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# Emergence and Evolution of Multidrug-Resistant *Klebsiella pneumoniae* with both *bla*<sub>KPC</sub> and *bla*<sub>CTX-M</sub> Integrated in the Chromosome

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**ABSTRACT** The extended-spectrum- $\beta$ -lactamase (ESBL)- and *Klebsiella pneumoniae* carbapenemase (KPC)-producing *Enterobacteriaceae* represent serious and urgent threats to public health. In a retrospective study of multidrug-resistant *K. pneumoniae*, we identified three clinical isolates, CN1, CR14, and NY9, carrying both *bla*<sub>CTX-M</sub> and *bla*<sub>KPC</sub> genes. The complete genomes of these three *K. pneumoniae* isolates were *de novo* assembled by using both short- and long-read whole-genome sequencing. In CR14 and NY9, *bla*<sub>CTX-M</sub> and *bla*<sub>KPC</sub> were carried on two different plasmids. In contrast, CN1 had one copy of *bla*<sub>KPC-2</sub> and three copies of *bla*<sub>CTX-M-15</sub> integrated in the chromosome, for which the *bla*<sub>CTX-M-15</sub> genes were linked to an insertion sequence, *ISEcp1*, whereas the *bla*<sub>KPC-2</sub> gene was in the context of a *Tn4401a* transposition unit conjugated with a *PsP3*-like prophage. Intriguingly, downstream of the *Tn4401a-bla*<sub>KPC-2</sub>-prophage genomic island, CN1 also carried a clustered regularly interspaced short palindromic repeat (CRISPR)-*cas* array with four spacers targeting a variety of *K. pneumoniae* plasmids harboring antimicrobial resistance genes. Comparative genomic analysis revealed that there were two subtypes of type I-E CRISPR-*cas* in *K. pneumoniae* strains and suggested that the evolving CRISPR-*cas*, with its acquired novel spacer, induced the mobilization of antimicrobial resistance genes from plasmids into the chromosome. The integration and dissemination of multiple copies of *bla*<sub>CTX-M</sub> and *bla*<sub>KPC</sub> from plasmids to chromosome depicts the complex pandemic scenario of multidrug-resistant *K. pneumoniae*. Additionally, the implications from this study also raise concerns for the application of a CRISPR-*cas* strategy against antimicrobial resistance.

**KEYWORDS** CRISPR-Cas, CTX-M, carbapenem-resistance, chromosomal beta-lactamases, *Klebsiella pneumoniae*, *bla*<sub>KPC</sub>

The acquisition and spread of  $\beta$ -lactamases among bacterial species has led to increased resistance to  $\beta$ -lactam antibiotics. Such resistance constitutes an urgent threat to patient management and public health (1). In the mid-1980s, extended-spectrum  $\beta$ -lactamases (ESBLs) were first detected. To date, the Centers for Disease Control and Prevention (CDC) estimate that ESBL-producing *Enterobacteriaceae* cause at least 26,000 health care-associated infections and 1,700 deaths per year in the United States (1). Among the many types of ESBLs reported (2, 3), CTX-M enzymes are the most prevalent (3). Although the dominant variants of CTX-M are geographically different,

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CTX-M-15 and CTX-M-14 are the most common ones identified worldwide (3). Moreover, the genes encoding CTX-M enzymes ( $bla_{CTX-M}$ ) can be horizontally mobilized by various genetic elements. An insertion sequence (IS), *ISEcp1*, is often associated with the region upstream of  $bla_{CTX-M}$  genes and provides promoters to regulate  $bla_{CTX-M}$  expression (3).

Carbapenem-resistant *Enterobacteriaceae* (CRE) have also emerged and rapidly spread worldwide. Each year, approximately 600 deaths result from CRE infections in the United States (1). Carbapenemases are a diverse group of  $\beta$ -lactamases that are active against oxyimino-cephalosporins and cephamycins as well as carbapenems. Among these, *Klebsiella pneumoniae* carbapenemase (KPC) is currently the most prevalent (4), with KPC-2 and KPC-3 being the most common variants in the United States (5). Genetically, the gene encoding KPC ( $bla_{KPC}$ ) has been identified within a  $\sim 10$ -kb Tn3-family transposon, Tn4401 (6). The mobile genetic element Tn4401 possesses a transposase gene (*tnpA*) and a resolvase gene (*tnpR*), in addition to two unrelated ISs, ISKpn6 and ISKpn7.

*Klebsiella pneumoniae* is an opportunistic pathogen causing severe infections in patients, especially neonates, the elderly, and immunocompromised individuals (7). Emergence and facilitated spread of multidrug-resistant (MDR) *K. pneumoniae*, such as the ESBL (8–10)- and KPC-producing strains (4, 5, 11), are often responsible for the failure of antibiotic treatment (1). *K. pneumoniae* isolates carrying both  $bla_{CTX-M}$  and  $bla_{KPC}$  were first reported in New York City in 2013 (12). However, the genetic structure of these coexisting  $\beta$ -lactamases and mechanism of transmission are unclear. Limited data suggest that  $bla_{KPC}$  and  $bla_{CTX-M}$  are most commonly carried by promiscuous plasmids that readily transfer among *Enterobacteriaceae* species (13–16). *K. pneumoniae* strains containing multiple copies of  $\beta$ -lactamase genes in the chromosome, especially the same  $\beta$ -lactamase gene, are rare.

We previously examined *K. pneumoniae* clinical isolates collected between 2005 and 2012 at the Westchester Medical Center (WMC), New York, for the presence of ESBL and KPC  $\beta$ -lactamase genes, including  $bla_{TEM}$ ,  $bla_{SHV}$ ,  $bla_{CTX-M}$ , and  $bla_{KPC}$ , by PCR and DNA sequencing (17). In a subsequent study using next-generation sequencing, we analyzed additional *K. pneumoniae* isolates collected at the WMC between 2012 and 2014 and at two New York City hospitals collected in 2013. We obtained the complete genome sequences of three *K. pneumoniae* clinical isolates harboring both  $bla_{CTX-M}$  and  $bla_{KPC}$ . In this report, we present the comparative genomic analysis data that reveal the emergence and potential evolution of *K. pneumoniae* strains carrying multiple chromosomal  $\beta$ -lactamase genes. We also investigated the molecular mechanism potentially associated with such chromosomal integration of antibiotic resistance genes.

