Chromosomal Integration of Huge and Complex $bla_{NDM}$-Carrying Genetic Elements in Enterobacteriaceae

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In this study, a detailed genetic dissection of the huge and complex $bla_{NDM}$-carrying genetic elements and their related mobile genetic elements was performed in Enterobacteriaceae. An extensive comparison was applied to 12 chromosomal genetic elements, including six sequenced in this study and the other six from GenBank. These 12 genetic elements were divided into five groups: a novel IME Tn6588; two related IMEs Tn6523 (SGI1) and Tn6589; four related ICEs Tn6512 (R391), Tn6575 (ICE$_{Pvu}$ChnBC22), Tn6576, and Tn6577; Tn7 and its derivatives Tn6726 and 40.7-kb Tn7-related element; and two related IMEs Tn6591 (Glsul2) and Tn6590. At least 51 resistance genes, involved in resistance to 18 different categories of antibiotics and heavy metals, were found in these 12 genetic elements. Notably, Tn6576 carried another ICE Tn6582. In particular, the six $bla_{NDM}$-carrying genetic elements Tn6588, Tn6589, Tn6575, Tn6576, Tn6726, and 40.7-kb Tn7-related element contained large accessory multidrug resistance (MDR) regions, each of which had a very complex mosaic structure that comprised intact or residual mobile genetic elements including insertion sequences, unit or composite transposons, integrons, and putative resistance units. Core $bla_{NDM}$ genetic environments manifested as four different Tn125 derivatives and, notably, two or more copies of relevant Tn125 derivatives were found in each of Tn6576, Tn6588, Tn6589, and 40.7-kb Tn7-related element. The huge and complex $bla_{NDM}$-carrying genetic elements were assembled from complex transposition and homolog recombination. Firstly identified were eight novel mobile elements, including three ICEs Tn6576, Tn6577, and Tn6582, two IMEs, Tn6588 and Tn6589, two composite transposons Tn6580a and Tn6580b, and one integron In1718.

Keywords: Enterobacteriaceae, chromosomal integration, $bla_{NDM}$, multidrug resistance, mobile genetic elements
INTRODUCTION

New Delhi metallo-β-lactamase (NDM) is able to hydrolyze nearly all β-lactams except aztreonam and thus mediates resistance to penicillins, cephalosporins, and carbapenems (Yong et al., 2009). It is hypothesized that the blaNDM gene is originally integrated into the Acinetobacter chromosome from an unknown environmental species and then captured by two copies of ISAba125, giving rise to ISAba125-composite transposon Tn125 (Poirel et al., 2012). With the transposition of Tn125, blaNDM is disseminated among Acinetobacter, Enterobacteriaceae and Pseudomonas species; Tn125 and its blaNDM-carrying derivatives, with various truncations and deletions, can be found in the accessory resistance regions of bacterial plasmids or chromosomes (Wu et al., 2019). There are reports of chromosomal location of blaNDM in Enterobacteriaceae species including Escherichia coli (Pfeifer et al., 2011; Poirel et al., 2011; Shen et al., 2017; Reynolds et al., 2019), Providencia stuartii (Poirel et al., 2011), Proteus mirabilis (Girlich et al., 2015), Klebsiella pneumoniae (Sakamoto et al., 2018), and Proteus vulgaris (Kong et al., 2020), but few of them are subjective to detailed genetic dissection of blaNDM-carrying accessory resistance regions (Girlich et al., 2015; Sakamoto et al., 2018; Reynolds et al., 2019; Kong et al., 2020).

Integrative and conjugative elements (ICEs) and integrative and mobilizable elements (IMEs) (Bellanger et al., 2014; Delaval et al., 2017; Botelho and Schulenburg, 2021) are two different types of mobile genetic elements which are frequently integrated into bacterial chromosome, contributing to dissemination of resistance genes. ICEs have the ability to transfer between cells because of their self-encoded conjugation function. It is typically composed of attL (attachment site at the left end), int (integrase), xis (excisionase), rlx (relaxase), oriT (origin of conjugative replication), cpl (coupling protein), a P (TivB)- or F (TivF)-type T4SS machinery (mating pair formation), and attR (attachment site at the right end). IMEs are not self-transmissible, and they achieve the intercellular mobility with the help of other conjugative elements that encode proteins involved in complete conjugation function. IMEs typically have attL, int, rlx, oriT, and attR, but contained no conjugal transfer genes. Tn7 is a unit transposon with the ability to integrate into bacterial chromosomes and plasmids, and it encodes five core transposition determinants TnsA and TnsB (transposases), TnsC (regulator), and TnsD and TnsE (DNA-binding proteins), as well as three TnsB-binding sites and four TnsB-binding sites at its left and right ends, respectively (Peters, 2014).

