RESEARCH ARTICLE

Insertion Sequence IS*26* Reorganizes Plasmids in Clinically Isolated Multidrug-Resistant Bacteria by Replicative Transposition

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ABSTRACT **Carbapenemase-producing *Enterobacteriaceae* (CPE), which are resistant to most or all known antibiotics, constitute a global threat to public health. Transposable elements are often associated with antibiotic resistance determinants, suggesting a role in the emergence of resistance. One insertion sequence, IS*26*, is frequently associated with resistance determinants, but its role remains unclear. We have analyzed the genomic contexts of 70 IS*26* copies in several clinical and surveillance CPE isolates from the National Institutes of Health Clinical Center. We used target site duplications and their patterns as guides and found that a large fraction of plasmid reorganizations result from IS*26* replicative transpositions, including replicon fusions, DNA inversions, and deletions. Replicative transposition could also be inferred for transposon Tn*4401*, which harbors the carbapenemase bla_{KPC} gene. Thus, replicative transposition is important in the ongoing reorganization of plasmids carrying multidrug-resistant determinants, an observation that carries substantial clinical and epidemiological implications for understanding how such extreme drug resistance phenotypes evolve.**

IMPORTANCE **Although IS*26* is frequently reported to reside in resistance plasmids of clinical isolates, the characteristic hallmark of transposition, target site duplication (TSD), is generally not observed, raising questions about the mode of transposition for IS*26*. The previous observation of cointegrate formation during transposition implies that IS*26* transposes via a replicative mechanism. The other possible outcome of replicative transposition is DNA inversion or deletion, when transposition occurs intramolecularly, and this would also generate a specific TSD pattern that might also serve as supporting evidence for the transposition mechanism. The numerous examples we present here demonstrate that replicative transposition, used by many mobile elements (including IS*26* and Tn*4401*), is prevalent in the plasmids of clinical isolates and results in significant plasmid reorganization. This study also provides a method to trace the evolution of resistance plasmids based on TSD patterns.**

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Members of the family *Enterobacteriaceae* are major causes of severe and often lethal hospital-acquired infections. In the past two decades, carbapenemase-producing *Enterobacteriaceae* (CPE) have appeared that are resistant to most or all clinically available antibiotics, including carbapenems, which are often considered the antibiotics of last resort (1). It has been known for over 40 years that antibiotic resistance determinants are often associated with mobile genetic elements (2), a phenomenon that has been reinforced by genome sequencing of clinical isolates. For instance, bla_{CTX}, the coding gene of an extended-spectrum β-lactamase (ESBL), is often located downstream of insertion sequence (IS) IS*Ecp1* (3–6), and among carbapenemase-coding genes, bla_{KPC} is typically carried by the Tn*3* family transposon Tn*4401* (7, 8), whereas bla_{NDM} is frequently located downstream of IS*Aba125* (9, 10).

Mobile elements are also associated with formation of resistance gene clusters in which different determinants that give rise to a multidrug-resistant phenotype are found in close proximity. For example, specific recombination platforms, integrons (11), can recruit cassettes carrying additional resistance genes via site-specific recombination (12–14). These are, in turn, often associated with transposons and IS*CR*s (insertion sequences with a common region), a class of mobile elements that has been implicated in capturing genes or gene fragments (15). Mobile elements can also integrate control elements such as promoters upstream of resistance determinants, leading to increased expression (16–18). The horizontal dissemination of resistance between bacteria can occur via conjugative plasmids and integrative conjugative elements (ICEs) (19, 20).

ISs are the simplest autonomous mobile elements in bacterial genomes. They are typically composed of short terminal sequences, often arranged as inverted repeats (terminal inverted repeats [TIRs]) at their ends and at least one open reading frame (ORF) that encodes the transposase, the only protein essential for

mobility (21). The TIRs serve as specific recognition sites for the transposase, the enzyme that carries out the strand cleavage and transfer reactions which confer mobility. While the average IS DNA content of bacterial chromosomes is below 3%, in plasmids larger than 20 kb it is between 5 and 15%, with one extreme case exceeding 40% (22). The 4,240 different currently identified ISs can be divided into 30 families based on their overall genetic organization and specific genetic signatures (https://www-is.biotoul.fr/) (23). ISs within one family are assumed to use the same or a similar transposition mechanism to one another, although only a subset of IS families has been investigated experimentally, and the mechanisms of many remain poorly understood.

A vast majority of presently identified ISs encode transposases whose catalytic domain is homologous to those of RNase H and members of the retroviral integrase superfamily (24, 25), the so-called DDE transposases. Some ISs transpose by a cut-and-paste mechanism by excising from one genetic location and integrating into another (21). Others transpose by using a copy-in (26) or replicative transposition (21) pathway that requires extensive DNA replication and typically generates products known as cointegrates, which have a second copy of the IS at the target site while the original copy is left intact (27, 28).

During replicative transposition, a branched or forked "Shapiro intermediate" is formed between the donor IS and the target DNA (Fig. 1a) (28). When the IS and the target site are in two different replicons (intermolecular transposition) (Fig. 1a), subsequent DNA replication at the intermediate branch fuses the two replicons, duplicating both the transposable element and a short nucleotide sequence (target site duplication [TSD]) flanking the insertion site. The TSD becomes two new direct repeats (Fig. 1a, red arrows). Cointegrates can be subsequently resolved by recombination between the two directly repeated ISs, either by a dedicated resolvase or a host-mediated process, to regenerate the two replicons, each with a single IS copy, and the TSDs will flank the copy in the target replicon.

When the donor IS and target sites are present in the same replicon (intramolecular transposition) (Fig. 1b), there are two possible and very different outcomes. After the initial DNA cleavage steps, the liberated 3′-OH groups at the two IS ends can attack the target DNA in two different orientations, i.e., the 3′-OH of the "top" strand of the IS can attack either the "top" or "bottom" strand at the target site (27).

Attack in one orientation (Fig. 1b, *cis*) results in the deletion of the DNA between the IS and the target site, leaving only one copy of the IS. A circular product is also created that contains a second IS copy, but this will survive only if it contains an origin of replication. Neither of the resulting IS copies is flanked by TSDs: these are distributed between the two products of deletion.

Attack in the other orientation (Fig. 1b, *trans*) results in IS duplication in an inverted configuration, and the DNA segment between the original IS and the target site is inverted; in addition, the target site is duplicated. However, as the TSD is at one end of the original IS and the opposite end of the copy, again, neither of the two resulting IS copies is flanked by TSDs. In principle, *cis* and *trans* attacks are equally likely, given the pseudo twofold symmetry of double-stranded DNA (dsDNA) and the general lack of target sequence specificity of ISs.

Two identical IS copies within one replicon can generate a composite transposon in which cleavage at two outer ends can mobilize both ISs and the DNA segment between them. If the IS uses a replicative transposition pathway, an alternative parsimonious way could be used to mobilize the composite transposon. Transposition of one of the ISs can generate a cointegrate with three IS copies (cointegrate, Fig. 1c). Resolution of this cointegrate between two IS copies (arrows, Fig. 1c) will result in the transfer of the composite transposon into the target replicon (21). This pathway has been shown experimentally in *Escherichia coli* for IS*26* (38).

In 2011, the National Institutes of Health Clinical Center (NIHCC) experienced an outbreak of carbapenem-resistant *Klebsiella pneumoniae* infections initially involving 18 patients with a high fatality rate. Sequencing of all isolates recovered during the 2011 outbreak demonstrated that the outbreak was clonal (29). The NIHCC Hospital Epidemiology and Microbiology Services now routinely performs surveillance cultures of patients and the hospital environment, and other CPE isolates have since been recovered.

Here, we have analyzed CPE genomes from the NIHCC collection isolated between 2011 and the end of 2013, focusing largely on IS content and particularly on replicative transposons. Although the possible outcomes of replicative transposition have been described (27, 28), no systematic investigation on the genomic level has been undertaken to understand its impact in clinically relevant circumstances. Our analysis of the distinct patterns of sequences that flank these mobile elements allows us to propose mechanisms and pathways that explain several changes due to transposition events, and we demonstrate that IS*26* in particular actively remodels resistance plasmids by both inter- and intramolecular replicative transposition.