(Part of this study was presented in a poster at the Association of Molecular Pathology 2016 Annual Meeting, Charlotte, NC, 10 to 12 November 2016.)

## RESULTS

**Whole-genome sequences of *K. pneumoniae* CN1, NY9, and CR14.** Three *K. pneumoniae* isolates, CN1, NY9, and CR14, were confirmed to carry both  $bla_{CTX-M}$  and  $bla_{KPC}$  genes. CN1 and NY9 were recovered from two patients in New York City in 2013, whereas CR14 was cultured from a WMC patient in 2012. To obtain complete genome sequence for structural analysis, we employed both short- and long-read sequencing and conducted *de novo* assembly. The genomic characteristics based on final complete genome sequences of these three isolates are summarized in Table 1. Briefly, *K. pneumoniae* isolates CN1, NY9, and CR14 belong to ST392, ST340, and ST258, with corresponding genome sizes of 5.44, 5.88, and 6.06 Mb, respectively. Each strain contains a circular chromosome, with a GC content of about 57.5%, similar to those of other *K. pneumoniae* strains publicly available. A comparison of their chromosomal architectures is shown in Fig. 1A. Compared to CN1 and NY9, CR14 had a relatively larger genome, with a large insertion ( $\sim 171$ -kb) and a large inversion ( $\sim 245$ -kb) in the chromosome. While CR14 and NY9 contained five and six circular plasmids, respectively, CN1 had only two circular plasmids.

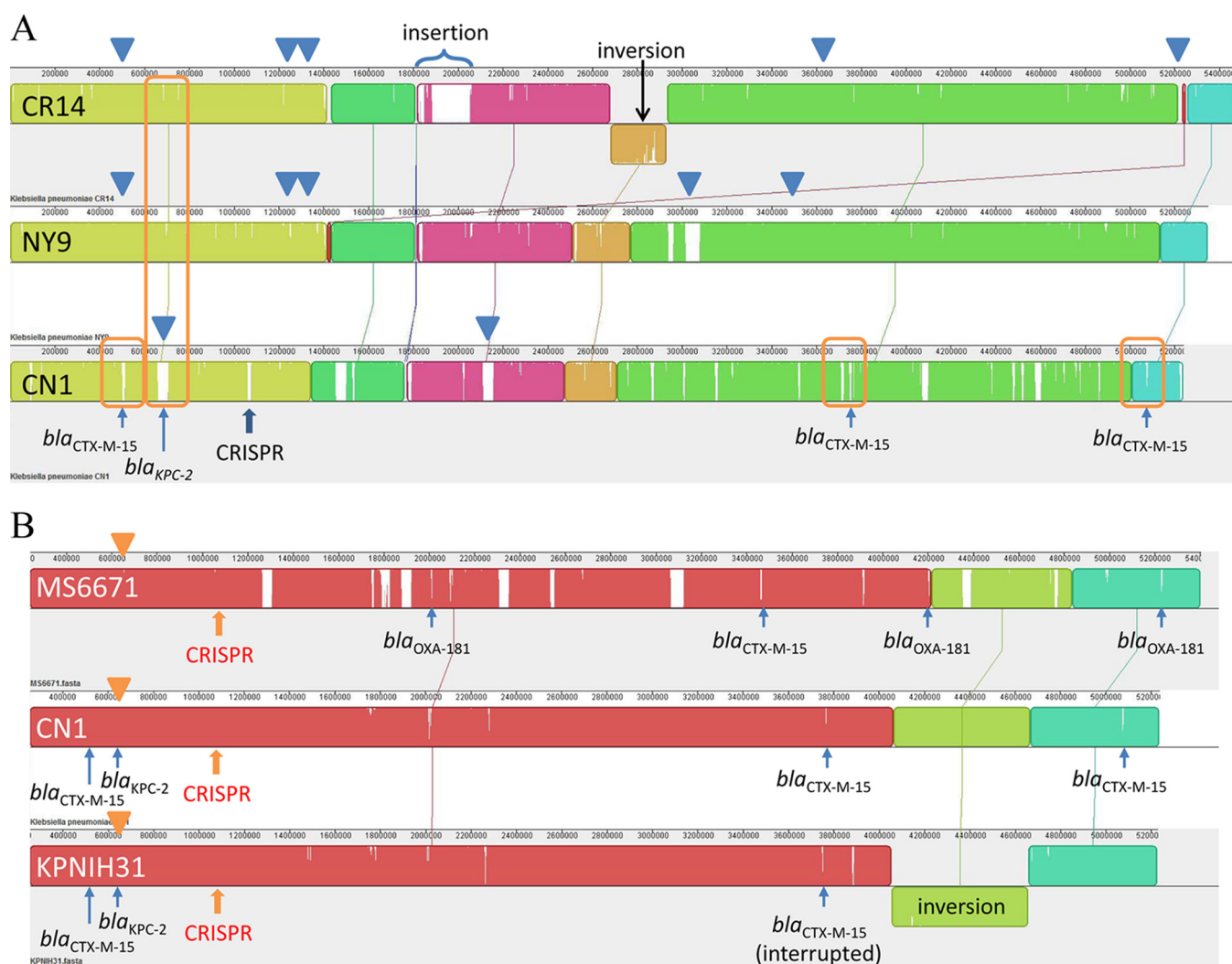
**TABLE 1** Genomic characteristics of *K. pneumoniae* clinical isolates CR14, NY9, and CN1 with both *bla*<sub>CTX-M</sub> and *bla*<sub>KPC</sub> genes