In this work, whole-genome sequencing identified four blaNDM-carrying genetic elements plus two additional genetic elements harboring other resistance genes in the chromosomes of four isolates of Providencia rettgeri, Proteus mirabilis, and K. pneumoniae. An extension sequence comparison was then applied to a collection of 12 chromosomal genetic elements (including the above six ones sequenced in this work) that could be grouped into ICEs, IMEs, and Tn7 unit transposon and its derivatives. Data presented here gave a detailed genetic dissection of the huge and complex blaNDM-carrying genetic elements and their related mobile genetic elements in multiple Enterobacteriaceae species.

MATERIALS AND METHODS

Bacterial Strains

The four chromosomal blaNDM-carrying isolates (Table S1) were screened from more than two hundred blaNDM-carrying Enterobacteriaceae isolates routinely collected from China hospitals and livestock farms. Providencia rettgeri 1701091 and Proteus mirabilis 1701092 (Table S1) were recovered in 2017 from the chicken intestinal contents in two different China livestock farms. K. pneumoniae QD23 and Providencia rettgeri 51003 were recovered from the urine specimens of two different patients with nosocomial infections in two Chinese public hospitals in 2015 and 2017, respectively. Bacterial species identification was performed using genome sequence-based average nucleotide identity analysis (http://www.ezbiocloud.net/tools/ani) (Richter and Rossello-Mora, 2009).

Sequencing and Sequence Assembly

Bacterial genomic DNA was isolated using the UltraClean Microbial Kit (Qiagen, NW, Germany) and sequenced from a sheared DNA library with average size of 15 kb (ranged from 10 to 20 kb) on a PacBio RSII sequencer (Pacific Biosciences, CA, USA), as well as a paired-end library with an average insert size of 350 bp (ranged from 150 to 600 kb) on a HiSeq sequencer (Illumina, CA, USA). The paired-end short Illumina reads were used to correct the long PacBio reads utilizing proovread (Hackl et al., 2014), and then the corrected PacBio reads were assembled de novo utilizing SMARTdenovo (https://github.com/ruanjue/smartdenovo).

Sequence Annotation and Comparison

Open reading frames (ORFs) and pseudogenes were predicted using RAST 2.0 (https://rast.nmpdr.org/) (Brettin et al., 2015) combined with BLASTP/BLASTN searches (Boratyn et al., 2013) against the UniProtKB/Swiss-Prot database (https://web.expasy.org/docs/swiss-prot_guideline.html) (Boutet et al., 2016) and the RefSeq database (https://www.ncbi.nlm.nih.gov/refseq/) (O’Leary et al., 2016). Annotation of resistance genes, mobile elements, and other features were carried out using the online databases including CARD (https://card.mcmaster.ca/browse) (Jia et al., 2017), ResFinder (https://cge.cbs.dtu.dk/services/ResFinder/) (Zankari et al., 2012), ISfinder (https://www-is.biotoul.fr/) (Siguier et al., 2006), INTEGRALL (http://integrall.bio.ua.pt/) (Moura et al., 2009) and Tn Number Registry (https://www.ucl.ac.uk/eastman/tn-number-registry) (Roberts et al., 2008). Multiple and pairwise sequence comparisons were performed using MUSCLE 3.8.31 (Edgar, 2004) and BLASTN, respectively. Gene organization diagrams were drawn in Inkscape 1.0 (https://inkscape.org/en/). Heatmaps were plotted with MeV 4.9.0 (Saeed et al., 2003).
Conjugal Transfer

Conjugal transfer experiments were carried out with rifampicin-resistant *Escherichia coli* EC600 or sodium azide-resistant *E. coli* J53 being used as a recipient, and the 1701092 or QD23 isolate as a donor. Three milliliters of overnight cultures of each of donor and recipient bacteria were mixed together, harvested and resuspended in 80 μl of Brain Heart Infusion (BHI) broth (BD Biosciences). The mixture was spotted on a 1 cm² hydrophilic nylon membrane filter with a 0.45 μm pore size (Millipore) that was placed on BHI agar (BD Biosciences) plate and then incubated for mating at 37°C for 12 to 18 h. Bacteria were washed from filter membrane and spotted on Muller–Hinton (MH) agar (BD Biosciences) plates, for selecting an *E. coli* transconjugant carrying bla<sub>NDM</sub> or carrying tetA(C). Then 200 mg/L sodium azide (for J53) or 1,000 mg/L rifampin (for EC600), together with 4 mg/L imipenem (for *E. coli*) was used for transconjugant selection.

PCR Identification

All the wild-type and transconjugant strains was subjected to PCR amplification followed by amplicon sequencing, for determining the sequences of bacterial 16S rRNA genes (Frank et al., 2016), the presence of key markers such as bla<sub>NDM</sub>, tetA(C), int, and parM, and also the location/boundary of mobile genetic elements such as Tn<sup>6577</sup>, Tn<sup>6589</sup>, Tn<sup>6576</sup>, Tn<sup>6577</sup>, and Tn<sup>6590</sup> (data not shown).

Phenotypic Assays

Activity of Ambler class A/B/D carbapenemases in bacterial cell extracts was determined by a modified CarbaNP test (Feng et al., 2016). Bacterial antimicrobial susceptibility was tested by BioMérieux VITEK 2 and interpreted as per the Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI, 2020).