RESULTS

IS annotations of the genomes of carbapenem-resistant *Klebsiella pneumoniae* isolates from the 2011 NIH outbreak. Since the 2011 outbreak, the NIHCC has routinely cultured CPE isolates from patients and from the clinical environment. In the subsequent 2 years, a number of CPE isolates, including *Klebsiella pneumoniae*, *Klebsiella oxytoca*, *Enterobacter cloacae* complex, *Citrobacter freundii* complex, *Escherichia coli*, and *Pantoea* spp. have been isolated from these clinical and surveillance cultures. The genomes of some of these strains have been sequenced at a very high coverage using Pacific Biosciences RS II single-molecule real-time (SMRT) technology which generates 14- to 40-kb-long reads (30). We therefore took advantage of these sequence data to investigate the diversity of ISs present in clinical isolates by first performing IS annotation of one representative isolate, KPNIH1 (29), from the clonal NIHCC *K. pneumoniae* outbreak. The assembled genome of this isolate contains one chromosome and three plasmids of >15 kb. Carbapenemase resistance is conferred by bla_{KPC} which is carried by Tn*4401* in plasmid pKpQIL, as previously observed in isolates from other hospitals (31, 32).

Whole-genome IS annotation of KPNIH1 using ISsaga (http://issaga.biotoul.fr/) (33) revealed 29 different ISs belonging to 12 different IS families (Fig. 2). Some of these are present either as a single copy or only in a partial form. In contrast, the IS*Kpn1*, IS*Kpn26*, IS*903B*, and IS*26* representatives are present in numerous copies, suggesting that they are currently active. The locations of these ISs vary: all IS*Kpn1* and most IS*Kpn26* copies are located in the chromosome, whereas most IS*26* copies are located in plasmids.

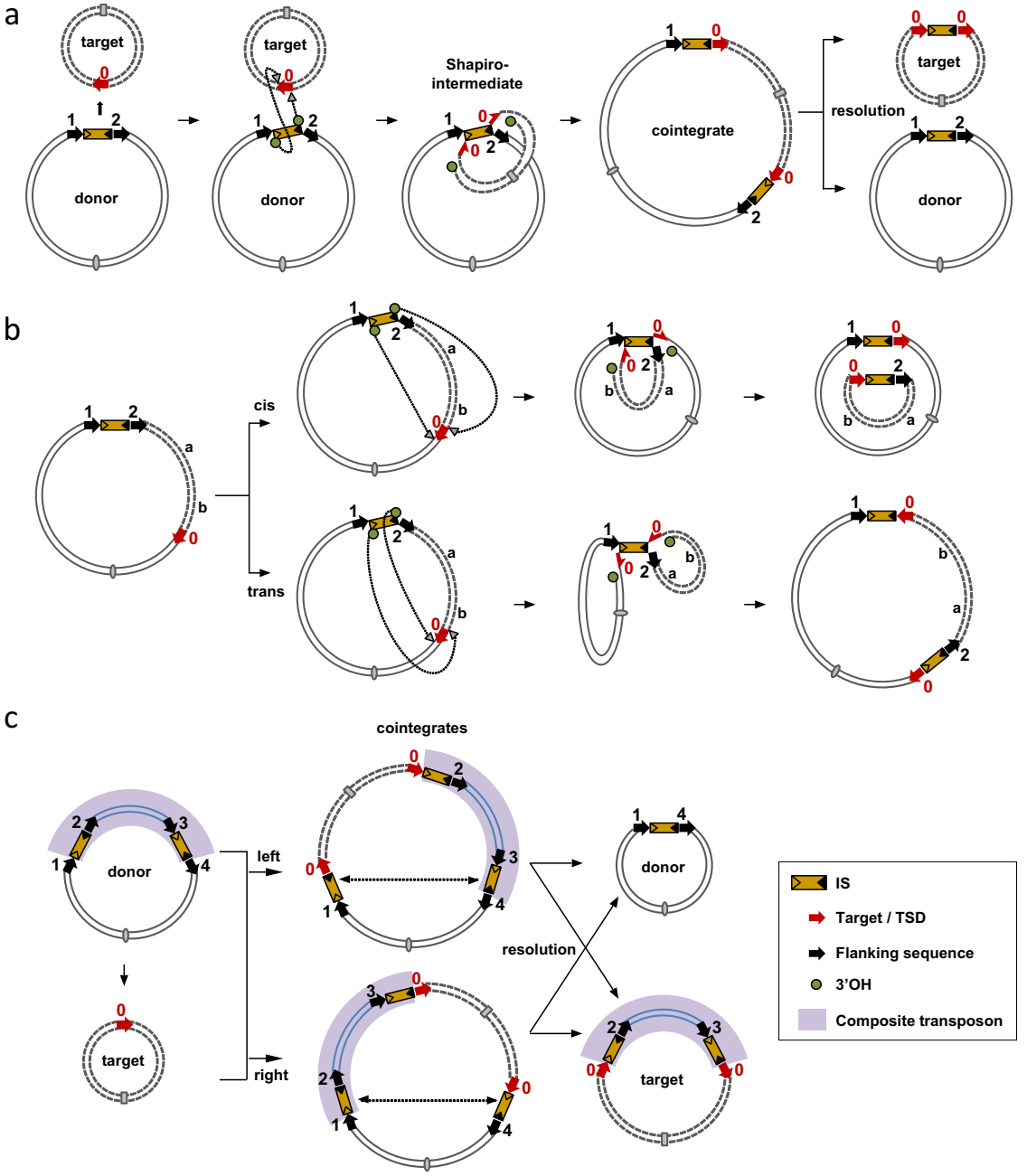

FIG 1 Schema of replicative transposition by an insertion sequence. (a) Intermolecular transposition. Cleavage at both TIRs of the IS results in nicks on both strands, generating 3′-OH groups which attack the target site, leading to the formation of a Shapiro intermediate. DNA replication generates the cointegrate containing a duplication of the IS and the target site. The cointegrate can be subsequently resolved into a plasmid identical to the original donor plasmid and a modified target plasmid carrying a copy of the IS flanked by TSDs arranged as direct repeats. (b) Intramolecular transposition. When an IS targets a target site in the same replicon, cleavages at both TIRs generate 3′-OH groups that can either attack the target site on the same strand (*cis*) or the opposite strand (*trans*). In the *cis* pathway, DNA between the IS and target site (dashed lines) becomes circularized and contains one IS copy and target site. In the *trans* pathway, DNA between IS and target site is instead inverted ("a b" becomes "b a"), bracketed by the original IS and a new copy in an inverted orientation. The target site is also duplicated but in inverted orientation, and each TSD is associated with one IS copy. (c) Intermolecular transposition of a composite transposon. A composite transposon consisting of two ISs (i.e., the DNA between TSDs 1 and 4 in the donor plasmid; purple) forms a cointegrate when either of the two flanking IS copies (left or right) transposes into a target plasmid (dashed lines), in which both the IS and the target site are duplicated. Resolution via homologous recombination yields a target plasmid carrying the whole composite transposon. IS, dark yellow rectangle; left and right TIRs, open and filled triangles; red arrows, targets for transposition; black arrows, potential TSDs from previous transposition events; gray oval or rectangle, origin of replication; filled green circle, 3′-OH. Arrowheads indicate orientation, and different numbers represent different sequences.

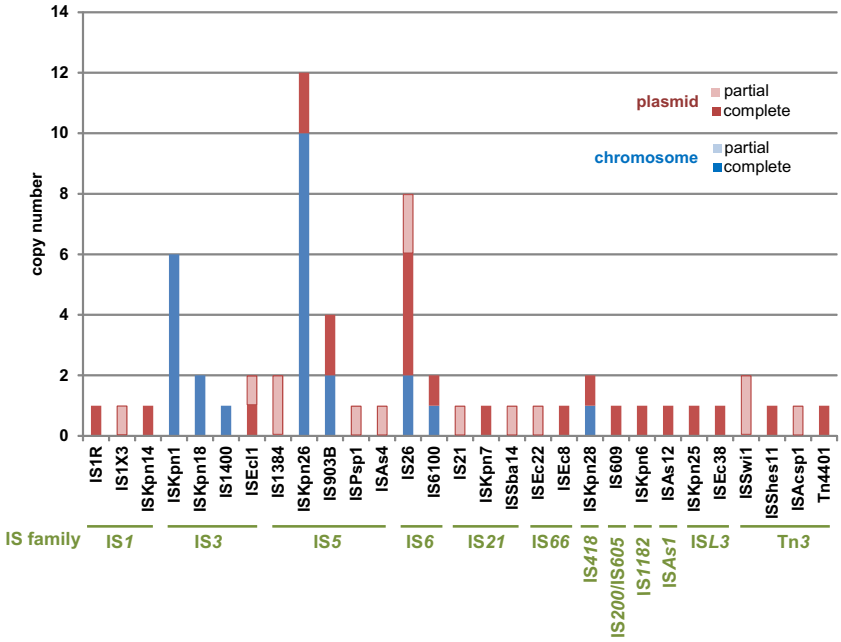

FIG 2 IS distribution in *Klebsiella pneumoniae* strain KPNIH1. IS copy numbers in the three plasmids and single chromosome are shown in red and blue, respectively. Pale color, partial IS; dark color, complete IS. IS family classifications (in green) were determined using ISfinder (https://www-is.biotoul.fr/).

