGE, located on the conjugative plasmid, translocated into the *cI* gene on pBACpAK, it would disrupt the expression of the *λ* repressor, conferring tetracycline resistance due to the derepression of the *P_tet* 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. *Cl*, chloramphenicol resistance; *Rif*, rifampicin resistance; *Amp*, ampicillin resistance; *Fus*, fusidic acid resistance; *Tetr*, tetracycline resistance.

**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 *cI* gene on pBACpAK, it would disrupt the expression of the *λ* repressor, conferring tetracycline resistance due to the derepression of the *P_tet* 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. *Cl*, chloramphenicol resistance; *Rif*, rifampicin resistance; *Amp*, ampicillin resistance; *Fus*, fusidic acid resistance; *Tetr*, tetracycline resistance.
TABLE 1 The details of carbapenemase-producing Enterobacteriaceae clinical strains

| Species            | Isolate  | No. of MGEs   | MGEs associated with plasmid- derived contigs | Resistance determinant(s) | Resistance phenotype |
|--------------------|----------|---------------|-----------------------------------------------|---------------------------|----------------------|
| K. pneumoniae      | 50675619 | 5             | 8                                             | catB3 (P)                 | S S S S             |
| K. pneumoniae      | 50877064 | 1             | 4                                             | No                         | S S S S             |
| K. pneumoniae      | 50825040 | 4             | 11                                            | No                         | S S S S             |
| E. coli            | 50676002 | 4             | 5                                             | tetA (C)                  | S S S 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). mlplasmid 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).

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

chloramphenicol, rifampicin, ampicillin, and fusidic acid (LB CRAFT 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 CRAFT 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 CRAFT 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-Tet-11, E. coli MDS-K46-62-Tet-21, E. coli MDS-50675619-Tet-4, and E. coli MDS-50825040-Tet-Tet-2-3) 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, IS50825040, IS50675619, and IS50627996) 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-Tet-2-3 and E. coli MDS-50627996-Tet-2 showed an IS5025 insertion between tet(A) and orfV 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-Tet-3-7 and E. coli MDS-50825040-Tet-4-38 each had an insertion of ISKpn25, one in the cl repressor gene and the other one between tet(A) and orfV. 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-Tet-11 and E. coli MDS-K46-62-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
| Strain (accession no.) | MGE/mutation | Location on pBACpAK (position) | Size (bp) | % similarity (ISFinder/NCBI) | BLASTN | BLASTX | Accession no. | Direct repeat |
|------------------------|--------------|--------------------------------|-----------|-----------------------------|--------|--------|---------------|---------------|
| *E. coli* MDS-K46-62-TC strains | | | | | | | | |
| Tet-11 | Tn7350 cI 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 arlK | 342 | 100 | ACI63129 | | | | | |
| | Zinc metalloproteinase Mpr | 465 | 99 | CDF32051 | | | | | |
| | ISSbo1 | 1,709 | 96 | CP001062 | | | | | |
| Tet-21 | Tn7351 cI 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 arlK | 342 | 100 | ACI63129 | | | | | |
| Zinc metalloproteinase Mpr | 465 | 99 | CDF32051 | | | | | |
| ISSbo1 | 1,709 | 94 | CP001062 | | | | | |
| *E. coli* MDS-50675619-TC-Tet-4 | ISbo1 cI repressor (2976) | 1,709 | 96 | 98 | CP001062 | | | GAAC |
| *E. coli* MDS-50825040-TC strains | | | | | | | | |
| Tet-2-1 | ISKpn14 cI repressor (3127) | 768 | 100 | 100 | CP000649 | | | CGGCCTTA |
| ISKpn25 | Between tet(A) and oriV (4976) | 8,154 | 100 | 100 | NC_009650 | | | TATTTATC |
| Deletion | cI repressor (3174–3179) | 6 | | | | | | | |
| Tet-3-1 (SAMN21542911) | K. pneumoniae blaKOM-1 plasmid p2 cI repressor (3126) | 118,959 | 99.99 | CP009115 | | | | TAACGCCG |
| Tet-3-7 (SAMN21542912) | ISKpn25 cI repressor (3224) | 8,154 | 100 | 100 | NC_009650 | | | CATTTATC |
| ISKpn25_1 | Between tet(A) and oriV (4980) | 8,154 | 100 | 100 | NC_009650 | | | TATTTATC |
| ISKpn25_2 | cI repressor (3224) | 8,154 | 100 | 100 | NC_009650 | | | AATTTATC |
| ISKpn25_1 | ISKpn25_2 | Between tet(A) and oriV (4941) | 8,154 | 100 | 100 | NC_009650 | | | AAAAGAAG |
| Tet-4-13 (SAMN21542913) | ISKpn25 cI repressor (3294) | 8,154 | 100 | 100 | NC_009650 | | | AATTTATC |
| ISKpn25_1 | ISKpn25_2 | Between tet(A) and oriV (4940) | 8,154 | 100 | 100 | NC_009650 | | | AAAAGAAG |
| Tet-4-38 (SAMN21542914) | ISKpn25 | cI repressor (3230) | 8,154 | 100 | 100 | NC_009650 | | | AAAAGAAG |
| Tet-4-52 (SAMN21542915) | ISKpn25 | cI repressor (3277) | 8,154 | 100 | 100 | NC_009650 | | | AAAAGAAG |
| *E. coli* MDS-50627996-TC-Tet-2 | IS26 sopA (8066) | 820 | 100 | 100 | X00011 | | | CAGATCTT |
| (SAMN21542918) | Deletion | cI repressor (2305–3090) | 786 | | | | | |
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 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.