Nucleotide Sequence Accession Numbers

The complete chromosome sequences of the 1701091, 1701092, QD23, and 51003 isolates were submitted to GenBank under accession numbers CP042860, CP042857, CP042858, and CP042861 respectively.

RESULTS

**Genome Sequencing for Dissection of Chromosomal bla<sub>NDM</sub>-Carrying Genetic Elements**

The complete genome sequences of four bla<sub>NDM</sub>-carrying isolates *Providencia rettgeri* 1701091, *Proteus mirabilis* 1701092, *K. pneumoniae* QD23, and *Providencia rettgeri* 51003 were determined in this work through high-throughput genome sequencing. A total of six chromosome-borne accessory resistance regions were identified: bla<sub>NDM</sub>-I<sup>3.5</sup>-carrying Tn<sup>6588</sup>, Tn<sup>6589</sup>, Tn<sup>6576</sup>, and 40.7-kb Tn<sup>7</sup>-related element from strains 1701091, 1701092, QD23, and 51003, respectively; tetA(C)- and bla<sub>CTX-M-14</sub>-carrying Tn<sup>6577</sup> were from strain 1701092; and strAB-carrying Tn<sup>6590</sup> was from strain 51003.

**TABLE 1 | Major features of genetic elements characterized in this work.**

| Group                  | Accession number | Genetic element Accession number | Presence (+) of bla<sub>NDM</sub> | Host bacterium | Reference |
|------------------------|------------------|----------------------------------|----------------------------------|----------------|-----------|
| Novel IME              | CP042860         | **bla<sub>NDM</sub>-related IME** | +                                | *Providencia rettgeri* 1701091 | This study |
| Tn<sup>6523</sup>-related OIEs | CP042857         | **Tn<sub>6523</sub>**             | +                                | *Proteus mirabilis* 1701092 | This study |
| Tn<sup>6576</sup>-related OIEs | CP042857         | **Tn<sub>6576</sub>**             | +                                | *K. pneumoniae* 1701092 | This study |
| Tn<sup>6590</sup>-related OIEs | CP042861         | **Tn<sub>6590</sub>**             | +                                | *K. pneumoniae* 51003 | This study |
| Tn<sup>7</sup>-related elements | CP042857         | **Tn<sub>7</sub>**                 | +                                | *K. pneumoniae* 51003 | This study |
| **bla<sub>NDM</sub>-CarbA** | CP042858         | **Tn<sub>6572</sub>**             | +                                | *K. pneumoniae* 51003 | This study |
| **tetA(C)**            | CP042859         | **Tn<sub>6577</sub>**             | +                                | *K. pneumoniae* 51003 | This study |
| **strAB**              | CP042859         | **Tn<sub>6577</sub>**             | +                                | *K. pneumoniae* 51003 | This study |
| **bla<sub>CTX-M-14</sub>** | CP042859         | **Tn<sub>6577</sub>**             | +                                | *K. pneumoniae* 51003 | This study |

For each group, all the fully sequenced and non-redundant bla<sub>NDM</sub>-carrying genetic elements available in GenBank (as last accessed on 15 December 2019) are included.
A detailed sequence comparison was applied to a collection of 12 chromosomal genetic elements, which included the above mentioned six genetic elements sequenced in this study, together with six additional ones from GenBank (four reference/prototype ones Tn6523, Tn6512, Tn7, and Tn6591, and two blaNDM-carrying ones Tn6575 and Tn6726). These 12 genetic elements could be further divided into five distinct groups: a novel IME Tn6588; two related IMEs Tn6523 and Tn6589; four related ICEs Tn6512, Tn6575, Tn6576 and Tn6577; Tn7 and its two derivatives Tn6726 and 40.7-kb Tn7-related element; and two related IMEs Tn6591 and Tn6590 (Table 1). Six (Tn6588, Tn6589, Tn6575, Tn6576, Tn6726, and 40.7-kb Tn7-related element) of them harbored blaNDM. At least 51 resistance genes, involved in resistance to 18 different categories of antibiotics and heavy metals, were identified in these 12 elements (Figure 1 and Table S2).

A Novel IME Tn6588

Tn6588 (100.0 kb in length) was inserted into the chromosomal opr1407 gene (cytochrome c551 peroxidase). Tn6588 had a 9.2-kb backbone (containing int) with insertion of two accessory modules: ISJ1A and a 90.1-kb multidrug resistance (MDR) region, and it had terminal 35-bp attl/attR pairs and were further bracketed by 5-bp direct repeats (DRs; target site duplication signals for transposition) (Figure 2A). The MDR region contained a total of 19 resistance genes including blaNDM1 (Figure 1 and Table S2), which were located at eight different resistance loci: In1718, IS26–mph(E)–IS26 unit, In1247, a truncated ISCR2–fleR unit, ISCR2–sul2 unit, ISEc59–aph(4)–Tn–aaccC4–IS26 unit, ΔTn4352 containing aphA1, and a 6.8-kb In27-carrying Tn6909-related region (Figure 2B).