| Genomic characteristic <sup>a</sup> | Finding for strain:  |  |                                    |
|-------------------------------------|--|--|------------------------------------|
|                                     | CR14   | NY9  | CN1                                |
| MLST                                | ST258  | ST340  | ST392                              |
| Chromosome size (Mb)                | 5.47   | 5.35   | 5.24                               |
| GC (%)                              | 57.3   | 57.5   | 57.5                               |
| No. of coding genes                 | 5,884  | 5,776  | 5,224                              |
| RNAs (no.)                          |  |  |                                    |
| rRNA                                | 25   | 25   | 25                                 |
| tRNA                                | 85   | 88   | 87                                 |
| Plasmids                            |  |  |                                    |
| No. of plasmids                     | 5  | 6  | 2                                  |
| Plasmid (size)                      | pCR14_1 (203 kb)<br>pCR14_2 (154 kb)<br>pCR14_3 (116 kb)<br>pCR14_4 (110 kb)<br>pCR14_5 (9 kb) | pNY9_1 (199 kb)<br>pNY9_2 (140 kb)<br>pNY9_3 (89 kb)<br>pNY9_4 (53 kb)<br>pNY9_5 (39 kb)<br>pNY9_6 (11 kb) | pCN1_1 (183 kb)<br>pCN1_2 (15 kb)  |
| ESBL and KPC $\beta$ -lactamases    |  |  |                                    |
| <i>bla</i> <sub>CTX-M</sub>         |  |  |                                    |
| Allele (no. of copies)              | <i>bla</i> <sub>CTX-M-2</sub> (1)  | <i>bla</i> <sub>CTX-M-15</sub> (1)   | <i>bla</i> <sub>CTX-M-15</sub> (3) |
| Genetic location                    | pCR14_2  | pNY9_3   | Chromosome                         |
| <i>bla</i> <sub>KPC</sub>           |  |  |                                    |
| Allele (no. of copies)              | <i>bla</i> <sub>KPC-2</sub> (1)  | <i>bla</i> <sub>KPC-3</sub> (1)  | <i>bla</i> <sub>KPC-2</sub> (1)    |
| Genetic location                    | pCR14_3  | pNY9_2   | Chromosome                         |
| CRISPR on chromosome                | No   | No   | Yes                                |

<sup>a</sup>Refer to supplemental materials for a complete list of antimicrobial resistance genes, including other non-ESBL and KPC  $\beta$ -lactamases (Table S1) and antimicrobial susceptibility profiles (Table S2) of these three clinical isolates. MLST, multilocus sequence typing; CRISPR: clustered regularly interspaced short palindromic repeats.

From the whole-genome sequences, we identified multiple antimicrobial resistance genes, in either chromosomes or plasmids (see Table S1 in the supplemental material). Among these, multiple  $\beta$ -lactamase genes were identified in each of the three *K. pneumoniae* isolates: CR14 carried *bla*<sub>CTX-M-2</sub>, *bla*<sub>OXA-2</sub>, and *bla*<sub>TEM-1B</sub> in plasmid pCR14\_2 (154 kb) with an A/C2 replicon, as well as *bla*<sub>KPC-2</sub>, *bla*<sub>OXA-9</sub>, and *bla*<sub>TEM-1A</sub> in another plasmid, pCR14\_3 (116 kb), with two replicons, FIB and FIIC. Isolate NY9 carried *bla*<sub>SHV-11</sub> in the chromosome; *bla*<sub>KPC-3</sub>, *bla*<sub>OXA-9</sub>, and *bla*<sub>TEM-1A</sub> in plasmid pNY9\_2 (140 kb) with two replicons, FIA and FII; and *bla*<sub>CTX-M-15</sub>, *bla*<sub>OXA-1</sub>, and *bla*<sub>TEM-1B</sub> in another plasmid, pNY9\_3 (89 kb), with an FII replicon. Interestingly, both *bla*<sub>KPC</sub>-carrying plasmids contained two replicons, whereas both *bla*<sub>CTX-M</sub>-carrying plasmids only had one. More remarkably, CN1 carried three copies of *bla*<sub>CTX-M-15</sub>, in addition to one copy of *bla*<sub>SHV-11</sub> and *bla*<sub>KPC-2</sub>, in the chromosome (Fig. 1A), with only *bla*<sub>OXA-1</sub> in plasmid pCN1\_1 (183 kb).

**Chromosomal *bla*<sub>CTX-M-15</sub> and *bla*<sub>KPC-2</sub> in *K. pneumoniae*.** The coexistence of chromosomal *bla*<sub>CTX-M-15</sub> and *bla*<sub>KPC-2</sub> is rare and raised our interest. In search of homologous genome sequence similar to that of the CN1 chromosome, we identified *K. pneumoniae* isolate KPNIH31, which was recovered from a patient in the United States in 2013 (15). Both CN1 and KPNIH31 belonged to ST392, with the same GC content of 57.5%. These two isolates shared a small, 15.1-kb plasmid (pCN1\_2 or pAAC154-a9e). Their largest plasmid (pCN1\_1, 183 kb; and pKPN-c22, 179 kb) and chromosome (CN1, 5.24 Mb; and KPNIH31, 5.23 Mb) also had similar sizes (Table S3) and showed high nucleotide sequence identity of more than 99% over the coverage of more than 98% of chromosomal and plasmid sequences. However, KPNIH31 carried an extra plasmid, pKPN-852, in which no antimicrobial resistance gene was found. Genome sequence alignment demonstrated that compared to CN1, KPNIH31 had a 60-kb



