Complete sequences of KPC-2-encoding plasmid p628-KPC and CTX-M-55-encoding p628-CTXM coexisted in Klebsiella pneumoniae

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A carbapenem-resistant Klebsiella pneumoniae strain 628 was isolated from a human case of intracranial infection in a Chinese teaching hospital. Strain 628 produces KPC-2 and CTX-M-55 encoded by two different conjugative plasmids, i.e., the IncFII plasmid p628-KPC and the IncF plasmid p628-CTXM respectively. blaKPC-2 is captured by a Tn1722-based unit transposon with a linear structure. ΔTn3-ISKpn27-blaKPC-2-ΔISKpn6-ΔTn1722 and this transposon together with a mercury resistance (mer) gene locus constitutes a 34 kb acquired drug-resistance region. blaKPC-2 has two transcription starts (nucleotides G and C located at 39 and 250 bp upstream of its coding region respectively) which correspond to two promoters, i.e., the intrinsic P1 and the upstream ISKpn27/Tn3-provided P2 with the core −35/−10 elements TAATCC/TTACAT and TTGACA/AATAAT respectively. blaCTX-M-55 is mobilized in an ISEcp1-blaCTX-M-55-Δorf477 transposition unit and appears to be the sole drug-resistant determinant in p628-CTXM. blaCTX-M-55 possesses a single transcription start (nucleotides G located at 116 bp upstream of its coding region) corresponding to the ISEcp1-provided P1 promoter with the core −35/−10 element TTGAAA/TACAAT. All the above detected promoters display a characteristic of constitutive expression. Coexistence of blaKPC and blaCTX-M in K. pneumoniae has been reported many times but this is the first report to gain deep insights into genetic platforms, promoters, and expression of the two coexisting bla genes with determination of entire nucleotide sequences of the two corresponding plasmids.

Keywords: Klebsiella pneumoniae, KPC-2, CTX-M-55, p628-KPC, p628-CTXM, promoter

Introduction

KPC-producing Klebsiella pneumoniae has spread worldwide and became an emerging pathogen with serious clinical and infection control implications (Tzouvelekis et al., 2012; Munoz-Price et al., 2013). Coexistence of blaKPC and blaCTX-M in K. pneumoniae has been reported in several countries, such as blaKPC-2/blaCTX-M-1 group, blaKPC-2/blaCTX-M-2 group, and
**Materials and Methods**

**Bacterial Strains and Identification**

*K. pneumoniae* strain 628 was isolated from the cerebrospinal fluid specimen of a 64-year-old male with intracranial infection in a Chinese teaching hospital in October 2010. Bacterial species identification was performed using Bruker MALDI Biotyper (Bruker Daltonics, Bremen, Germany) and 16s rRNA gene sequencing (Frank et al., 2008). The major carbapenemase and extended-spectrum beta-lactamase (ESBL) genes were detected by PCR, followed by sequencing on an ABI Sequencer (Applied Biosystems, Foster City, CA, USA) (Chen et al., 2015). Bacterial antimicrobial susceptibility was tested by using VITEK 2 and judged by CLSI standard (CLSI, 2012).

**Plasmid Transfer**

Plasmid conjugal transfer experiments were carried out with Escherichia coli EC600 (LacZ\(^{-}\), Na\(^{+}\), Rif\(^{R}\)) being used as recipient and strain 628 as donor. Three milliliter of overnight culture of each of donor and recipient bacteria were mixed together, harvested and resuspended in 80 µl of Brain Heart Infusion broth (BD Biosciences, San Jose, CA, USA). The mixture was spotted on a 1 cm\(^2\) filter membrane that was placed on Brain Heart Infusion agar (BD Biosciences, San Jose, CA, USA) plate, and then incubated for mating at 37\(^{\circ}\)C for 12 to 18 h. Bacteria were washed from filter membrane and spotted on Muller-Hinton agar (BD Biosciences, San Jose, CA, USA) plate containing 1000 µg/L rifampin (Merck, Darmstadt, Germany) and 200 µg/L ampicillin (Merck, Darmstadt, Germany) for selection of bla\(_{\text{CTX-M-}}\) or bla\(_{\text{KPC-}}\)-positive *E. coli* transconjugants.

**Determination of Plasmid DNA Sequence**

Plasmid DNA was isolated from the cell culture of *E. coli* transconjugant using Qiagen large construct kit (Qiagen, Hilden, Germany) and then sequenced by using whole-genome shotgun strategy in combination with Illumina HiSeq 2500 (Illumina, San Diego, CA, USA) sequencing technology. The contigs were assembled with Velvet and the gaps were filled through combinatorial PCR and Sanger Sequencing on ABI 3730 Sequencer. The genes were predicted with GeneMarkSTM and further annotated by BLASTP and BLASTN against UniProt and NR databases.