The four IS families with multiple copies present in KPNIH1 are all reported to generate TSDs (ISfinder tool, at https://www-is.biotoul.fr/). TSDs are genomic signatures of transposition events carried out by DDE transposases, and their length is a characteristic property of the transposon, reflecting the particular structure of the transpososome (the molecular assembly composed of transposase and transposon ends that catalyzes transposition) (34). Among the IS families with multiple representatives, most chromosome-located ISs had directly abutted TSDs consistent with ISfinder records, yet those located on plasmids rarely had them (see Table S1 in the supplemental material). Interestingly, none of the six full IS*26* copies, including two found on the chromosome, had directly flanking TSDs. Lack of TSDs has also been recently reported in another U.S. isolate of *K. pneumoniae*, ATCC BAA-2146, which carries a bla_{NDM} carbapenemase gene and in which none of the 11 genomic IS*26* copies has TSDs (35). Although the lack of TSDs was attributed by those authors to homologous recombination following IS*26* transposition—and this is certainly possible—we also suspect that, since IS*26* transposes via a cointegrating mechanism, its movement might in itself generate copies without flanking TSDs.

Transposition mechanism of IS*26*. The evidence that IS*26* transposes by using replicative transposition and cointegrate formation is compelling. IS*26*, a member of the IS*6* insertion sequence family, is 820 bp long and has 14 bp TIRs (36). It is a component of Tn*2680*, in which two IS*26* copies bracket a 5-kb DNA segment, including a kanamycin resistance cassette on the *Proteus vulgaris* R plasmid Rtsl (36). Tn*2680* transposition was observed from Rtsl into the bacteriophage P1 genome in *Escherichia coli*, indicating that the two IS*26* copies were responsible for mobility (37). Movement of IS*26* from bacteriophage P1 to a pBR322 plasmid resulted in a cointegrate between the entire circular prophage genome and the target plasmid and generated an additional IS*26* copy. An 8-bp TSD was observed at the junction of IS*26* and the pBR322 insertion site. RecA was required to resolve the cointegrate to generate pBR322 containing the insertion (36, 38). Another study involving IS*15*, the product of insertion of an IS*26*-like element into itself, also demonstrated that a cointegrate is the transposition product and that resolution requires RecA-initiated homologous recombination (39).

IS*26* in the genomes of *Enterobacteriaceae* strains isolated in the NIHCC from 2011 to 2013. When we extended our ISsaga analysis of NIHCC clinical strains by annotating eight additional fully assembled genomes (for a total of 36 replicons [see Table S2 in the supplemental material]), IS*26* was observed to be the most predominant IS in the plasmids of these strains without any apparent species specificity. Although these eight genomes contained about 70 IS*26* copies, only 6 were chromosomal. Furthermore, not a single IS*26* copy exhibited flanking 8-bp TSDs (Table 1). On the other hand, we observed several identical flanking sequences at a single end of two different IS*26* copies (highlighted with the same color label in Table 1). These were identified not only in IS copies within the same replicon but also in different replicons. These identical sequences are therefore "tracers" and allow us to follow the replicative transposition of IS*26* and its consequences in the form of DNA rearrangements within these plasmids.

Intermolecular transposition events in the analyzed NIH Clinical Center strains. When we examined the pairs of identical 8-bp flanking sequences of different IS*26* copies in their genomic contexts, it became clear that these sequences provide evidence for replicative transposition. For example, we detected the hallmark of replicative transposition—cointegrate formation—when we compared pAAC154-a50 from the KPNIH1 strain and pKPN-819 from strain KPNIH24 from a patient hospitalized in NIHCC in July 2012. pAAC154-a50 is a small 15-kb plasmid that carries an IS*Swi1* transposon derivative of the Tn*3* family, IS*Swi1*-m2, which appears to be a descendant of IS*Swi1* (Fig. 3a). IS*Swi1* encodes a

TABLE 1 The 8-bp flanking sequences of IS*26* in nine genomes of *Enterobacteriaceae*[a]

Strain	Replicon	Left	Right	Strain	Replicon	Left	Right
KPNIH1	chromosome	GGTTTTCG	TTCTACGG	KONIH1	pKOX-137	GTCCGGAG	CTCGAAAA
		GAGATTGG	GGTTTTCG		pKOX-86d	GAGATTGG	TAAAATAA
	pKpQIL	CAAATTCG	AGGCAGGC			ATTGCTGA	ATCTTTCT
		CGCCGATG			pKPC-727	AAGCAGAC	AGAAAATA
	pAAC154-a50	TTGGAAAC	CTGATAAA			AGAAAATA	AGCTGCGG
	pKPN-498	CATCAGAG	GGATTGAA			GTTTCAGG	GGAAGGAA
			AATTTATG				
		ATTGTTTT	GGTCTTAA	ECNIH2	chromosome	CGGCAGAG	CCGCTGTA
					pKEC-39c	GTGGACTG	CGGTAGAT
KPNIH24	chromosome	GGTTCGCG	GGTTTTCG			CGGTAGAT	GTTTAACC
		GGTTCGCG	CTGATAAA			GAGATTGG	TAAAATAA
		GAGATTGG	CTGATAAA			ATTGCTGA	TTCTACGG
		GGTTTTCG	TTCTACGG			GCTGCGCT	CCCTGTAG
	pKPC-484	ATTGTTTT	GGATTGAA			GAAAAAAC	ATATCCTG
		CAAATTCG	AGGCAGGC			TTTTAAGC	TTTCGCCC
		TTGGAAAC	CTGATAAA			GCTTCCAC	GGGAGGAC
		CGATCAAC	CATTTATG		pKPC_272	GCTGTTGC	ATACTGTG
	pKPN-819	GGTTCGCG	ACAATGTG			AGAGTAGA	GATAGATC
		TTGGAAAC	CTGATAAA				
		CCGGCGTT	GGTTCGCG	ECNIH3	pENT-576	ATATTCCT	CGAATGAC
						CGAATGAC	AAAAAAAC
KPNIH27	pKEC-dc3	GTGGACTG	CGGTAGAT			CATCGCCG	TTCGCCCG
		CGGTAGAT	GTTTAACC		pENT-8a4	CTCATTGT	GGCTCTAC
		GAGATTGG	TAAAATAA			AGAGTAGA	AGTGAGTT
		ATTGCTGA	TTCTACGG			GGCTCTAC	AAGCTGGC
		GCTGCGCT	CCCTGTAG			CCGGCGTT	GCGCGACG
		GAAAAAAC	ATATCCTG			CTAAACAG	CGAGCCCC
		TTTTAAGC	TTTCGCCC			AAAGAAAT	TTCTGAAA
		GCTTCCAC	GGGAGGAC		pKPC_47e	GTGGACTG	ATATCCTG
	pKPN-068	ATTGTGAT	GCTCCATT			CGCTGGAC	
		CCGGCGTT	ATTGTGAT				
		CAATTTCT	CCCTGTTG	ECR091	pENT-08e	CTCATTGT	GGCTCTAC
		GCTCCATT	GTTCAACG		pKPC-47e	GTGGACTG	ATATCCTG
	pKPN-262	CAAAAAAC	AAGGCATT			CGCTGGAC	
		CTTACGCC	TAAGTCAG				
	pKPN-b0b	GAAAAGCA	CATTACGT	CFNIH1	chromosome	GTCCAGAC	CTTTCCTG
		TATTAGAA	ATTTTTCC		pKEC-a3c	GTGGACTG	CGGTAGAT
		ATTTTTCC	CACCGGAG			CGGTAGAT	GTTTAACC
						GAGATTGG	TAAAATAA
KPR0928	pKPN-294	TTGGAAAC	CTGATAAA			ATTGCTGA	TTCTACGG
	pKpQIL-531	CAAATTCG	AGGCAGGC			GCTGCGCT	CCCTGTAG
		CGCCGATG				GAAAAAAC	ATATCCTG
						TTTTAAGC	TTTCGCCC
						GCTTCCAC	GGGAGGAC

[a] Identical sequences are shown in the same color pattern. A blank implies a partial *IS26* copy.

transposase (*tnpA*), a resolvase (*tnpR*), and a TEM beta-lactamase (bla_{TEM-1}) and is present in plasmids pZM-3 from *Salmonella enterica* serovar Wien (40) and pNJST258N4 from *K. pneumoniae* NJST258_1 (41). IS*Swi1*-m2 most likely arose from another IS*Swi1* derivative, IS*Swi1*-m1 (also called Tn*1331* [42–44]), which is present in plasmid pNJST258N5 of *K. pneumoniae* NJST258_1 due to an IS*26* insertion into bla_{TEM-1} of parental IS*Swi1* and adjacent DNA deletion.