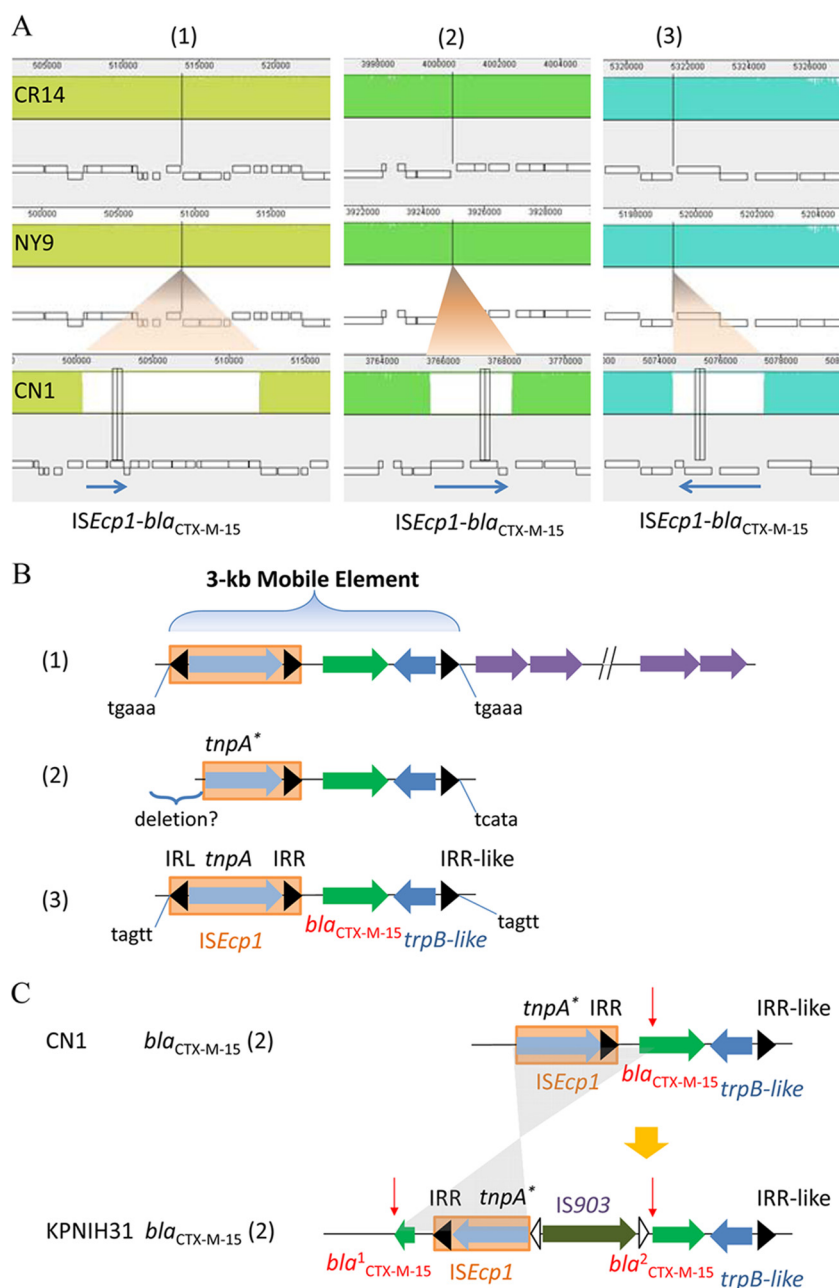
**FIG 1** Overview of whole-chromosome sequence comparisons using progressive Mauve. (A) Chromosome comparison of *K. pneumoniae* isolates CR14, NY9, and CN1. Indicated are locations of  $\beta$ -lactamase genes (*bla*<sub>CTX-M-15</sub> and *bla*<sub>KPC-2</sub>), prophages (▼), and the CRISPR site, as well as a large inversion in the CR14 chromosome. The boxed areas and CRISPR site are shown in more detail in later figures. (B) Chromosome comparison of *K. pneumoniae* isolates MS6671, CN1, and KPNIH31. Indicated are multiple  $\beta$ -lactamase genes, *bla*<sub>CTX-M-15</sub>, *bla*<sub>KPC-2</sub>, and *bla*<sub>OXA-181</sub>, and a large inversion in the KPNIH31 chromosome. A homologous Psp3-like prophage (▼) and a similar CRISPR-cas array (labeled in red font) are shared by these chromosomes. For a better view of sequence alignment and chromosomal architecture in comparison, genome sequences of KPNIH31 (2013, United States) and MS6671 (2014, United Arab Emirates) are adjusted to *dnaA* as the first gene.

inversion in the chromosome (Fig. 1B). Interestingly, KPNIH31 also harbored both *bla*<sub>CTX-M-15</sub> and *bla*<sub>KPC-2</sub> in the chromosome.

Insertions of three copies of *bla*<sub>CTX-M-15</sub> in the CN1 chromosome were found to be associated with the same mobile element, *ISEcp1*, despite their different insertion sizes (Fig. 2A and B). This feature was consistent with transposition events of *bla*<sub>CTX-M-15</sub> in plasmids, as seen in plasmid pNY9\_3 of isolate NY9. In the context of these *bla*<sub>CTX-M-15</sub> genes, we identified a 5-bp target site duplication (TSD) sequence for each *ISEcp1* copy (Fig. 2B). Interestingly, the second *ISEcp1*-*bla*<sub>CTX-M-15</sub> copy in isolate CN1 was not complete, with the left TSD eliminated and the *ISEcp1* transposase truncated at the 5' end (Fig. 2B), suggesting a sequence deletion occurred after *ISEcp1*-*bla*<sub>CTX-M-15</sub> insertion. Unlike isolate CN1, KPNIH31 had only one complete copy of the *ISEcp1*-*bla*<sub>CTX-M-15</sub> unit. While the third *ISEcp1*-*bla*<sub>CTX-M-15</sub> unit was totally eliminated in KPNIH31, the second *ISEcp1*-*bla*<sub>CTX-M-15</sub> unit was also truncated and further interrupted by an insertion of IS903 in the middle of *bla*<sub>CTX-M-15</sub>, followed by an inversion (Fig. 2C).