In1718 was a concise class 1 integron with a gene cassette array (GCA) aacA4cr–blaOXA-1–catB3–arr-3. In1247 was a complex class 1 integron containing VR1 (variable region 1=GCA: aadA2–lnuF), five copies of 6.4-kb repeat [VR2 (ISCR1–bleMBL–blaNDM1–arr-3 unit) plus 3’-CS2 (a second 3’-consoed segment)], and VR3 (ISCR1–aphA6 unit). In1247 plus a Tn21 core transposition module trpAR–res–trnM in this MDR region were genetically related to the Tn3-family unit transposon Tn6727 (Partridge et al., 2001), which was initially found in Proteus vulgaris and originally associated with Tn21. The 6.8-kb blaNDM90-related region looked like a truncated version of Tn3-family unit transposon Tn6909 that was originally associated with Tn1696 and Tn21 (Partridge et al., 2001).

Two Related IMEs Tn6523 and Tn6589

Tn6523, a 42.4-kb IME initially found in Salmonella enterica serovar Typhimurium DT104 (Boyd et al., 2000), had a 27.2-kb backbone (containing attl, int, xis, rlx, oriT, and attR) with insertion of a single accessory module In127. Besides the GCA (aadA2), In127 captured additional two resistance loci: ISCR3–tetA(G)–fleR unit and a blaCARB2-carrying In167. The backbone of Tn6589 was almost identical to Tn6523 but integrated with a 66.4-kb MDR region instead of In127 (Figure 3A). This MDR region contained a total of 16 resistance genes including blaNDM1 (Figure 1 and Table S2), which were located at seven different resistance loci: In27, ISCR3–tetA(G)–fleR unit, Tn2–rmtB region, ISCR2–fleR unit, ISPa13–erm42–IS26 unit, aaccC2–tmrB region, and In363 with a GCA dfrA1–gcuC (Figure 3B). In27 in this MDR region was a complex class 1 integron, which carried VR1 (GCA: dfrA12–gcuC–aadA2), and two copies of 11.8-kb repeat region [VR2 (ISCR1–bleMBL–blaNDM1–arr-3 unit) + 3’-CS2 + VR3 (ISCR1–qnrA1 unit) + 3’-CS3] (Figure 3B).

Four Related ICEs Tn6512, Tn6575, Tn6576, and Tn6577

Tn6512, an 88.5-kb ICE initially found in Providencia rettgeri 107 (Bolten et al., 2002), was composed of an 82.4-kb backbone with insertion of three accessory modules: IS15DI-composite transposon Tn6578 (containing aphA1), ISPpre1 and ISPpre2 (Figure S1). Tn6512, Tn6575 (146.9 kb in length) (Kong et al., 2020), Tn6576 (534.1 kb in length), and Tn6577 (137.5 kb in length)
FIGURE 2  | Shown are the organization of Tn6588 (A), and MDR region from Tn6588 (B). Genes are denoted by arrows. Genes, mobile elements, and other features are colored based on their functional classification. Shading denotes regions of homology (nucleotide identity \( \geq 95\% \)). Numbers in brackets indicate nucleotide positions within the chromosome of strain 1701091. The accession numbers of Tn6727, ISCR2-flR unit, Tn4352 (Wrighton and Strike, 1987), Tn6581c, and Tn6909 used as reference are CP047346, CP042857, CP042858, CP042857, and CP032168, respectively.
length) had similar backbones and, especially, shared the core backbone genes attL, int, xis, rlx, ori, cpl, a TivF-type T4SS gene set, and attR (Figure S1). All these four ICEs were integrated at the same site within the chromosomal gene prfC (peptide chain release factor 3).

Each of Tn6575, Tn6576, and Tn6577 had two accessory modules. Firstly, a 85.0-kb MDR region, a novel 406.4-kb ICE Tn6582, and a 11.1-kb blaHMS-sul2 region were inserted at the same site within umuC of Tn6575, Tn6576, and Tn6577, respectively, which led to different truncations of surrounding backbone regions of Tn6575 and Tn6577 but not Tn6576. Secondly, ISPpu12, Tn6580b (55.2 kb in length), and Tn6580a (50.0 kb in length) were integrated at the same site downstream of orf714 of Tn6575, Tn6576, and Tn6577, respectively (Figure S1).

The 85.0-kb MDR region of Tn6575 contained a total of 22 resistance genes including blaNDM-1 (Figure 1 and Table S2), which were located at 12 different resistance loci: two copies of a truncated IS6559-aph(3′)-Ia-aacC4-Is26 unit, an unnamable In element (harboring a long GCA aacA4cr-blaOXA-1-catB3-arr-3-aacA4cr-arr-3 but lacking the whole 5′-CS), aphA1-containing Tn4352, IS26-cfr-IS26 unit, IS26-composite transposon Tn6581 containing bleO, In525 (GCA: dfrA32-creA-aadA2), tetA(C)-containing Tn6309, blaNDM-1-containing Tn125-2, a 6.6-kb In0-carrying Tn6911-related region, a truncated ISCR2-strAB unit, a truncated ISCR2-sul2 unit, and strAB-containing ΔTn5393c (Figure 4). The Tn6911-related region in this MDR region looked like a truncated version of Tn3-family unit transposon Tn6911 that was originally associated with Tn21 (Partridge et al., 2001).