**RNA Isolation and Primer Extension Assay**

Bacteria were cultured overnight in Mueller-Hinton broth (BD Biosciences, San Jose, CA, USA). Total RNAs were extracted from harvested bacterial cells using TRIzol Reagent (Life Technologies, Carlsbad, CA, USA). RNA quality was monitored by agarose gel electrophoresis, and RNA quantity was determined by spectrophotometry. Each of the \([\gamma-\text{32P]}\) ATP end-labeled primers GCTCAGTGGAACGAAAAC, AGCCGGCAAAGTCTCGGTGC, and CATGGAGTCCCTTATTCTG, which corresponded to bla\(_{\text{KPC-}}\) promoter P2, bla\(_{\text{KPC-}}\) promoter P1, and bla\(_{\text{CTX-M-55}}\) promoter P1 respectively, was annealed with total RNA sample for primer extension assay as described previously (Zhang et al., 2011). For different cell cultures in a single experiment, equal amounts of total RNA were used as starting materials. The corresponding end-labeled primers were also used for sequencing the PCR ampiclons generated by the primer pairs TCAGGACATCGTCAACCGTGCTGTTTCCCTTATGCCCC, TCAGGAGTCCCTTATTCTG, and AGACCTTTGTGTTGAATGTAG/AGCTTATTCATCGCCTACGTGTTTCCCTTTAGCC, which corresponded to bla\(_{\text{KPC-}}\) promoter P2, bla\(_{\text{KPC-}}\) promoter P1, and bla\(_{\text{CTX-M-55}}\) promoter P1 respectively. DNA sequencing was carried out using AccuPower & Top DNA Sequencing Kit (Bioneer, Daejeon, Korea). Primer extension products and sequencing materials were analyzed on 8 M urea-6% polyacrylamide gel electrophoresis. Radioactive species were detected by autoradiography.

**Nucleotide Sequence Accession Numbers**
The complete sequences of plasmids p628-KPC and p628-CTXM were submitted to GenBank under accession numbers KP987218 and KP987217 respectively.

**Results and Discussion**

**Characterization of *K. pneumoniae* Strain 628**

Strain 628 harbors bla\(_{\text{KPC-2}}\), bla\(_{\text{CTX-M-55}}\), bla\(_{\text{SHV}}\), and bla\(_{\text{TEM}}\) genes. Both bla\(_{\text{KPC-2}}\) and bla\(_{\text{CTX-M-55}}\) are located plasmids p628-KPC and p628-CTXM respectively. Conjugative transfer of p628-KPC or p628-CTXM into EC600 generates the transconjugant 628-KPC-EC600 (bla\(_{\text{KPC-2}}\), bla\(_{\text{CTX-M-55}}\), bla\(_{\text{SHV}}\), and bla\(_{\text{TEM}}\)) or 628-CTXM-EC600 (bla\(_{\text{KPC-2}}\), bla\(_{\text{CTX-M-55}}\), bla\(_{\text{SHV}}\), and bla\(_{\text{TEM}}\)) respectively. All of 628, 628-KPC-EC600 and 628-CTXM-EC600 are resistant to ampicillin, ampicillin/sulbactam, penicillin, monobactam, and cephalosporins tested (Table 1). 628 and 628-KPC-EC600 (but not 628-CTXM-EC600) are resistant to piperacillin/tazobactam. 628 and 628-KPC-EC600 (but not 628-CTXM-EC600) are carbapenem-resistant.

**Complete Nucleotide Sequence of p628-KPC**
The entire nucleotide sequence of p628-KPC is 105,008 bp in length, forming a circular plasmid with an average G+C content of 33.22 and a total of 127 open reading frames (ORFs) annotated.
Wang et al. Coexistence of p628-KPC and p628-CTXM in K. pneumoniae