We noted that pAAC154-a50 is highly similar to a portion of pKPN-819 (Fig. 3b; see also Fig. S1a and b for further details). The indication that IS*26* is involved is that the sequence in pKPN-819 homologous to pAAC154-a50 is bordered by two IS*26* copies in the same orientation and is flanked by the same 8 bp at one side of each of the copies (labeled "0" in Fig. 3b). The most parsimonious explanation for the homology is that an IS*26* in the ancestor of pKPN-819 inserted into the target sequence "0" in pAAC154-a50 to generate an additional IS*26* copy and TSDs (Fig. 3b). Therefore, pKPN-819 appears to be an IS*26*-mediated cointegrate of two plasmids.

There are three IS*26* copies in pKPN-819, and it appears that two of these form an active composite transposon (Fig. 3b, highlighted in purple) able to insert into the *K. pneumoniae* genome. The IS*26* copies bracket a 5-kb DNA segment, and the proposed composite transposon can be perfectly aligned to a chromosomal locus in KPNIH24 (see Fig. S1c in the supplemental material). Furthermore, the 5-kb DNA segment is duplicated at the chromo-

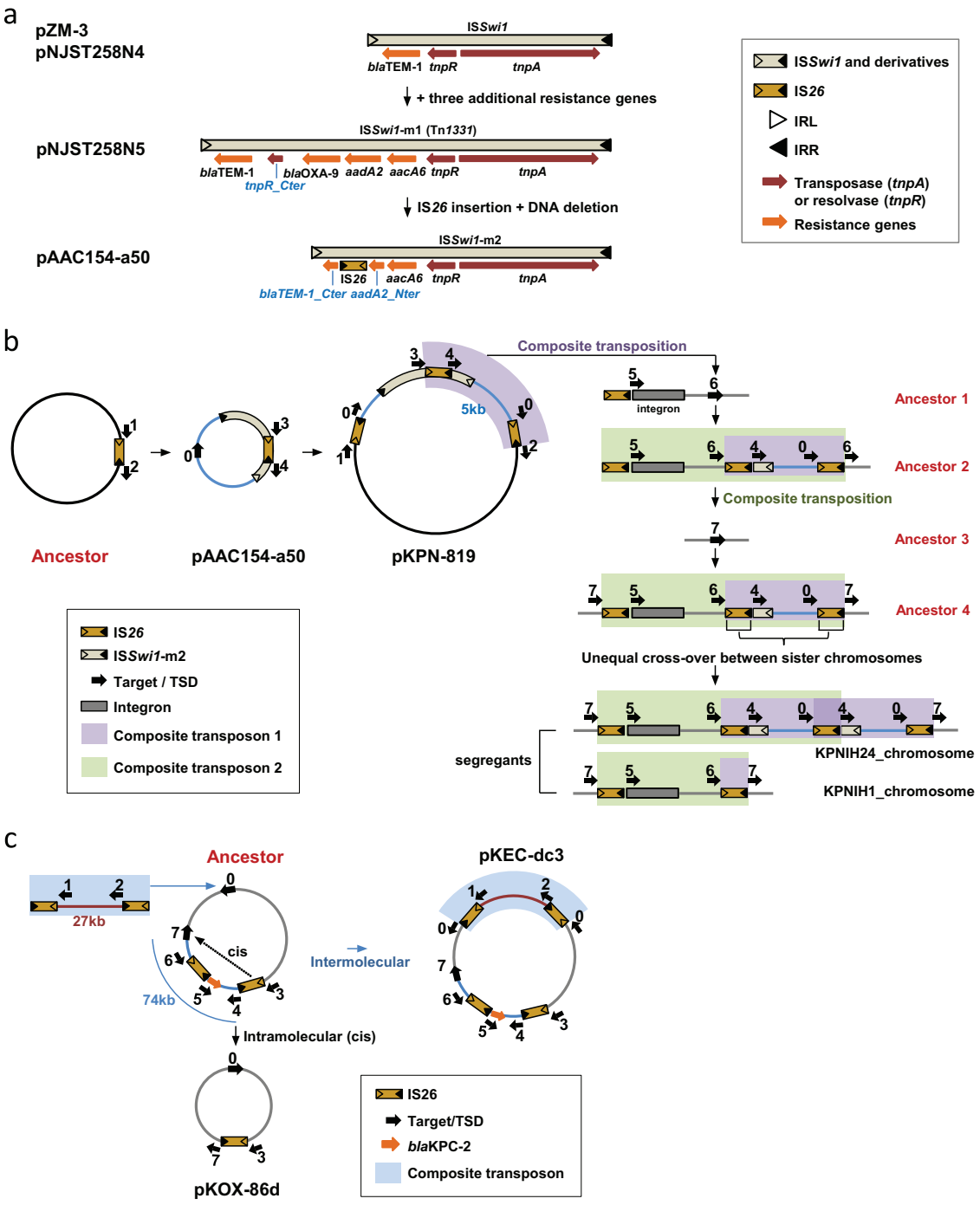

FIG 3 Examples of intermolecular transposition events observed within plasmids from NIHCC isolates. (a) Alignment of three IS*Swi1* derivatives from different plasmids. IS*Swi1* is shown in light beige, and IS*26* is shown in dark yellow. (b) Intermolecular transposition of IS*26*, leading to replicon fusion and composite transposition. The composite IS*26*-IS*26* transposon is highlighted with a purple background. A second composite transposon derived from ancestor 2 is highlighted with a green background. (c) IS*26*-mediated plasmid rearrangements. Transposition of a composite IS*26*-IS*26* transposon (background in blue) into an ancestor plasmid to generate pKEC-dc3 is shown. pKOX-86d is derived from an intramolecular IS*26* transposition (*cis* refers to the insertion orientation in Fig. 1b) in this ancestor. The sequences of the different flanking sequences 0 to 7 are listed in Fig. S2 in the supplemental material. Plasmid names are shown in black. Hypothetical ancestor plasmids are labeled in red. ISs are shown as rectangles (different colors represent different ISs), with triangles representing the TIRs. Full-length genes are labeled in black, and truncated genes are labeled in blue. Left TIR, open triangle; right TIR, closed triangle; integron, gray rectangle; transposase or resolvase (*tnpA* and *tnpR*), dark red arrows; resistance genes, dark orange arrows. The different flanking 8-bp sequences of IS*26* are labeled with different numbers, and their sequences are listed in figures in the supplemental material. Arrowheads indicate the orientation. These symbols and color patterns are applied in all subsequent figures.

somal locus as tandem repeats separated by an IS*26* and bracketed by two additional IS*26* copies. This remarkable DNA structure suggests that the chromosomal locus is likely the result of IS*26* composite transposition (Fig. 1c) followed by nonreciprocal recombination at the IS*26* copies (unequal crossover [45, 46]) (see Fig. S1d). Further supporting evidence for this proposed pathway is the absence of the tandem repeat in the chromosomal locus of KPNIH1. Instead, we found an IS*26* flanked by octanucleotides 6 and 7 (Fig. 3b) that flank the tandem repeats in KPNIH24. Therefore, one possibility (Fig. 3b) is that the KPNIH1 and KPNIH24 chromosomes are two segregants of an unequal crossover event that occurred at the chromosomal IS*26* composite transposon and resulted in a duplication and a deletion segregant. Alternatively, recombination between two IS*26*s in KPNIH24 could have given rise to the pattern of IS*26*s seen in KPNIH1.

If the composite transposon in KPNIH24 arose via the pathway in Fig. 1c, TSDs would be expected at the target replicon, whereas the flanking sequences 6 and 7 in KPNIH24 are different. However, sequence 7 is observed elsewhere in the KPNIH24 and KPNIH1 chromosomes as the flanking 8-bp sequence of another IS*26* upstream of an integron. This sequence pattern thus suggests a second composite transposon, embracing the original composite transposon much like nested Russian dolls, transposed prior to unequal crossover.