Tn6582 had a complete set of core ICE backbone determinants and, moreover, a lot of accessory modules: two MDR (MDR-1 and MDR-2) regions, sil-cop region as found in IncHI2 plasmid R478 (Gilmour et al., 2004), floR-strAB-sul2

FIGURE 3 | Shown are two Tn6523-related transposons (A), and MDR region from Tn6589 and In127 from Tn6523 (B). Genes are denoted by arrows. Genes, mobile elements, and other features are colored based on their functional classification. Shading denotes regions of homology (nucleotide identity ≥95%). Numbers in brackets indicate nucleotide positions within Tn6523 (Boyd et al., 2000) and the chromosome of strain 1701092. The accession number of Tn6523 used as reference is AF261825.
The 38.9-kb MDR-1 region (Figure 5) comprised ΔTn6029 (containing sul2 and strAB), Tn6581b, an unnamable In37-like element (harboring the In37 GCA aacA4cr–arr3 but lack of the whole 5′-CS), In27 (GCA: dfrA12-gcuF–aadA2), and two copies of 11.0-kb repeat (each copy harbored a truncated aacC2–tmrB region, Tn4352, IS26–mpb(A)–IS6000 unit, and chrA-orf98 unit). The 43.7-kb MDR-2 region (Figure 6) comprised ΔTn21 (containing mer), aacC2–tmrB region, and two copies of 21.4-kb repeat (each copy harbored IS26–mpb(A)–IS6100 unit, chrA-orf98 unit, In1021, and a truncated aacC2–tmrB region). In1021 was a complex class 1 integron containing VR1 (GCA: aacA4cr–arr3–dfrA27–aadA16) and VR2 (ISCR1–bleM–blaNDM–3–arr3 unit).

The ISPPu12-composite transposon Tn6580a contained a total of 17 resistance genes (Figure 1 and Table S2), which were located at nine different resistance loci: In525 (GCA: dfrA12–gcuF–aadA2), Tn4352, IS26–mpb(A)–IS6000 unit, and chrA-orf98 unit). The 43.7-kb MDR-2 region (containing ΔTn5393c and a truncated ISCR2–floR unit), and multiple intact or residue IS elements (Figure S2). The 38.9-kb MDR-1 region (Figure 5) comprised ΔTn6029 (containing sul2 and strAB), Tn6581b, an unnamable In37-like element (harboring the In37 GCA aacA4cr–blaoxa-1–catB3–arr3 but lack of the whole 5′-CS), In27 (GCA: dfrA12-gcuF–aadA2), and two copies of 11.0-kb repeat (each copy harbored a truncated aacC2–tmrB region, Tn4352, IS26–mpb(A)–IS6000 unit, and chrA-orf98 unit). The 43.7-kb MDR-2 region (containing ΔTn5393c and a truncated ISCR2–floR unit), and multiple intact or residue IS elements (Figure S2).
dfrA32–ereA–aadA2), a truncated chrA–orf98 unit, Tn6581c, the unnamable In37-like element as described above, ISec59–aph(4)Ia–aacC4–IS26 unit, ISCR2–sul2 unit, ISCR2–floR unit, blaCTX-M-14-containing D\(\text{Tn}_{6503a}\), and tetA(C)-containing D\(\text{Tn}_{6309}\) (Figure 7). Three major modular differences were recognized in \(\text{Tn}_{6580b}\) relative to \(\text{Tn}_{6580a}\): i) replacement of \(\text{Tn}_{6581c}\) by \(\text{Tn}_{6581b}\); ii) inversion of the unnamable In37-like element; and iii) insertion of IS26–fosA3–IS26 unit together with \(\text{Tn}_{4352}\) at a site between \(\text{D}_{\text{Tn}_{6503a}}\) and \(\text{D}_{\text{Tn}_{6309}}\).

### Three Related Unit Transposons Tn7, Tn6726, and the 40.7-kb Tn7-Related Element

All of Tn7 (14.1 kb in length), Tn6726 (175.8 kb in length) (Sakamoto et al., 2018), and 40.7-kb Tn7-related element were integrated at a site downstream of the chromosomal gene \(\text{glmS}\) (glutamine-fructose-6-phosphate aminotransferase). Tn6726 differed from Tn7 by acquisition of In2-3 (GCA: dfrA1) instead of In2-4 (GCA: dfrA1–aadA1) and, moreover, a 162.6-kb IS\(\text{Kpn}_{26}\)-composite transposon \(\text{Tn}_{6728}\) was inserted at a site within intI2 of In2-3 (Figure 8). Tn6726 harbored a 40.9-kb MDR region as well as an array of \(\text{Inc}_{\text{HI3}}\) core backbone genes (Figure S3). This 40.9-kb MDR region included a 9.4-kb \(\text{Tn}_{6909}\)-related region together with \(\text{D}_{\text{Tn}_{1548}}\), and \(\text{aphA1}\)-carrying \(\text{D}_{\text{Tn}_{4352}}\).