**TABLE 1** | Antimicrobial drug susceptibility profiles.

| Antibiotics                     | 628   | 628-KPC-EC600 | 628-CTXM-EC600 | EC600 |
|---------------------------------|-------|---------------|---------------|-------|
| Ampicillin                      | ≥32/R | ≥32/R         | ≥32/R         | 16/I  |
| Ampicillin/sulbactam            | ≥32/R | ≥32/R         | ≥32/R         | 4/S   |
| Piperacillin                    | ≥128/R| ≥128/R        | ≥128/R        | ≤4/S  |
| Piperacillin/tazobactam         | ≥128/R| ≥128/R        | ≤4/S          | ≤4/S  |
| Aztreonam                       | ≥64/R | ≥64/R         | ≥64/R         | ≤1/S  |
| Cefazolin                       | ≥64/R | ≥64/R         | ≥64/R         | ≤1/S  |
| Cefuroxime sodium               | ≥64/R | ≥64/R         | ≤1/S          | ≤1/S  |
| Cefuroxime axetil               | ≥64/R | ≥64/R         | ≤1/S          | ≤1/S  |
| Ceftriaxone                     | ≥64/R | ≥64/R         | ≤2/S          | ≤2/S  |
| Ceftazidime                     | ≥64/R | ≥64/R         | ≤2/S          | ≤2/S  |
| Imipenem                        | ≥16/R | ≥16/R         | ≤1/S          | ≤1/S  |
| Meropenem                       | ≥16/R | 2/R           | ≤0.25/S       | ≤0.25/S|
| Ciprofloxacin                   | ≥4/R  | ≤0.25/S       | ≤0.25/S       | ≤0.25/S|
| Levofloxacin                    | ≥8/R  | 0.5/S         | 0.5/S         | 0.5/S |
| Macrolactin                     | ≥512/R| ≤1/S          | ≤16/S         | ≤16/S |
| Amikacin                        | ≤2/S  | ≤2/S          | ≤2/S          | ≤2/S  |
| Tobramycin                      | ≤1/S  | ≤1/S          | ≤1/S          | ≤1/S  |
| Trimethoprim/sulfamethoxazole   | 40/S  | ≤20/S         | ≤20/S         | ≤20/S |

**FIGURE 1** | Schematic maps of p628-KPC (A) and p628-CTXM (B). Genes are denoted by arrows and colored based on gene function classification. The innermost circle presents GC-Skew [(G-C)/(G+C)] with a window size of 500bp and a step size of 20bp. The blue circle presents GC content. Shown also are backbone and accessory module regions. (Figure 1A). p628-KPC belongs to the IncFIIK incompatibility group and harbors IncFIIK repA and the second IncFIB-like repA2, both of which encode replication initiation proteins. The p628-KPC backbone, 67,515 bp in length, is composed of DNA regions for plasmid replication (repA and repA2) and stability (parAB, stbAB, ssb, etc), and conjugal transfer (tra, trb,
Wang et al. Coexistence of p628-KPC and p628-CTXM in K. pneumoniae

**FIGURE 2 | Linear comparison of sequenced plasmids.** Genes are denoted by arrows and colored based on gene function classification. Shading regions denote regions of homology (>98% nucleotide similarity). Included are p628-KPC (A) and p628-CTXM (B) and their closely related plasmids.

**TABLE 2 | Genetic surroundings of bla\textsubscript{KPC}−2 from China.**

| bla\textsubscript{KPC}−2 Genetic environment | Plasmid | Bacterium | Host | References |
|------------------------------------------|---------|-----------|------|------------|
| Core structure                           | Transposon | Name | Incomparability group | Accession number | |
| IS\textsubscript{Kpn}27−bla\textsubscript{KPC}−2−ΔIS\textsubscript{Kpn6} | Tn1722-based unit transposon\textsuperscript{a} | pKPC-NY79 | IncX3 | JX104759 | K. pneumoniae | Human patient | Ho et al., 2013b |
| IS\textsubscript{Kpn}27−bla\textsubscript{KPC}−2−ΔIS\textsubscript{Kpn6} | Tn3-based Tn4401 | pKP048 | IncFlK\textsuperscript{a} | KF628167 | K. pneumoniae | Human patient | Shen et al., 2009 |
| IS\textsubscript{Kpn}27−bla\textsubscript{KPC}−2−ΔIS\textsubscript{Kpn6} | Tn3-related | pKP048 | IncFlK\textsuperscript{a} | KF628167 | K. pneumoniae | Human patient | Shen et al., 2009 |
| IS\textsubscript{Kpn}27−bla\textsubscript{KPC}−2−ΔIS\textsubscript{Kpn6} | IS\textsuperscript{26}-based composite transposon\textsuperscript{a} | pKPC-LKeC | Inc\textsuperscript{a}/IncN/RepIC | KC788405 | E. coli | Human patient | Chen et al., 2014b |
| IS\textsubscript{Kpn}27−bla\textsubscript{KPC}−2−ΔIS\textsubscript{Kpn6} | IS\textsuperscript{26}-based composite transposon\textsuperscript{a} | pECN580 | IncN | KF914891 | E. coli | Human patient | Chen et al., 2014a |
| IS\textsubscript{Kpn}27−bla\textsubscript{KPC}−2−ΔIS\textsubscript{Kpn6} | IS\textsuperscript{26}-based composite transposon\textsuperscript{a} | pKo6 | IncN | KC988437 | E. coli | Human patient | Chen et al., 2014a |

Included are all the bla\textsubscript{KPC}-carrying plasmids with determined genome sequences from China.