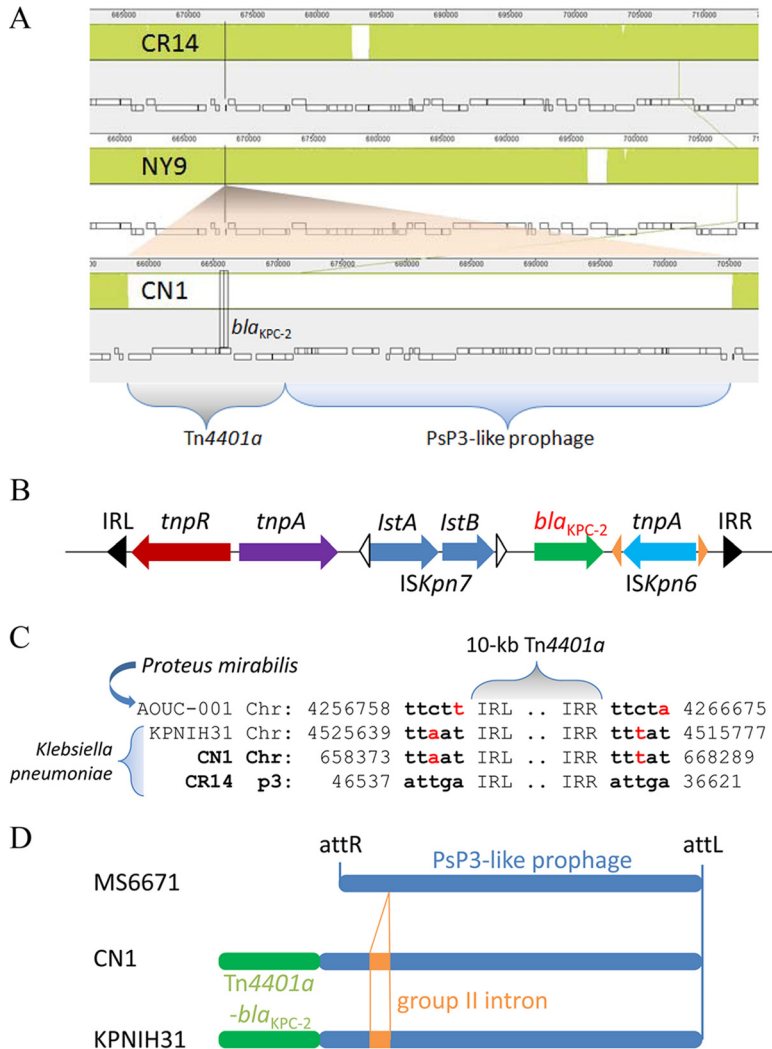
In addition to the three insertions of *ISEcp1*-*bla*<sub>CTX-M-15</sub>, the CN1 chromosome retained an insertion of Tn4401a-*bla*<sub>KPC-2</sub> in a genomic island (Fig. 3A). There were only





**FIG 2** Integration of *bla*<sub>CTX-M-15</sub> in the chromosome by mobile element *ISEcp1*. (A) A zoomed-in view of three *bla*<sub>CTX-M-15</sub> genes inserted in the CN1 chromosome, compared with chromosomes of CR14 and NY9. The blue arrow indicates a mobile element of *ISEcp1-bla*<sub>CTX-M-15</sub>. (B) A schematic view of genetic elements in 3-kb mobile elements of *ISEcp1-bla*<sub>CTX-M-15</sub> in the CN1 chromosome. IRL, left inverted repeat; IRR, right inverted repeat; *tnpA*, gene encoding transposase A type; *trpB*-like, gene encoding tryptophan synthase subunit beta-like protein. The target site duplication (TSD) sequences are shown at the ends. The suggested deletion was marked on the second copy of *ISEcp1-bla*<sub>CTX-M-15</sub>. (C) A schematic view of the second copies of *ISEcp1-bla*<sub>CTX-M-15</sub> in CN1 and KPN1H31 chromosomes. The second copy of *ISEcp1-bla*<sub>CTX-M-15</sub> in KPN1H31 was disrupted by IS903 insertion. Indicated with a red arrow is the interruption site. Insertion of IS903 interrupts the *bla*<sub>CTX-M-15</sub> gene in the middle and further inverts a truncated part of *ISEcp1* (shaded gray). IRR, right inverted repeat.

two additional *Enterobacteriaceae* strains, the aforementioned *K. pneumoniae* KPN1H31 (15) and *Proteus mirabilis* AOU-001, isolated from Italy in 2013 (18), which carry the chromosomal *bla*<sub>KPC-2</sub> gene. The *bla*<sub>KPC-2</sub> gene in these chromosomes shared the same mobile genetic element as the plasmids, a 10-kb Tn4401a flanked by two 38-bp imperfect but conserved inverted repeats (IRL and IRR) (Fig. 3B). However, we observed