In2-16 carried VR1 (GCA: lnu(F)–dfrA1–aadA1a) and two

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**FIGURE 5** | Organization of MDR-1 region from Tn6582, and comparison to related regions. Genes are denoted by arrows. Genes, mobile elements, and other features are colored based on their functional classification. Shading denotes regions of homology (nucleotide identity \(\geq 95\%\)). Numbers in brackets indicate nucleotide positions within the chromosome of strain QD23. The accession numbers of Tn6029 (Cain et al., 2010), aacC2–tmrB region (Partridge et al., 2012), and In37 (Wang et al., 2003) used as reference are GQ150541, JX101693, and AY259086, respectively.

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**FIGURE 8** | \(\text{MDR-1 region from Tn6582}\) (4609332.4624390).
copies of 5.6-kb repeat [VR2 (ISCR1–bleMBL–blaNDM-1 unit) plus Δ3’CS2] (Figure 10).

ISec29–mph(E)–IS26/IS15DI unit and ISCR1–armA unit were present in both ΔTn1548 from Tn6728 and that from 40.7-kb Tn7-related element, whereas In27 was found in the former ΔTn1548 rather than the later one; in addition, ISec29–mph(E)–IS26/IS15DI unit was interrupted by insertion of two different IS elements ISKpn21 and ISABA24, respectively, in these two ΔTn1548 (Figures 9 and 10).

Two Related IMEs Tn6591 and Tn6590
The 15.5-kb IME Tn6591 (GIsul2) (Wei et al., 2003), initially found in Shigella flexneri 2457T, was integrated into the chromosomal gene guaA (glutamine-hydrolyzing GMP synthase) and had a 12.6-kb backbone (containing attL, int, oriT and attR) with insertion of a single accessory module ISCR2–sul2 unit (Figure 11). Tn6590 (15.6 kb in length) was integrated at the same chromosomal site, and Tn6590 differed from Tn6591 by only truncation of ISCR2–sul2 unit due to insertion of strAB-carrying ΔTn5393c.

Plasmids of the Four Strains Sequenced in This Study
Proteus mirabilis 1701092 carried no plasmids, and all accessory resistance regions (Tn6577 and Tn6589) were located in the chromosome. Besides chromosome-borne accessory resistance regions, an IncFII plasmid p701091-FII (carrying no resistance genes), an IncI plasmid pQD23-CTXM [harboring blaCTX-M-104 and erm(B)], and an IncFII plasmid p51003-FII (containing blaTEM-1B and blaCTX-M-3) together with an Col3M plasmid p51003-qrnD (having qnrD) were identified in Providencia spp. 1701091, K. pneumoniae QD23, and Providencia spp. 51003, respectively. Coexistence of a large array of resistance genes in both chromosome and plasmids of a single bacterial isolate makes it tends to become extensively resistant.

Transferability and Antimicrobial Susceptibility
This work identified three ICEs Tn6577, Tn6582 and Tn6576 in total, all of which had essential conjugal transfer genes. Notably, Tn6582 was located within Tn6576. As for conjugation experiments, Tn6577 was transferred from the wild-type isolate (susceptible to rifampin) into E. coli EC600, generating the transconjugant Tn6577–TETA(C)–EC600; Tn6582 could be transferred from the wild-type isolate (non-susceptible to rifampin but susceptible to sodium azide) in E. coli J53 to obtain Tn6582–NDM–J53, but repeated conjugation attempts failed to transfer Tn6576 into E. coli J53. Tn6577–TETA(C)–EC600 was highly resistant to tetracycline and ceftriaxone owing to presence of tetA(C) and blaCTX-M-14. Tn6582–NDM–J53 was highly resistant to ceftriaxone and imipenem resulted from production of NDM enzyme (data not shown). The Ambler class B carbapenemase activity was detected in Tn6582–NDM–J53 and its wild-type isolate.

DISCUSSION
Since the blaNDM gene was initially identified in India in 2009 (Yong et al., 2009), it spread rapidly all over the world (Dortet et al., 2014). Although blaNDM was initially discovered in a plasmid of K. pneumoniae (Yong et al., 2009), the chromosomal location of blaNDM in Enterobacteriaceae species has been reported in recent years (Pfeifer et al., 2011; Poirel et al., 2011; Girlich et al., 2015; Shen et al., 2017; Sakamoto et al., 2018; Reynolds et al., 2019; Kong et al., 2020). There were few reports related to a detailed genetic dissection of different kinds of blaNDM-carrying accessory resistance regions in the chromosomes (Girlich et al., 2015; Sakamoto et al., 2018; Reynolds et al., 2019; Kong et al., 2020), but none of them had

FIGURE 6 | Organization of MDR-2 region from Tn6582. Genes are denoted by arrows. Genes, mobile elements, and other features are colored based on their functional classification. Shading denotes regions of homology (nucleotide identity ≥95%). Numbers in brackets indicate nucleotide positions within the chromosome of strain QD23.
a systematic summary for these *bla<sub>NDM</sub>*-carrying mobile genetic elements.