**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 pK65-67VIM 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.
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 IS\textsubscript{Kpn14} elements in pBACpAK, it fit the definition of a composite transposon (21) and was named Tn\textsubscript{7359} (Fig. 4). It was also highly similar to the bla\textsubscript{NDM-1}-containing plasmid p2 found in *K. pneumoniae* (accession number CP009115) (Fig. S1B) (22). Tn\textsubscript{7359} contained a Tn\textsubscript{21}-like structure (merCAD, mercury resistance genes, sur\textsubscript{2}, and tni\textsubscript{A} genes) and multiple ARGs within an IS\textsubscript{26}-based pseudo-compound transposon (PCT)-like structure (23), as one of the IS\textsubscript{26} elements was disrupted by the tni\textsubscript{A} gene, a conjugative module, and a plasmid stability/replication module (Fig. 4). The IS\textsubscript{26} PCT-like structure contained multiple β-lactamase genes (bla\textsubscript{TEM-1}, bla\textsubscript{OXA-9}, bla\textsubscript{CTX-M-15}, and bla\textsubscript{NDM-1}), aminoglycoside resistance genes (ant\textsubscript{1}, aac\textsubscript{4}, and aph\textsubscript{4}), the ble bleomycin resistance gene, and the qnrS1 quinolone resistance gene. It also carried several IS elements, such as IS\textsubscript{Ecp1}, IS\textsubscript{Ec36}, IS\textsubscript{Spu2}, IS\textsubscript{Kpn19}, IS\textsubscript{Kpn8}, and IS\textsubscript{Kpn25}.

**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 (Tn\textsubscript{7350} and Tn\textsubscript{7351}) 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 Tn\textsubscript{7351} compared to Tn\textsubscript{7350}, are flanked by GAAC inverted or direct repeats. GAAC represents the conserved target site of IS\textsubscript{91} insertion sequences (29, 30). IS\textsubscript{Sbo1}, which is present on both Tn\textsubscript{7350} and Tn\textsubscript{7351}, is a member of the IS\textsubscript{91} family of insertion sequences. 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 IS\textsubscript{Kpn14} elements in pBACpAK, it fit the definition of a composite transposon (21) and was named Tn\textsubscript{7359} (Fig. 4). It was also highly similar to the bla\textsubscript{NDM-1}-containing plasmid p2 found in *K. pneumoniae* (accession number CP009115) (Fig. S1B) (22). Tn\textsubscript{7359} contained a Tn\textsubscript{21}-like structure (merCAD, mercury resistance genes, sur\textsubscript{2}, and tni\textsubscript{A} genes) and multiple ARGs within an IS\textsubscript{26}-based pseudo-compound transposon (PCT)-like structure (23), as one of the IS\textsubscript{26} elements was disrupted by the tni\textsubscript{A} gene, a conjugative module, and a plasmid stability/replication module (Fig. 4). The IS\textsubscript{26} PCT-like structure contained multiple β-lactamase genes (bla\textsubscript{TEM-1}, bla\textsubscript{OXA-9}, bla\textsubscript{CTX-M-15}, and bla\textsubscript{NDM-1}), aminoglycoside resistance genes (ant\textsubscript{1}, aac\textsubscript{4}, and aph\textsubscript{4}), the ble bleomycin resistance gene, and the qnrS1 quinolone resistance gene. It also carried several IS elements, such as IS\textsubscript{Ecp1}, IS\textsubscript{Ec36}, IS\textsubscript{Spu2}, IS\textsubscript{Kpn19}, IS\textsubscript{Kpn8}, and IS\textsubscript{Kpn25}.

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**FIG 4** The structure of Tn\textsubscript{7359} 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 cI 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).
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 over-expression 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 (cI) 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

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IS2-family PCTs) and unconventional circularized structures (UCSs) (39, 40). However, the estimation of copy numbers of the \textit{tet}(A) resistance gene in \textit{E. coli} MDS-50825040-TC-Tet-3-7 and \textit{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 \textit{tet}(A) was unlikely 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 \textit{Tn7359} from \textit{E. coli} MDS-50825040-TC-Tet-3-7 and \textit{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 \textit{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.