There have also been several IS*26*-mediated plasmid rearrangements in the pKEC plasmid series from the NIHCC CPE isolates (Fig. 3c). Three isolates—*K. pneumoniae* KPNIH27, *C. freundii* CFNIH1, and *E. cloacae* ECNIH2—carry highly similar pKEC series plasmids (see Fig. S2a in the supplemental material). In all three, we found a 27-kb DNA segment bracketed by two IS*26* copies in the same orientation (Fig. 3c, highlighted in blue; for simplicity, only pKEC-dc3 from KPNIH27 is shown) and with identical 8-bp TSDs arranged as direct repeats at one side of each copy ("0"). This 27-kb DNA segment encodes many conjugative transfer and plasmid stability proteins, the highly mutagenic polymerase UmuDC, as well as anti-restriction-modification proteins. This entire region is absent in the otherwise-highly homologous pKOX-86d from *K. oxytocia* strain KONH1 (Fig. 3c, bottom left), but the "0" 8-bp sequence is present. This suggests either intermolecular IS*26* transposition resulting in replicon fusion (pathway in Fig. 1a, if the 27-kb region contained a replication origin) or transposition by an IS*26*-IS*26* composite transposon followed by resolution (pathway in Fig. 1c). Another difference between these two plasmids is a 74-kb region in pKEC-dc3—missing in pKOX-86d—that is next to an IS*26* copy (Fig. 3c; see also Fig. S2b in the supplemental material). This is probably due to IS*26*-mediated deletion in the ancestral plasmid upon intramolecular transposition in *cis*.

Intramolecular transposition leading to DNA inversions. Matched pairs of 8-bp sequences flanking different IS*26* copies within the same plasmid provide evidence for intramolecular replicative transposition in *trans*, leading to DNA inversion; four examples are shown in Fig. 4. Plasmid pKPN-068 from KPNIH27 (Fig. 4a, right) carries multiple antibiotic resistance genes for two beta-lactamases ($bla_{\text{TEM-1}}$ and $bla_{\text{SHV-12}}$), two aminoglycoside modification enzymes (*ant2* and *cata1*), and a quinolone resistance gene (*qnrB*). This 80-kb plasmid has seven ISs, including four IS*26* copies, one copy of IS*Ecp1* (an IS*1380* family member), an IS*Acsp1*-like IS (a Tn*3* family member), and one IS*Swi1* copy that is disrupted into three segments (pA, pB, and pC). This disruption pattern is likely the result of IS*Acsp1* insertion and IS*26*-mediated intramolecular transposition giving rise to inversion. The first disruption is clearly made by insertion of the IS*Acsp1*-like IS (shown in green) into the *ISSwi*1 transposase gene (*tnpA*) (see Fig. S3a in the supplemental material) as indicated by the flanking 5-bp TSDs characteristic of IS*Acsp1* (shown as unfilled arrows in pKPN-068). The second disruption is an inversion of pA relative to the rest of IS*Swi1* and its separation from pB by ~30 kb. In pKPN-068, two copies of IS*26* adjacent to the pA and pB segments are in opposite orientation and are flanked by identical 8-bp repeats inverted relative to each other ("0" in red). This arrangement is consistent with intramolecular transposition in *trans* of a single IS*26* into a "0" target site in an assumed hypothetical ancestor at the junction of the pB and pA segments of IS*Swi1*, resulting in the inversion of the intervening DNA.

The hypothetical ancestor plasmid shown on the left in Fig. 4a would have carried three copies of IS*26*, two with identical 8-bp flanking DNAs ("1") and in the same orientation. This was probably a consequence of intermolecular transposition of an IS*26*-IS*26* composite transposon (grey background) carrying the $bla_{\text{SHV-2}}$, *qnrB*, and *cata1* resistance genes, followed by resolution (pathway in Fig. 1c). These steps would have brought two antibiotic resistance determinants, $bla_{\text{TEM-1}}$ and *ant2*, previously 30 kb apart, into the immediate neighborhood of $bla_{\text{SHV-12}}$, thereby augmenting an existing resistance cluster. This illustrates an IS*26*-mediated mechanism of resistance cluster formation different from and independent of integron- or IS*CR*-mediated rearrangements.

We also found a pattern of diagnostic repeats within the plasmid pKPC-727 from *K. oxytoca* KONIH1 (Fig. 4b), where two IS*26* copies located next to a split conjugal transfer operon include two 8-bp flanking sequences ("0" in red) in an inverted configuration. This is consistent with IS*26*-mediated transposition resulting in intramolecular inversion, leading to the remodeling of the operon. We found a similar pattern of IS*26* elements and flanking repeats in plasmid pENT-576 of *E. cloacae* ECNIH3 (Fig. 4c), where a 16-kb DNA region that included ABC transporter and urease genes was probably inverted due to intramolecular IS*26* transposition into an intergenic region.

Examples of IS*26*-mediated intramolecular plasmid rearrangements extend beyond the NIHCC isolates. We detected similar IS*26*-related patterns in a series of pKpQIL-like carbapenemase bla_{KPC3}-carrying plasmids embedded in strains of *K. pneumoniae*, *E. coli*, and *Enterobacter aerogenes* isolates from New York and New Jersey hospitals (47). Those authors noted an inversion bracketed by two IS*26* copies in pKpQIL-234, but only one of these IS*26* copies is present in pKpQIL-10 (Fig. 4d; see also Fig. S4 in the supplemental material). This was interpreted as an insertion of a second IS*26* followed by subsequent DNA inversion between two IS*26* elements. However, it seems more likely that pKpQIL-234 is a product of a one-step intramolecular transposition in pKpQIL-10 by IS*26* into the target "0" octanucleotide. Those authors also noted another rearrangement in pKpQIL-Ec (Fig. 4d, bottom), a deletion of 8 kb of DNA starting from the right flank of IS*Kpn14* (an IS*1* family element). We suggest that this is consistent with intramolecular replicative transposition in *cis* of IS*Kpn14* resulting in DNA deletion, as IS*1* family members also appear to use replicative transposition (48).

IS*Swi1* is a hot spot for transposition. As shown in Fig. 1b, one possible outcome of intramolecular transposition is DNA seg-