Data presented here involved a total of six chromosomal *bla<sub>NDM</sub>*-carrying genetic elements Tn6575, Tn6726, Tn6588, Tn6589, Tn6576, and 40.7-kb Tn7-related element, and the last four were sequenced in this work. These six genetic elements belonged to three different categories: ICEs (Tn6575 and Tn6576), IMEs (Tn6588 and Tn6589), and two derivatives (Tn6726 and 40.7-kb Tn7-related element) of Tn7 unit transposon. Notably, Tn6576 carried another ICE Tn6582. These ICEs and IMEs would have the intercellular self-mobility as they carried essential conjugal transfer genes (Bellanger et al., 2014; Botelho and Schulenburg, 2021).

Tn6726 would have the intracellular mobility as it had a complete core transposition module *tnsABCDE*, while 40.7-kb Tn7-related element would lose its mobility due to lesion in *tnsABCDE*.

Tn6512-related ICEs were frequently reported in *Vibrio*, *Proteus*, and *Shewanella* (Burrus et al., 2006; Nonaka et al., 2012; Lei et al., 2016; Fang et al., 2018). Tn6575 and Tn6576 were the only two *bla<sub>NDM</sub>*-carrying Tn6512-related ICEs (last accessed 15 December 2019). Tn6523-related IMEs were frequently reported in *Salmonella* and *Proteus mirabilis* (Hall, 2010; Siebor and Neuwirth, 2013; Sung et al., 2017). This study presented Tn6589, the first *bla<sub>NDM</sub>*-carrying Tn6523-related IME. Tn7, and its derivatives had the ability to integrate into
bacterial plasmids and chromosomes (Peters, 2014). There were several reports of Tn7 derivatives located in bacterial chromosomes (Chen et al., 2018; Chen et al., 2019). To date, Tn6726 and 40.7-kb Tn7-related element were the only two blaNDM-carrying Tn7 derivatives integrated into chromosomes. Different to 40.7-kb Tn7-related element, Tn6726 carried a series of backbone genes of IncHI3 plasmid, which means that blaNDM together with its surrounding genetic environment in Tn6726 might be originated from a IncHI3 plasmid. In summary, Tn6512-related ICEs, Tn6523-related IMEs, and Tn7 derivatives recently began to be a reservoir of blaNDM genes in Enterobacteriaceae.

Each of these six blaNDM-carrying genetic elements had large accessory resistance regions: i) Tn6575, Tn6588, Tn6589, and 40.7-kb Tn7-related element; each had a single MDR region, 85.0 kb, 90.1 kb, 66.4 kb, and 33.8 kb in length, respectively; ii) Tn6726 contained a 162.6-kb ISKpn26-composite transposon Tn6728 integrated with a 40.9-kb MDR region; and iii) Tn6576 harbored a 406.4-kb ICE Tn6576 (containing two distinct MDR-1 and MDR-2 regions, 38.9 kb and 43.7 kb in length, respectively), and additionally a 55.2-kb ISPpu12-composite Tn6580b that as a whole could be considered as a MDR region. Each of these large MDR regions had a very complex mosaic structure, which was composed of intact or residue mobile genetic elements including ISs, unit or composite transposons, integrons and putative resistance units, and likely assembled from complex transposition and homologous recombination.

Four different Tn125 derivatives, namely ΔTn125-1, ΔTn125-2, ΔTn125-3, and ΔTn125-4 (Figure 12), were identified from the relevant MDR regions of these six blaNDM-carrying genetic elements. ΔTn125-1 from Tn6726 and ΔTn125-2 from Tn6575 highly resembled the prototype Tn125. ΔTn125-1 resulted from the insertion of ISEc33 into the left copy of ISAba125, while terminal truncation of both copies of ISAba125 generated ΔTn125-2. It was thought that ΔTn125-1 and ΔTn125-2 were generated from transposition of Tn125 into Tn6726 and Tn6575, followed by further modular modifications such as insertion and truncation. ΔTn125-3 from 40.7-kb Tn7-related element and ΔTn125-4 from Tn6576, Tn6588 and Tn6576 had very short blaNDM-1/-3-carrying structures. ΔTn125-3 or ΔTn125-4 was captured by ISCR1, generating ISCR1–bleMBL–blaNDM-1 or ISCR1–bleMBL–blaNDM-1/3–arr-3 unit, respectively. Furthermore, the former unit was integrated into In2-16 (Figure 10) while the latter one into In1247_Tn6588 (Figure 2), In27_Tn6589 (Figure 3) and In1021_Tn6576 (Figure 6), manifesting as the VR2 regions of these integrons. Notably, two or more copies of blaNDM-1/3 genes were found in each of Tn6576, Tn6588, Tn6589, and 40.7-kb Tn7-related element, which resulted from the presence of multiple ≥5.6-kb repeats (each harboring a
Tn125 derivative and the other components) in these four genetic elements.