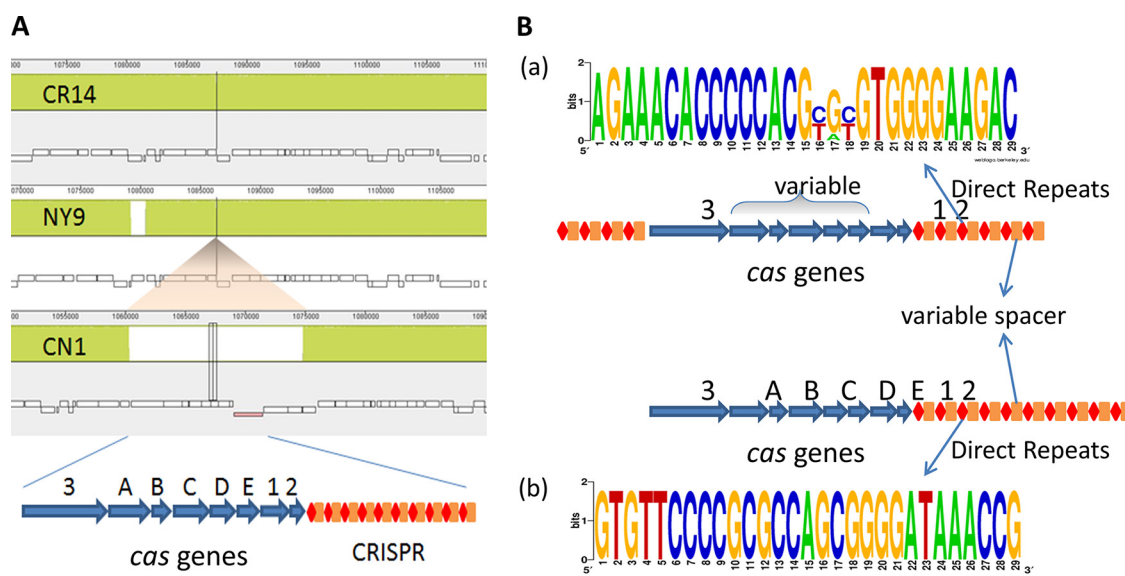


**FIG 3** Integration of *bla*<sub>KPC-2</sub> in the chromosome by a mobile element, Tn4401a. (A) A zoomed-in view of *bla*<sub>KPC-2</sub> insertion in the CN1 chromosome by a 10-kb Tn4401a conjugated with a 43-kb PsP3-like prophage and compared with chromosomes of CR14 and NY9. (B) A schematic view of genetic elements in a 10-kb mobile element of Tn4401a-*bla*<sub>KPC-2</sub>, including transposase *tnpA*, resolvase *tnpR*, and insertion sequences *ISKpn6* and *ISKpn7*. IRL, left inverted repeat; IRR, right inverted repeat. (C) Comparison of target site duplication (TSD) sequences of Tn4401a-*bla*<sub>KPC-2</sub> transposon in plasmid pCR14\_3 (p3) with those in chromosomes of *K. pneumoniae* CN1 and KPNIH31 (2013, United States) and *Proteus mirabilis* AOUC-001 (2013, Italy). The numbers shown indicate nucleotide positions at either chromosome or plasmid. (D) A schematic view of PsP3-like prophage region in *K. pneumoniae* MS6671, CN1, and KPNIH31. MS6671 contained both 32-bp phage attachment sites (attL and attR) at the ends, whereas CN1 and KPNIH31 lost attR at the site of Tn4401a-*bla*<sub>KPC-2</sub> (green bar) conjugating with the PsP3-like prophage (blue bar). Additionally, CN1 and KPNIH31 had identical sequences in the region, and both contained an insertion of group II intron (orange bar) in the prophage.

that the paired 5-bp TSD sequences of transposon Tn4401a-*bla*<sub>KPC-2</sub> in chromosomes did not match perfectly those in plasmids, such as in pCR14\_3 of isolate CR14 (Fig. 3C). This feature of chromosomal Tn4401a-*bla*<sub>KPC-2</sub> differed from that of chromosomal *ISEcp1-bla*<sub>CTX-M-15</sub>.

Both CN1 and KPNIH31 had an identical genomic island, with an aforementioned 10-kb Tn4401a-*bla*<sub>KPC-2</sub> transposon conjugated to a 43-kb *Enterobacteriaceae* phage PsP3-like prophage. The whole prophage region contained about 50 genes coding for one tRNA and 49 proteins. Among these, 44 proteins (90%) had high similarity to phage proteins, and 30 proteins (61%) were highly homologous to the proteins in bacteriophage PsP3, isolated from *Salmonella*. Sequence homology search further revealed that this prophage region could also be found in the chromosomes of *K. pneumoniae*





**FIG 4** CRISPR-*cas* system in *K. pneumoniae*. (A) A zoomed-in view of CRISPR-*cas* array insertion in the CN1 chromosome, showing the *cas* operon of eight *cas* genes followed by sequence repeats with spacers. (B) A schematic view of two subtypes (a and b) of the CRISPR-*cas* system in *K. pneumoniae*. The consensus sequences were generated in WebLogo in analysis of direct repeats collected from 22 CRISPR arrays and grouped into two subtypes. Numbers and sizes of direct repeats, spacers, and *cas* genes are not to scale.

MS6671 (2014, United Arab Emirates), SKGH01 (2015, United Arab Emirates), and AATZP (2014, United States) but without a 2-kb insertion of a retroelement containing a group II intron reverse transcriptase (Fig. 3D). Notably, these chromosomes contained both 32-bp phage attachment sites (*attL* and *attR*) with a consensus sequence of TGGGTTT GAACCAACGACCAAGCGATTATGAG. In contrast, with the Tn4401a-*bla*<sub>KPC-2</sub> transposon integrated into the chromosomes of CN1 and KPNIH31, one phage attachment site (*attR*) was eliminated (Fig. 3D).

**Association of CRISPR with chromosomal integration of multiple  $\beta$ -lactamase genes.** About 360 kb downstream of the prophage site in CN1 chromosome (1069045..1071450), we identified a CRISPR locus with a consensus direct repeat (DR), CGGTTTATCCCCGCTGGCGCGGGGAACAC (29 bp in length), and 39 spacers. Proximate to and upstream of the CRISPR sequences, there was a cluster of eight CRISPR-associated *cas* genes (*cas* operon) (Fig. 4A). We then conducted a survey of the CRISPR-*cas* system on 64 complete genomes of *K. pneumoniae* publicly available at the NCBI genome database (as of May 2016, including our own three genomes). We identified 22 (34%) strains harboring the CRISPR-*cas* array in the chromosome. All of the CRISPR-*cas* arrays in *K. pneumoniae* strains belonged to type I-E in the classification of CRISPR-*cas* systems (19, 20). On the basis of their distinguishable features, we further classified them into two distinctive subtypes, a and b (Table S4 and Fig. 4B). In subtype a, two CRISPRs were separated by the *cas* operon; the DR sequences were either 28 or 29 bp in length and had consensus with 2 to 3 variables in the middle; also, the *cas* operons were variable in *cas* genes and their order, occasionally with a transposase gene integrated in the middle (Fig. 4B). In contrast, subtype b was quite consistent and stable, containing the same DR (29 bp in length) and *cas* operon with only one CRISPR downstream of the *cas* operon (Fig. 4B). In both subtypes a and b, the number and sequence of spacers varied in each strain, which might represent its own established immune system.