\textsuperscript{a}See reference (Roberts et al., 2008) for classification of transposons.

\textsuperscript{b}In addition to the IncFlK\textsuperscript{a}, the plasmid contains the second IncFIB-like repA2.

\textsuperscript{c}This plasmid harbors a repB putative replication initiation region but, surprisingly, lacks the IncFlK\textsuperscript{a} repA.

etc), which show >98% sequence identity to the corresponding regions of the IncFlK plasmids pKP4 (GenBank accession number CP000649), pKP048 (Jiang et al., 2010), and pKPHS2 (CP003224) (Figure 2A). The overall structure of p628-KPC is most similar to that of pKPHS2 (91% query coverage and 98% maximum nucleotide identity) (Figure 2A). pKP4 is recovered from clinical K. pneumoniae MGH 78578 and represents the reference IncFlK plasmid, carrying \textit{bla}\textsubscript{SHV}−12 (cephalosporin
Coexistence of p628-KPC and p628-CTXM in K. pneumoniae

**FIGURE 3 | Linear comparison of** \( \text{bla}^{\text{KPC-2}} \) **genetic surroundings.** Genes are denoted by arrows and colored based on gene function classification.

 resistance), and \( aac(6') \) and \( aadA \) (aminoglycoside resistance). pKP048 and pKPHS2 are from two KPC-2-producing clinical K. pneumoniae isolates from China.

As shown in **Figures 1A, 2A**, p628-KPC contains three distinct accessory modules: a 34 kb drug-resistance region, a 1038 bp \( \text{ISKpn28} \)-based element, and a 2302 bp region of unknown function [identical sequences can be found in \( \text{bla}^{\text{KPC-2}} \)-carrying plasmid pKPCAPSS (KP008371) and \( qnrS1 \)-harboring pE66An (HF545433)]. The 34 kb region harbors two drug-resistance loci, the \( \text{mer} \) locus (mercury resistance) and the \( \text{bla}^{\text{KPC-2}} \) locus, and it is almost the same as the counterpart of pKPHS2 (Figure 2A). A 71 kb multi-drug-resistance region in pKP048 (Jiang et al., 2010) is composed of the 34 kb region of p628-KPC and the extra part (carrying \( \text{bla}^{\text{DHA-1}}, qnrB4 \), and \( \text{armA} \) encoding resistance to cephalosporins, fluoroquinolones, and aminoglycosides respectively) absent from p628-KPC (Figure 2A).

**Complete Nucleotide Sequence of p628-CTXM**

The p628-CTXM genome consists of an 85,338 bp circular DNA molecule with an average G+C content of 49.71 and harbors a total of 92 ORFs annotated (Figure 1B). p628-KPC belongs
to the IncI1 incompatibility group expressing the replication initiation protein RepZ. The p628-CTXM backbone, 82,357 bp in length, contains DNA regions for plasmid replication (repY, repZ, and inc), conjugal transfer (tra, trb, pil, etc) and transfer leading (imp, yfa to yfh, yga to ygg, etc), which show >98% sequence identity to the corresponding regions of the IncI1 plasmids R64 from *Salmonella enterica* serovar Typhimurium (Sampei et al., 2010), pKHSB1 from *Shigella sonnei* (Holt et al., 2013) and pEK204 from *E. coli* O25:H4-ST131 clone (Woodford et al., 2009) (Figure 2B). Another backbone component is the plasmid stability region, composed of three genes yafA, yafB, and yagA, which is highly conserved among p628-CTXM, pKHSB1, and pEK204; by contrast, the corresponding segment of R64 is a 17.8 kb region which harbors at least 18 genes and especially include those encoding site-specific recombination (resD and yefA) and partition (parAB) of replicated DNA into daughter cells during cell division (Sampei et al., 2010) (Figure 2B).