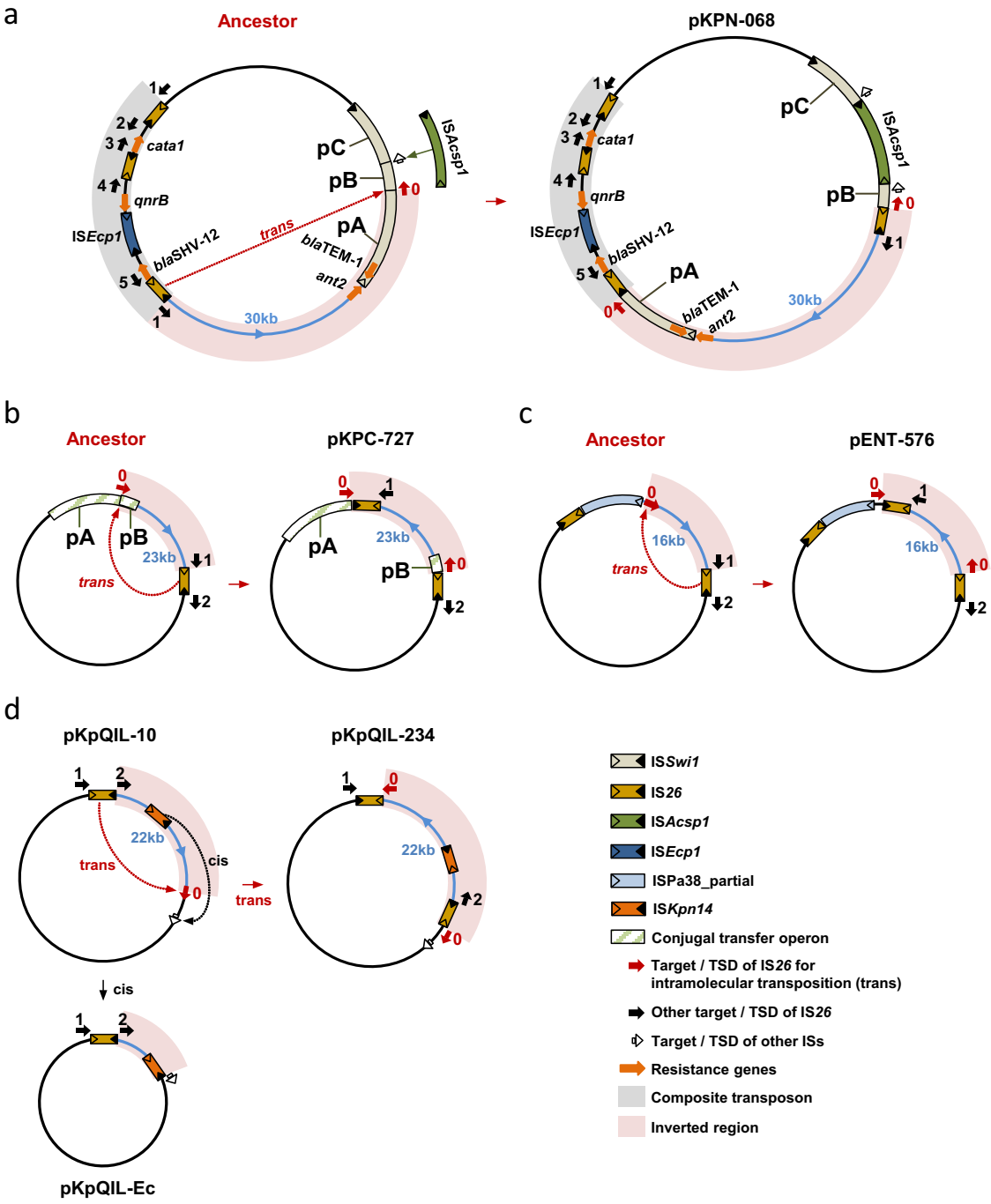

FIG 4 Intramolecular transposition (*trans*) resulting in DNA inversion. Cases of deduced intramolecular transposition events within hospital isolated strains are shown. Inverted regions are highlighted with a pink background. The *cis* and *trans* notations indicate the insertion orientation, as shown in Fig. 1b. Different ISs are shown in different colors. (a) Intramolecular transposition leading to pKPN-068. pA, pB, and pC represent the disrupted segments of IS*Swi*1. Further details, including sequences of the target and the flanking sequences 1 to 5 can be found in Fig. S3a in the supplemental material. (b) Intramolecular transposition leading to pKPC-727. pA and pB represent the partial DNAs constituting the conjugal transfer operon; further details, including the sequences of the target and the flanking 1 and 2 sequences can be found in Fig. S3b. (c) Intramolecular transposition leading to pENT-576. The sequences of target and flanking sequences 1 and 2 can be found in Fig. S3c. (d) Intramolecular transposition events among a series of pKpQIL plasmids isolated from New York and New Jersey hospitals. Further details, including sequences 1 and 2, can be found in Fig. S4 in the supplemental material.

ment deletion without generating telltale abutted TSDs. Deletion events are difficult to identify unless replicons both before and after the transposition event have been detected. Although this is rare, deletion can be inferred when a partial gene is left behind. As over half the 8-bp flanking sequences in Table 1 are unique, we believe some of these are due to transposition-mediated deletion.

Figure 5a depicts the many ways that the 16 copies of the IS*Swi1* locus in different plasmids in the analyzed NIHCC genomes have

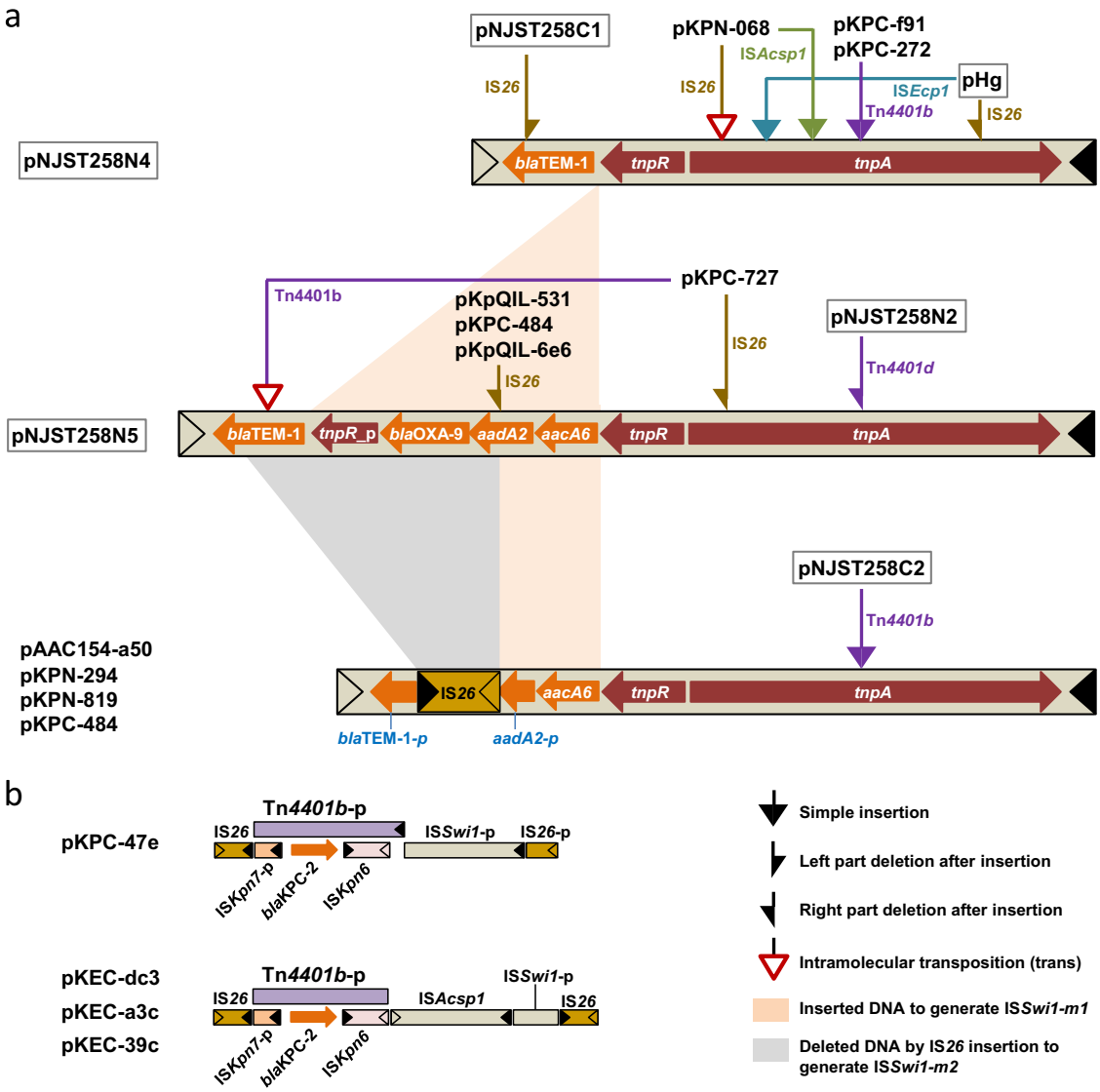

FIG 5 IS*Swi1* derivatives and IS*26*-mediated gene deletions. (a) IS*Swi1* serves as an insertion hot spot for IS*26* and other ISs. Plasmids isolated from hospitals other than the NIHCC are boxed by rectangles in gray, and the detailed genetic context of different IS*Swi1* derivatives in these plasmids is shown in Fig. S5 in the supplemental material. Three IS*Swi1* derivatives and the corresponding plasmid names are shown. (Top) Intact IS*Swi1*; (middle) IS*Swi1*-m1; (bottom) IS*Swi1*-m2. Inserted DNA to generate IS*Swi1-m1* is highlighted in light orange. Deleted DNA by IS*26* insertion to generate IS*Swi1-m2* is highlighted in gray. Arrows show the other disruptions, by either IS*26* or other ISs in the indicated plasmids; note that the IS*26* insertions often disrupt the antibiotic resistance gene locus (aminoglycoside modification genes or $bla_{\text{TEM-1}}$). Complete filled arrows indicate simple insertions, and half arrows indicate that one part of IS*Swi1* was deleted after IS insertion. Red empty arrows indicate disruption was caused by intramolecular transposition in *trans* leading to DNA inversion. (b) Tn*4401* truncations by IS26s and other ISs.

been disrupted. IS*Swi1* is also found in hospital-isolated strains ATCC BAA-2146 (35), NJST258_1, and NJST258_2 (41) and, again, most of the copies are disrupted. The disruption pattern is usually different among the different copies but, remarkably, it is always caused by other transposable elements, such as IS*26* and Tn*4401* (interestingly, the three isoforms of Tn*4401* we observed [a, b, and d] give rise to the highest bla_{KPC} expression among five reported Tn*4401* isoforms [49]). Thus, it appears that IS*Swi1* might be a hot spot targeted by these elements. The mechanism responsible for this targeting is not clear, but as IS*Swi1* carries one $bla_{\text{TEM-1}}$ gene and Tn*4401* carries carbapenemase gene bla_{KPC}, the result is often the clustering of these genes. When the disruptions are caused by IS*26*, deletions are often observed at the antibiotic resistance gene locus. We suspect that these observations indicate ongoing plasmid streamlining that continues even after the acquisition of resistance determinants.