Eight of 22 (36.4%) *K. pneumoniae* isolates harboring the CRISPR-*cas* array carried multiple  $\beta$ -lactamase genes in their chromosomes. In contrast, multiple chromosomal  $\beta$ -lactamase genes were detected in only 3 of 42 (7.1%) *K. pneumoniae* strains without CRISPR-*cas* in their chromosomes ( $P = 0.0164$ ) (Table S5).

There were five *K. pneumoniae* strains containing both the PsP3-like prophage site and subtype b CRISPR-Cas array in the chromosome, i.e., CN1, KPNIH31, AATZP,

MS6671, and SKGH01. Intriguingly, four of them carried multiple  $\beta$ -lactamase genes in the chromosome. Instead of multiple copies of *bla*<sub>CTX-M-15</sub> observed in CN1 and KPNIH31, three copies of *bla*<sub>OXA-181</sub> (a carbapenem-hydrolyzing oxacillinase) were carried by MS6671 and SKGH01, in addition to one *bla*<sub>CTX-M-15</sub> copy and one *bla*<sub>SHV-11</sub> copy, in the chromosome (Table S4). Strain AATZP was exceptional, carrying only one chromosomal *bla*<sub>SHV-11</sub> copy but *bla*<sub>OXA-9</sub> and *bla*<sub>TEM-1A</sub> in one plasmid (pKPN-041) and *bla*<sub>CTX-M-15</sub>, *bla*<sub>OXA-1</sub>, and *bla*<sub>NDM-1</sub> in another (pNDM-1fa) (21). Upon investigating spacer sequences in these five *K. pneumoniae* strains, we found that they shared identical spacers, except that four “old” spacers in the 3′ extremities of CRISPR arrays were eliminated from CN1 and KPNIH31 (Table S6). Sequence homology search further demonstrated that four spacers (1, 28, 29, and 38 in CN1) targeted a variety of *K. pneumoniae* plasmids, of which the “newest” one (1; GAGCAGGCACCCGCCGAACGACGAAGAGCGC) at the 5′ extremities of CRISPR arrays targeted more than 200 plasmids. Some of these plasmids were hit more than one time, and most of them carried antimicrobial resistance genes, including *bla*<sub>KPC-2</sub> (e.g., p628-KPC) and *bla*<sub>CTX-M-15</sub> (e.g., pKpN01-CTX). Notably, of 43 spacers in AATZP, none were homologous to any sequence of its plasmid pKPN-1fa carrying *bla*<sub>CTX-M-15</sub>.

## DISCUSSION

To date, the ESBL and KPC  $\beta$ -lactamase genes *bla*<sub>CTX-M</sub> and *bla*<sub>KPC</sub> have been reported to be carried on separate plasmids in most *Enterobacteriaceae* strains (13–16), just as we observed in clinical *K. pneumoniae* isolates CR14 and NY9 in this study. Since plasmids can be horizontally transferred across strains, species, and even genera, plasmid-borne antimicrobial resistance is of concern. Moreover, we report here a clinical *K. pneumoniae* isolate, CN1, with multiple copies of *bla*<sub>CTX-M-15</sub> and *bla*<sub>KPC-2</sub> integrated in the chromosome. Our whole-genome sequence analysis suggests that these chromosomal *bla*<sub>CTX-M-15</sub> and *bla*<sub>KPC-2</sub> elements mobilized from plasmids. Such integration and dissemination of antimicrobial resistance genes illustrates the currently complex pandemic scenario of MDR *K. pneumoniae* isolates.

It is uncommon for *K. pneumoniae* to harbor multiple  $\beta$ -lactamase genes in its chromosome, especially multiple copies of the same  $\beta$ -lactamase gene. Most recently, it has been reported that strain KPNIH31 carries two chromosomal *bla*<sub>CTX-M-15</sub> copies with *ISEcp1* (although one is disrupted by *IS903*, as shown in Fig. 2C) (15), KPNIH33 carries two chromosomal *bla*<sub>KPC-3</sub> copies with *Tn4401b* (14, 15), and MS6671 carries three chromosomal *bla*<sub>OXA-181</sub> copies with *ISEcp1* (Fig. 1B) (22). We describe that the CN1 chromosome carries three *bla*<sub>CTX-M-15</sub> copies with *ISEcp1* and one *bla*<sub>KPC-2</sub> copy with *Tn4401a*. Most interestingly, our comparative genomic analysis demonstrates that strains KPNIH31, MS6671, and CN1 share the same chromosomal architecture feature: multiple copies of a  $\beta$ -lactamase gene linked with *ISEcp1*, a large *Psp3*-like prophage sequence, and a CRISPR-*cas* array with the same DR sequence. The CRISPR-*cas* system is a prokaryotic immune system that confers resistance to foreign genetic elements, such as those present within plasmids and phages, and provides a form of acquired immunity in its spacers (19, 20). Coexistence of a CRISPR-*cas* array and multiple copies of the same  $\beta$ -lactamase genes in the chromosome lead us to hypothesize a mechanism for antimicrobial resistance genes jumping from plasmids to the chromosome, along with their associated mobile genetic elements. We postulate that the novel acquired spacer in the CRISPR-*cas* array could induce degradation of its targeting plasmids in the host or in an environmental niche and promote antimicrobial resistance gene mobilization from plasmids into the chromosome when the bacteria are under the selective force of antimicrobial agents. Notably, isolate CN1 has relatively fewer plasmids than isolates CR14 and NY9.