The entrapment of \textit{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 pS0825040 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 ISKpn19-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 ISKpn25 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 Xhol plasmid digestion to compare with other tetracycline-resistant transconjugants. However, Xhol 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 ISKpn25 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 Fung::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 miplasmids (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
mlplasmids (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 cI 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 μL 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 μL 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 cI-tet(A) PCR with c-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 μL of the overnight culture was plated onto LB agar supplemented with 20 μg/mL rifampicin and incubated overnight. The colonies grown on the selective plates were subcultured onto another fresh rifampicin

| TABLE 3 Bacterial strains used in this study |
|---------------------------------------------|
| **Strain** | **Characteristics and MLST** | **Resistance phenotype** | **Reference of source** |
| Donor strains | | | |
| K. pneumonia strains | | | |
| K57-33 | ST461, isolated in 2009 | Amp’ | 20 |
| K68-18 | ST147, isolated in 2010 | Amp’ Chl’ | 20 |
| K46-62 | ST2134, isolated in 2007 | Amp’ | 20 |
| 50825040 | ST17, isolated in 2014 | Amp’ | 20 |
| 50877064 | ST37, isolated in 2014 | Amp’ | 20 |
| 50675619 | ST336, isolated in 2012 | Amp’ | 20 |
| 50627996 | ST11, isolated in 2012 | Amp’ Chl’ Tet’ | 20 |
| E. coli 50676002 | ST131, isolated in 2012 | Amp’ Tet’ | 20 |
| Recipient strains | | | |
| E. coli strains | | | |
| MDS | Electrocompetent; reduced genome including deletion of mobile DNA | | Scarab Genomics, USA |
| MDS:pBACpAK | E. coli MDS containing pBACpAK | Chl’ | This study |
| MDS Rif::pBACpAK | E. coli MDS::pBACpAK with spontaneous rifampicin resistance | Chl’ Rif’ | This study |
| MDS Rif Fus::pBACpAK | E. coli MDS::pBACpAK with spontaneous fusidic acid resistance; recipient for filter-mating expt | Chl’ Rif’ Fus’ | This study |
| Transconjugant strains | | | |
| E. coli strains | | | |
| MDS-K57-33-TC | Transconjugant from filter mating between E. coli MDS Rif Fus::pBACpAK and K. pneumoniae K57-33 | Chl’ Rif’ Fus’ Amp’ | This study |
| MDS-K46-62-TC | Transconjugant from filter mating between E. coli MDS Rif Fus::pBACpAK and K. pneumoniae K46-62 | Chl’ Rif’ Fus’ Amp’ | This study |
| MDS-50825040-TC | Transconjugant from filter mating between E. coli MDS Rif Fus::pBACpAK and K. pneumoniae 50825040 | Chl’ Rif’ Fus’ Amp’ | This study |
| MDS-50877064-TC | Transconjugant from filter mating between E. coli MDS Rif Fus::pBACpAK and K. pneumoniae 50877064 | Chl’ Rif’ Fus’ Amp’ | This study |
| MDS-50675619-TC | Transconjugant from filter mating between E. coli MDS Rif Fus::pBACpAK and K. pneumoniae 50675619 | Chl’ Rif’ Fus’ Amp’ | This study |
| MDS-50627996-TC | Transconjugant from filter mating between E. coli MDS Rif Fus::pBACpAK and K. pneumoniae 50627996 | Chl’ Rif’ Fus’ Amp’ | This study |
| MDS-50676002-TC | Transconjugant from filter mating between E. coli MDS Rif Fus::pBACpAK and E. coli 50676002 | Chl’ Rif’ Fus’ Amp’ | This study |

*Multilocus sequence types (MLST) were reported in a previous study (20).

Chl’, chloramphenicol resistance; Rif’, rifampicin resistance; Fus’, fusidic acid resistance; Amp’, ampicillin resistance.
selective plate to confirm their rifampicin resistance phenotype. *E. coli* MDS Rif Fups:pBACpAK was then generated from *E. coli* MDS Rif:pBACpAK with the same process but with LB agar supplemented with 400 μg/mL fusidic acid. Rifampicin and fusidic acid resistance in *E. coli* MDS Rif Fups: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 Fups:pBACpAK transconjugants.

**Filter mating between clinical isolate donors and *E. coli* MDS Rif Fups:pBACpAK recipient.** The frequency of spontaneous mutation of *E. coli* MDS Rif Fups:pBACpAK exposed to tetracycline was determined by spreading an overnight culture of *E. coli* MDS Rif Fups: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 Fups: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 (OD₆₀₀) of 0.1 and incubated at 37°C until mid-exponential phase (OD₆₀₀ 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-tet-A1 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 CRAF agar). One of them was incubated at 37°C, while the other 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 CRAF 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 scored for colony growth every day for a week and any resulting colonies were subcultured on fresh LB CRAF 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× 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× 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 Geneiz, 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 Xhol plasmid digestion pattern with the wild-type Xhol 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. All transconjugants were checked for colony growth every day for a week, and any resulting colonies were subcultured on fresh LB CRAF broth and then mixed together and 150 μL spread onto plates containing LB agar supplemented with chloramphenicol, rifampicin, fusidic acid, and tetracycline and incubated at 37°C until mid-exponential phase (OD₆₀₀ 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-tet-A1 and ERIS primers) to confirm that they were recipient cells (Table S1).

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**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.

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SUPPLEMENTAL MATERIAL
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