Tn*4401* is partially deleted by IS*26* in pKPC-47e and the pKEC series plasmids (Fig. 5b) and is flanked by two IS*26* copies, suggesting the possibility of a functional composite transposon. If this were the case, this composite transposon could be a new vehicle for bla_{KPC} dissemination. As there are many more copies of IS*26* than of Tn*4401*, we speculate that this composite element may have higher transposition activity than the original Tn*4401* and could be an example of IS*26*-mediated mobility enhancement of a critical resistance determinant.

Intramolecular transposition by Tn*4401* creates an antibiotic resistance gene cluster. Within the NIHCC strains, replicative transposition is not restricted to IS*26*; Tn*4401* also appears to

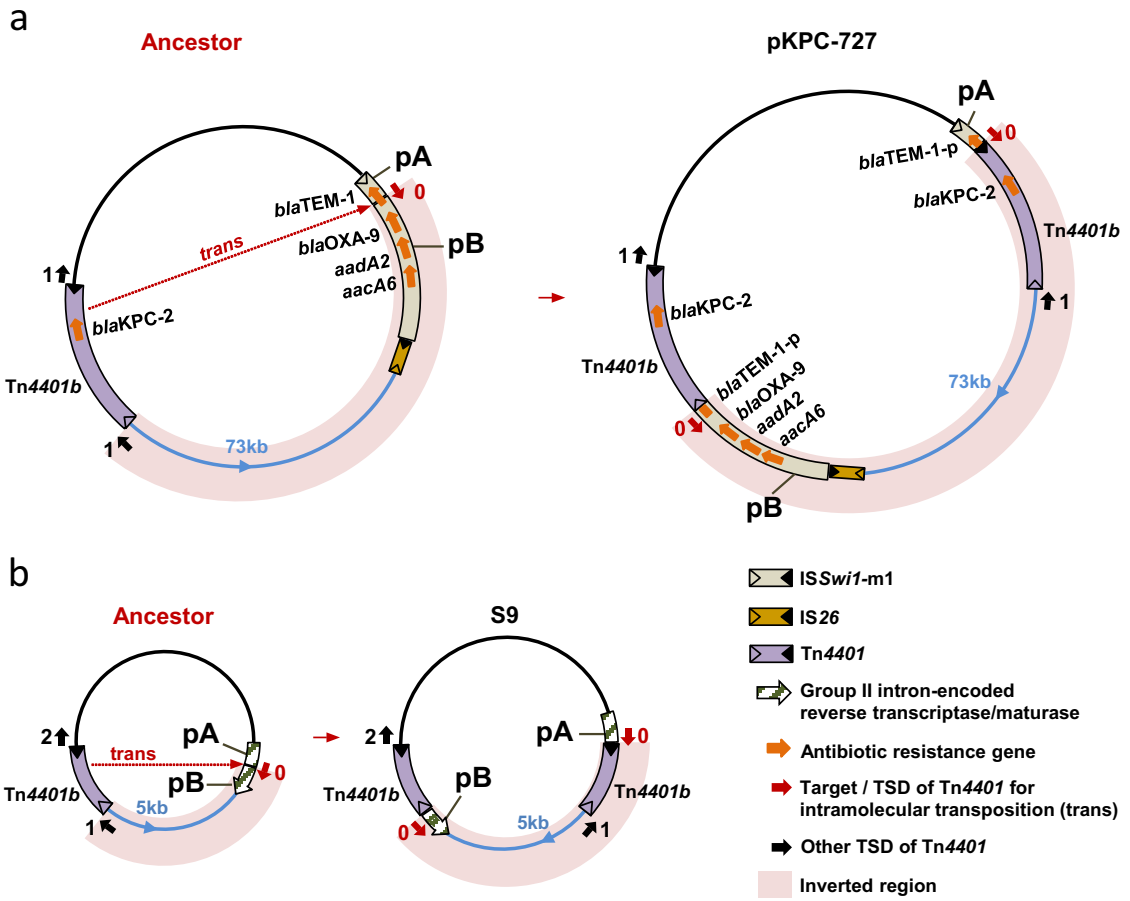

FIG 6 Examples of intramolecular transposition of Tn*4401*. (a) Deduced intramolecular transposition event leading to pKPC-727. pA and pB represent DNA segments which partially reconstitute IS*Swi1-m1*. Reversed regions are highlighted with a pink background. The notation *trans* indicates the insertion orientation shown in Fig. 1b. Further details, including the sequences 0 and 1 are shown in Fig. S6 in the supplemental material. (b) Deduced intramolecular transposition event leading to a plasmid from *K. pneumoniae* S9. Details, including the sequences 0, 1, and 2 are shown in Fig. S7 in the supplemental material.

play a role in plasmid reorganization. Although Tn*3* family elements such as Tn*4401* are known to use replicative transposition (27), they are believed to be constrained, as they often display transposition immunity (50), a regulatory mechanism preventing the insertion of a second copy into a replicon already containing a copy of the same transposable element. Consistent with this, most replicons analyzed here carry only a single copy of Tn*4401*. pKPC-727 from *K. oxytoca* KONIH1 is an exception (Fig. 6a, right). It carries two inverted Tn*4401* copies, 73 kb apart. Neither copy has a characteristic 5-bp abutted TSD (51). Interestingly, the two Tn*4401* copies are located next to two partial IS*Swi1* elements (pA and pB) which are inverted and flanked by the same 5-bp sequence ("0" in red), an expected length for Tn*3* family transposons and present in an inverted orientation. The DNA structures in pKPC-727 are thus consistent with a single step of Tn*4401* intramolecular transposition in *trans*. Tn*4401* in the hypothetical ancestor would have had perfect pentanucleotide TSDs ("1"), indicating a conventional intermolecular transposition event as shown in Fig. 1a. Although this intramolecular transposition event disrupted $bla_{\text{TEM-1}}$, it duplicated $bla_{\text{KPC-2}}$ and simultaneously brought several other antibiotic resistance determinants close to one $bla_{\text{KPC-2}}$ copy, thereby forming a resistance cluster.

Tn*4401* also appears to have carried out intramolecular transposition within a plasmid from a *K. pneumoniae* isolate S9 from a New York City Hospital (Fig. 6b) (8). S9 carries two Tn*4401* elements in inverted orientation and without detectable abutted TSDs. As group II intron fragments were detected at both ends of Tn*4401*, those authors hypothesized that Tn*4401* duplication was due to self-splicing by the intron. However, we suggest that the duplication is much more likely due to the intramolecular transposition of Tn*4401* into a target site within the group II intron.

DISCUSSION

We have carried out a global analysis of bacterial insertion sequences in the genomes of nine CPE isolates from the NIH Clinical Center collected from the 2011 outbreak to the end of 2013. As these genomes have been fully sequenced with long reads and with high coverage (30), we were able to precisely analyze their mobile elements using ISsaga. Analyzing multiple isolates was key to facilitating the identification of DNA rearrangements within the bacterial genomes. Although the chromosomes of these strains appear relatively stable, the plasmids where most of the resistance determinants reside are dynamic and display a large degree of interstrain variation. The presence of a highly active IS*26* is consistent with previous reports on other clinical isolates (52–54), but here we have been able to trace its impact by taking into account its

replicative transposition mechanism and using its expected TSD patterns as guides. In several cases, our findings allow us to trace not only precisely how resistance plasmids are reorganized by this mobile element, but also the temporal relationship between related plasmids.

High activity of IS*26* in the analyzed strains is suggested by its high copy number. Although target site duplication is usually considered a fingerprint of transposition, within the genomes analyzed we did not find a single IS*26* copy flanked by a matching pair of TSDs. However, the lack of flanking TSDs cannot, by itself, be interpreted as lack of recent IS*26* activity or be arbitrarily assumed to indicate an unconventional transposition mechanism, as intermolecular replicative transposition without resolution does not generate abutted TSDs flanking a single IS copy. Also, the various possible consequences of intramolecular transposition must be taken into account. Nevertheless, the lack of flanking TSDs does imply that either intermolecular transposition by IS*26* occurs at a very low frequency or that the resolution step of this process is very inefficient. As resolution is believed to be dependent on RecA-mediated homologous recombination (39), it seems unlikely that this host machinery is limiting.

It is clear that intramolecular transposition in *trans* that results in DNA inversion occurs fairly often, leading to large DNA rearrangements. This pathway generates not only additional IS*26* copies but also an inverted DNA segment bracketed between two copies of IS*26* which in principle can act as a composite transposon, mobilizing the DNA segment between them in subsequent transposition events.