R64 carries a single accessory module, a 17 kb IS2-based transposon, which interrupts *arsA1* (a member of the *arsR1-arsD1-arsA1-arsB-arsC* operon) (Sampei et al., 2010). p628-CTXM harbors a single accessory module, a 2980 bp IS*Ecp1*-related element, which interrupts *yagA* (a member of the plasmid stability region) (Figures 1B, 2B). Two distinct Tn3-related elements, 7935 and 8014 bp in length, are inserted downstream of *yagA* in pKHSB1 and pEK204 respectively. There are still additional accessory modules including IS*Cro1* and IS*421* for pKHSB1, and IS*66* for pEK204.

**Genetic Surroundings of *bla*<sub>KPC-2</sub>**

As characterized in European and American countries, the *bla*KPC genes are located in a Tn3-family transposon named Tn4401, which is present on a wide variety of plasmids varying in size, structure and replicon (Naas et al., 2008; Kitchel et al., 2009, 2010; Chen et al., 2012; Bryant et al., 2013; Chmelnitsky et al., 2014). At least eight isoforms of Tn4401 have been named, i.e., Tn4401a to Tn4401g and a separate Tn4401d (Table S1 in Supplementary Material). Several unnamed Tn4401 isoforms have been also reported recently (Cuzon et al., 2011; Li et al., 2011; Ho et al., 2013b; Naas et al., 2013; Perez-Chaparro et al., 2014). Tn4401b is considered as the prototype one, and the other
isoforms result from occurrence of distinct deletion or insertion events at different sites.

As shown in Table 2 and Figure 3, the \( \text{bla}_{\text{KPC}-2} \) genetic environments from China can be assigned into three main categories: Tn\( \text{4401} \) with the IS\( \text{Kpn7} \)-\( \text{bla}_{\text{KPC}-2} \)-IS\( \text{Kpn6} \) core structure (pKPC-NY79), the Tn\( \text{1722} \)-based unit transposons with the IS\( \text{Kpn27} \)-\( \text{bla}_{\text{KPC}-2} \)-\( \Delta \text{IS} \text{Kpn6} \) core structure (pKP048, p628-KPC, pH5062105-3, pKPHS2, pKPC-LK30, and pH5102707; IS\( \text{Kpn27} \) is initially named in the ISfinder database (Siguier et al., 2006)), and the IS\( \text{26} \)-based composite transposons with the IS\( \text{Kpn27} \)-\( \text{bla}_{\text{KPC}-2} \)-\( \Delta \text{IS} \text{Kpn6} \) core structure (pKPC-LKEc, pECN580, and pKo6). The Tn\( \text{4401} \) of pKPC-NY79 is a novel isoform of Tn\( \text{4401a} \) with \( \text{tnpR} \) truncated. The prototype Tn\( \text{1722} \)-based transposon as observed in pKP048 has a linear structure \( \Delta \text{mcp-Tn3-ISKpn7-blakPC-2-ISKpn6} \), for which presence of two IS\( \text{26} \) elements at both ends truncates IS\( \text{Kpn6} \) and Tn3, notably, different lengths of truncated IS\( \text{Kpn6} \) can be observed for these IS\( \text{26} \)-based transposons from different plasmids.

### Genetic Surroundings of \( \text{bla}_{\text{CTX-M-55}} \)

R64, p628-CTXM, pKHSB1, and pEK204 carry a 17 kb IS\( \text{2} \)-based mobile element, a 2980 bp ISEcp1-based transposition unit, a 7935 bp Tn3-based element, and an 8014 bp Tn3-based element respectively; each of them is the sole determinant for antibiotics resistance of the corresponding plasmid (Figure 4). For R64, stepwise insertions occur to eventually assemble the IS\( \text{2} \)-based element: insertion of IS2 into \( \text{ars}A1 \), that of Tn6082 into IS2, that of IS1133 into Tn6082, and finally that of Tn10 into IS1133; the \( \text{tet} \) locus carried by Tn10 and the \( \text{strAB} \) operon carried by Tn6082 account for resistance to tetracycline and streptomycin respectively.

A lot of \( \text{bla}_{\text{CTX-M-1}} \) group genes such as \( \text{bla}_{\text{CTX-M-55}} \), \( \text{bla}_{\text{CTX-M-15}} \) and \( \text{bla}_{\text{CTX-M-3}} \) are often connected with ISEcp1 (upstream; responsible for capture and mobilization of \( \text{bla}_{\text{CTX-M}} \) and \( \Delta \text{orf477} \) (downstream), constituting an ISEcp1-\( \text{bla}_{\text{CTX-M}} \)-\( \Delta \text{orf477} \) transposition unit (Lartigue et al., 2006).