Multiple copies of bla<sub>NDM</sub> located in a single plasmid or chromosome were reported in previous studies (Jovčić et al., 2013; Shen et al., 2017; Feng et al., 2018), and all these bla<sub>NDM</sub> genes were around IS<sub>CR1</sub>. Similarly, Tn6576, Tn6588, Tn6589, and 40.7-kb Tn7-related element in this study also contained IS<sub>CR1</sub>-around bla<sub>NDM</sub> genes. It was confirmed that IS<sub>CR1</sub> captured adjacent genes (frequently including antibiotic resistance genes) at the end of its initiation of replication (ori<sub>IS</sub>) through rolling-circle transposition (Toleman et al., 2006). Our sequencing data suggested that IS<sub>CR1</sub> might experience multiple rounds of capturing bla<sub>NDM</sub> and further integrating bla<sub>NDM</sub> into the integrons, resulting in the presence of multiple copies of IS<sub>CR1</sub>-associated bla<sub>NDM</sub> genes in a single genetic element.

There were eight novel (firstly identified in this study) mobile genetic elements, including three ICEs Tn6576, Tn6577, and Tn6582, two IMEs Tn6588 and Tn6589, two composite transposons Tn6580<sub>a</sub> and Tn6580<sub>b</sub>, and one integron In1718. Additional 12 genetic elements (IME: Tn6590; composite...
FIGURE 10 | Organization of MDR region from 40.7-kb Tn7-related element, and comparison to related regions. Genes are denoted by arrows. Genes, mobile elements, and other features are colored based on their functional classification. Shading denotes regions of homology (nucleotide identity ≥95%). Numbers in brackets indicate nucleotide positions within the chromosome of strain 51003. The accession number of Tn1548 (Galimand et al., 2005) used as reference is AF550415.

FIGURE 11 | Organization of two related IMEs Tn6590 and Tn6591, and comparison to related regions. Genes are denoted by arrows. Genes, mobile elements, and other features are colored based on their functional classification. Shading denotes regions of homology (nucleotide identity ≥95%). Numbers in brackets indicate nucleotide positions within the chromosome of strain 51003. The accession number of Tn5393c (L. Abel-Lund and Sørum, 2000) used as reference is AF262622.
transposons: Tn6578, Tn6581a, Tn6581b, Tn6581c, and Tn6728; unit transposons: Tn6726, Tn6727, Tn6909, and Tn6911; IS: ISPvu1; and 40.7-kb Tn7-related element) were newly designated (firstly designated in this study, but with previously determined sequences). The four previously designated ICEs/IMEs SGI1, R391, ICEPvuChnBC22, and GIsul2 were renamed as standard Tn designations Tn6523, Tn6512, Tn6575, and Tn6591, respectively. All the putative resistance units presented in this work were annotated and collected in a custom and yet unpublished database.

CONCLUSION

This study dealt with an extensive sequence comparison of 12 chromosomal genetic elements, including six blaNDM-carrying ones. All these blaNDM-carrying genetic elements had huge and complex MDR regions. The core blaNDM genetic environments manifested as four different Tn125 derivatives. Notably, two or more copies of blaNDM were found in each of the four genetic elements. Eight novel mobile elements were firstly identified, including three ICEs Tn6576, Tn6577, and Tn6582, two IMEs Tn6588 and Tn6589, two composite transposons Tn6580a and Tn6580b, and one integron In1718. This study would provide a deeper genetic insight into the chromosomal integration of blaNDM-carrying genetic elements in Enterobacteriaceae.

DATA AVAILABILITY STATEMENT

The datasets generated for this study can be found in the complete chromosomal nucleotide sequences of 1701091, 1701092, QD23 and 51003, which were submitted to GenBank under accession numbers CP042860, CP042857, CP042858 and CP042861, respectively.
ETHICS STATEMENT
This study used the bacterial isolates obtained from the Chinese livestock farm and public hospitals as listed in Table S1. The local legislation did not require the study to be reviewed or approved by an ethics committee, because the bacterial isolates involved in this study was part of the routine laboratory procedures. The research involving biohazards and all related procedures were approved by the Biosafety Committee of the Beijing Institute of Microbiology and Epidemiology.

AUTHOR CONTRIBUTIONS
DZ and HY conceived the study and designed experimental procedures. XL, YJ, and FC performed the experiments. XL, YJ, and LZ analyzed the data. LH, DW, and YS contributed to reagents and materials. XL and ZY wrote the original draft. DZ and HY reviewed the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL
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