Recently, Hall and coworkers investigated IS*26* transposition in RecA-deficient *E. coli* (55) and reported that the presence of an IS*26* in a target plasmid greatly increased the frequency of additional IS*26* insertions into that plasmid. Using a PCR-based assay, they found replicon fusions between IS*26*-containing donor and target plasmids, but the resulting cointegrate did not contain an additional copy of IS*26* as would be expected for replicative transposition. Despite the absence of RecA, the observed cointegrate was structurally equivalent to the recombination product between the two IS*26* copies in the donor and target plasmids. Although we cannot exclude RecA-independent mechanisms of template switching (56), the transposase of IS*26* might be responsible for this homology searching and pairing. One possibility is that two IS ends from different IS copies in separate replicons form a synapse in the same transpososome (see Fig. S8 in the supplemental material), a process that resembles transposition of a composite transposon which involves TIRs from two different IS copies located in the same replicon. Strand exchange followed by replication would thus result in the observed replicon fusion. Such in *trans* synapsis thus allows the possibility of "self-targeted" insertion. A similar mechanism has been invoked to explain "targeted" insertion of IS*3* and IS*30* family members into TIRs (57–59). This process would be expected to be independent of RecA but, in the case of the IS*3* family member IS*911*, it is known to depend on the RecG helicase (60). However, in these cases, the strand exchange reaction is expected to produce tandem copies of the element, due to their different transposition mechanisms. IS*911* is known to use a copy-and-paste mechanism (21), whereas IS*30* has been proposed to use both direct insertion and cointegrate formation (59). It would be certainly interesting to investigate experimentally whether such events occur and how often, or if IS*26* in some circumstances transposes via mechanisms different from replicative transposition.

As we observed, intramolecular transposition can lead to an increase in the IS*26* copy number within a genome (*trans* [Fig. 1b]), but if the opposite strand of the target site is used (*cis* [Fig. 1b]), the outcome is DNA deletion. More than half the 70 IS*26* copies in the nine genomes we analyzed have flanking 8-bp sequences not associated with any of the other IS*26* copies. These may well be the result of intramolecular transposition, implying that it is relatively common, as might be expected if there is no strand preference during the strand transfer step of transposition. Intramolecular transposition accompanied by deletion is clearly a useful mechanism for keeping bacterial genome size under control during the onslaught of exogenous DNA acquired, for instance, through horizontal transfer. As the horizontal acquisition of resistance determinants is clearly important, it is possible that IS*26*-mediated genome trimming plays a significant role in this process, enabling the maintenance of a reasonable genome size and therefore viability (of course, we do not exclude the possibility that other ISs or transposons can accomplish the same thing). Deletions could also eliminate certain antigens to allow escape from the host immunity system, as seen in the *Bordetellae*, where IS activity resulted in a genome size reduction concomitant with gene inactivation (61). We have also seen that *cis*-mediated disruptions and deletions can occur within antibiotic resistance genes (Fig. 5). Most of these involve aminoglycoside modification enzymes or narrow-spectrum beta-lactamases. Perhaps this is yet another sign of genome optimization to increase fitness by eliminating "narrow-spectrum" resistance genes no longer needed once a broader-spectrum resistance gene in the same class has been acquired (for example, elimination of an ESBL gene once a carbapenemase gene is acquired).

In addition to playing a role in the organization of resistance genes, we observed that transposition of IS*26* remodeled some plasmid-borne genes involved in conjugational transfer and plasmid stability, for instance. As this may have conflicting effects on plasmid maintenance and stability, some mechanisms would be needed to rectify conflicts. Furthermore, replicative transposition could lead to plasmid fusion or plasmid integration into the chromosome in the absence of homologous sequences. For example, this has been observed in *Yersinia pseudotuberculosis* due to the activity of the IS*6* family member IS*Yps1* (62).

Replicative transposition is not restricted to the IS*6* family, and other presumed replicative mobile elements, such as Tn*4401* (Tn*3* family), IS*903B* (IS*5* family), and IS*Kpn14* (IS*1* family), also exist (27, 48, 63). In contrast to the IS*26* examples identified here, we found several copies of these family members flanked by direct repeats of the expected lengths, suggesting intermolecular transposition followed by resolution. While Tn*3* family members such as Tn*4401* often encode their own resolvase (64), IS*903B* and IS*Kpn14* do not (see the ISfinder database). Perhaps resolution of cointegrates generated by these elements relies on RecA-mediated homologous recombination, as suggested for IS*26*, or these IS elements may be able to transpose by simple insertion (65, 66).

Results of our analysis suggest that IS*26* prefers to transpose within plasmids rather than into the chromosome. This is not a general feature of ISs, as there are many other ISs present at very high copy number in the chromosomes analyzed here. For example, up to 17 copies of IS*Kpn26* were observed in certain chromosomes (e.g., KONIH1 [data not shown]). As the chromosomal IS

copies tend to have conserved flanking TSDs more frequently than those in the plasmids, it seems that most chromosomal transposition events employ simple transposition mechanisms, such as cut-and-paste or copy out-and-paste (26), which might be better tolerated, as they cause less overall disruption than replicative transposition. Although there are several chromosomal IS*26* copies within the nine analyzed NIHCC strains, these are exclusively located in a prophage-carrying region and may have transposed into the phage genome before its integration into the host chromosome. We also believe that selection bias might be involved, which would constrain the IS*26* copy number in the chromosome so as to avoid potentially harmful deletions. However, we cannot exclude the possibility of replicative transposition within chromosomes: IS*903B* may have deleted a 15-kb region from the KPNIH1 chromosome (region 2716602 to 2731997 in CP008827) that is rich in transporter genes and whose loss results in the absence of this region in NJST258_1 (data not shown). This is consistent with the reported intramolecular replicative transposition activity of IS*903B* (63).

The availability of whole-genome sequencing carried out with new tools, such as Pacific Biosciences RS II SMRT technology, makes possible the unambiguous identification of mobile elements, and we expect many more clinical isolates to be sequenced. Combining these sequence data with IS annotation tools like ISsaga provides robust and reliable data from which to derive mechanistic information about the activity of mobile elements in their native hosts and therefore to understand the evolution of their host genomes. This in turn may open up a new avenue to trace IS dissemination and host adaptation. In particular, following transposition events of mobile elements such as IS*26* is a way to understand the evolution of resistance plasmids, revealing changes that may optimize the viability of the pathogen in a hospital environment. The ability to follow these events clearly establishes directionality as the rearrangements generated by replicative transposition events are not reversible.

It is not obvious why IS*26*, among the large variety of mobile genetic elements, gained such prevalence in the resistance plasmids in the examined enterobacterial strains. One possibility is a founder effect, in which the first IS*26* element inserted in proximity to resistance genes, which allowed it a foothold with these particular genes. Alternatively, there might be some currently unknown host factor involved in IS*26* mobility, making it highly active and perhaps influencing the choice toward intramolecular pathways. By extending these investigations to other clinically isolated pathogens, we hope to uncover common features of mobile genetic element-driven resistance plasmid reorganizations and to understand their effects in a mechanistic framework.

MATERIALS AND METHODS

Genomic sequences of the nine NIHCC isolates as well as other clinical isolates analyzed in this paper are from the NCBI public database (see Table S2 in the supplemental material for a list of the analyzed genomes). IS annotation for these genomes was carried out with the ISsaga program (insertion sequence semi-automatic genome annotation; at http://issaga-.biotoul.fr/ISsaga/issaga_index.php) (33). SnapGene 2.5 was used to visualize the annotation results. Mauve 2.3.1 was used to perform comparative genome alignments.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.00762-15/-/DCSupplemental.

Figure S1, EPS file, 2 MB.
Figure S2, PDF file, 0.8 MB.
Figure S3, EPS file, 1.5 MB.
Figure S4, EPS file, 2.3 MB.
Figure S5, EPS file, 1.7 MB.
Figure S6, EPS file, 1.3 MB.
Figure S7, EPS file, 1.8 MB.
Figure S8, EPS file, 0.9 MB.
Table S1, DOCX file, 0.02 MB.
Table S2, DOCX file, 0.02 MB.

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S.H., A.B.H., M.C., J.P.D., and F.D. contributed to project design and cowrote the paper. S.H. carried out genome IS annotations and data analysis. P.S. performed the IS annotations of the NJST258_1 and NJST258_2 genomes. A.M.V. adapted the ISsaga annotation tool to allow batch genome analysis.

We declare no competing financial interests.

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