Recently, the CRISPR-*cas* system has been proposed and tested for application against antimicrobial resistance using bacteriophage as a vector (23–25). However, our whole-genome analysis reveals that both CRISPR-*cas* array and bacteriophage could be integrated into the chromosome, as well as the antimicrobial resistance genes along with their mobile genetic elements. This suggests that CRISPR targeting on the plas-

mids carrying antimicrobial resistance leads to resistance genes being mobilized into the chromosome. Efficient CRISPR targeting should be on sequences specific to the antimicrobial resistance gene itself. It also suggests that vector sequences, such as bacteriophages or plasmids, invade the chromosome. Thus, the genetic components of the vector should be carefully considered to avoid long-term genetic contamination when designing the CRISPR-*cas* strategies against antimicrobial resistance. Additionally, the existing CRISPR-*cas* array in some bacteria should be taken into account and utilized wisely.

In conclusion, this study describes three complete genomes of MDR *K. pneumoniae* carrying both *bla*<sub>CTX-M</sub> and *bla*<sub>KPC</sub>. Consistent with previous observations, transpositions of *bla*<sub>CTX-M-15</sub> and *bla*<sub>KPC-2</sub> in chromosomes or plasmids are linked to the mobile genetic elements *ISEcp1* and *Tn4401a*, respectively. Such mobile elements provide opportunities for horizontal gene transfer of antibiotic resistance genes among plasmids and between plasmid and chromosome, posing complications for the efficient prevention and control of MDR bacterial infections in health care. Our comparative genomic analysis further reveals that genomic characteristics of CRISPR-*cas* arrays, *ISEcp1-bla*<sub>CTX-M-15</sub> and *Tn4401a-bla*<sub>KPC-2</sub> mobile elements, and prophage sites in *K. pneumoniae* strains allow differentiation and tracking of the prevalent MDR *K. pneumoniae* strains and give us insights into the molecular mechanisms of bacterial evolution and adaptation.

## MATERIALS AND METHODS

**Clinical isolates.** Three *K. pneumoniae* isolates carrying both *bla*<sub>CTX-M</sub> and *bla*<sub>KPC</sub> (CR14, NY9, and CN1) were included in this study. These three isolates were identified from 394 nonduplicate *K. pneumoniae* clinical isolates from the WMC (2005 to 2014; *n* = 374) and two New York City hospitals (2013; *n* = 20) that were analyzed by next-generation sequencing. The clinical isolates from two academic hospitals in metropolitan New York City, Bellevue Hospital (*n* = 11) and New York-Presbyterian Hospital (*n* = 9), were included for purposes of comparison. All *K. pneumoniae* isolates were recovered from patient specimens and identified by standard microbiology procedures at the clinical microbiology laboratories of the three hospitals. The WMC is a 643-bed academic tertiary-care medical center in Westchester County, New York. The Institutional Review Board of New York Medical College approved this study.

**Antimicrobial susceptibility testing.** Antimicrobial susceptibility testing was performed for all antimicrobial agents, other than those listed below, with the MicroScan Walk-Away 96 system (Beckman Coulter) (17). Etests (bioMérieux) were employed for susceptibility assessment of cefotaxime, ceftazidime, imipenem, meropenem, tigecycline, and colistin, and results were interpreted according to the Clinical and Laboratory Standards Institute (CLSI) guidelines (26).

**Whole-genome sequencing.** Genomic DNA was extracted from each isolate using a QIAamp DNA kit (Qiagen) and subjected to both short- and long-read massively parallel sequencing. Short-read sequencing was performed in the MiSeq system using the Nextera XT sample preparation kit (Illumina), reaching an averaged depth of more than 250× in coverage for each isolate. Long-read sequencing was performed using the Pacific Biosciences (PacBio) RSII single-molecule real-time (SMRT) sequencing system after processing the SMRTbell library using g-TUBE fragmentation (Covaris), the BluePippin size selection system for DNA fragments of 7 to 50 kb (Sage Science), and the SMRTbell template preparation kit (PacBio), reaching an averaged depth of more than 50× in coverage.

**Whole-genome *de novo* assembly.** Sequence assembly was conducted *de novo* on Illumina short reads using Velvet v.1.2.10 (27) and on the PacBio long reads using the Hierarchical Genome Assembly Process 3, version 2.3.0, pipeline (HGAP3) and Quiver tools in SMRT Analysis v.2.3.0 (PacBio) (28). Sequence assembly was also conducted with the SPAdes Genome Assembler (v3.5.0) for the combination of both Illumina short reads and PacBio long reads (29). Both BWA (30) and Bowtie2 (31) algorithms were employed for short reads aligned to the draft of the whole-genome assembly, whereas BLASR (32) was employed for long-read alignment. The final whole-genome sequences were reviewed under the Integrative Genomics Viewer (33, 34), manually modified, and trimmed into circular chromosome and plasmids.

**Whole-genome analysis.** The whole-genome sequences were annotated by the NCBI Prokaryotic Genome Automatic Annotation Pipeline (35). Multilocus sequence typing of each isolate was analyzed using SRST2 (36). Chromosome architecture comparisons between strains were carried out using Mauve 2.3.1 (37). Insertion sequences, CRISPR, and prophage sites were characterized by IslandViewer 3 (38), CRISPRFinder (39), and PHASTER (40), respectively. Antibiotic resistance genes were scanned by ResFinder 2.1 (41). Plasmid incompatibility (Inc.) groups were assessed by BLASTn and by using the PlasmidFinder database from the Center for Genomic Epidemiology (42). BLASTn was also used for other sequence homolog searches.

**Nucleotide sequence accession numbers.** The complete genome sequences have been deposited in GenBank under BioProject identifier [PRJNA319426](#) and accession numbers [CP015382](#) to [CP015397](#).

## SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/AAC.00076-17>.

**SUPPLEMENTAL FILE 1**, PDF file, 0.4 MB.

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We have no conflicts of interest to declare.

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