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**FIGURE 5 | Organization and expression of \( \text{bla} \) genes.** (A) Gene organization. Boxed arrows stand for length/direction of indicated genes. Broken-line arrows represent transcription starts for indicated \( \text{bla} \) genes. (B) Primer extension. Primer extension assay of the RNA transcript of each \( \text{bla} \) gene was performed for strain 628 cultured with addition of increasing amounts of imipenem or ampicillin. Lanes C, T, A, and G represent Sanger sequencing reactions. Lanes 0, 3, and 6 stand for 0, 3, and 6 mg/L ampicillin. Lanes 0, 200, and 400 stand for 0, 200, and 400 mg/L ampicillin. Transcription start of each \( \text{bla} \) gene is indicated by arrow with nucleotide, and minus number under arrow indicates nucleotide position upstream of indicated \( \text{bla} \) gene. Representative data from at least two independent biological replicates are shown.
2006; Zong et al., 2010). In p628-KPC, the plasmid backbone gene yagA is disrupted by ISecp1-blaCTX-M-55-Δorf477. In pKHSB1 and pEK204, a blaTEM-1-carrying Tn3 transposon is inserted at the site downstream of yagA and the Tn3 tnpA gene is further disrupted by ISecp1-blaCTX-M-15-Δorf477 and ISecp1-blaCTX-M-3-Δorf477, respectively. In addition, these two inserted ISecp1-based structures differ from each other with respect to targeting sites and oriented directions (Figure 4).

**Expression of blaKPC-2 and blaCTX-M-55**

Each of the blaKPC-2 genes in Tn4401a, b, d, f, and g has two transcription starts, i.e., nucleotides G and C located at 39 and 289 bp upstream of blaKPC-2, which correspond to the two promoters P1 and P2 (re-designated P2ISKpn7 herein) with core −35/−10 elements TAATCC/TTACAT and TTGACA/AATAAT respectively (Figures 5, 6). The first 74 bp fragments upstream of blaKPC-2Tn4401b and blaKPC-2Tn1722-based are essentially identical; the P1 promoter is located within this 74 bp region and thereby shared by blaKPC-2Tn4401b and blaKPC-2Tn1722-based (Figure 6). The next 280 bp region upstream of the above 74 bp fragment for blaKPC-2Tn4401b is dramatically divergent at nucleotide level from the counterpart for blaKPC-2Tn1722-based, these two distinct 280 bp regions contain P2ISKpn7 and P2ISKpn27/Tn3 respectively. The −35 element of P2ISKpn7 is provided by ISKpn7 inserted at 319 bp upstream of blaKPC-2Tn4401b, while the −35 and −10 elements of P2ISKpn27/Tn3 are provided by ISKpn27 and Tn3 inserted at 281 and 75 bp upstream of blaKPC-2Tn1722-based respectively (Figure 6).

Spacer regions between ISecp1 and blaCTX-M-55 from different ISecp1-blaCTX-M-55 isoforms display three different lengths, namely 45 bp (e.g., blaCTX-M-55p1081-CTXM) (Qu et al., 2014), 48 bp (e.g., blaCTX-M-55p628-CTXM), and 127 bp (e.g., blaCTX-M-55pQ343851). Two promoters, TTGAGG-CTACGTTACGAATTAAC-ΔG (organized as −35 element/-10 element/transcription start; named P1) and TTGACAT-TTACGTTACGAATTAAC-ΔG (organized as −35 element/-10 element/transcription start; named P2), are experimentally identified for blaCTX-M-3.15p550415 with a 127 bp spacer and...
Coexistence of p628-KPC and p628-CTXM in K. pneumoniae

FIGURE 7 | Alignment of $bla_{CTX-M}$ upstream sequences. The 153–235 bp upstream sequences together with the start codon of the $bla_{CTX-M}$ genes were aligned by CLUSTALW. Shown were core promoter regions, $-35$ and $-10$ elements, transcription starts, SD sequences for ribosome recognition, and translation starts. Asterisks indicate the identical nucleotides.

Moreover, the $ISEcp1$-provided promoter P1 is stronger and more important than the intrinsic P2 promoter in the 127 bp spacer (Ma et al., 2011). The above result is applicable to the $bla_{CTX-M-55}$ genes with the 127 bp spacer (Figure 7), because their $ISEcp1$+spacer region is identical to the counterpart of $bla_{CTX-M-3}$AF550415.

In the present study, the primer extension assays detected a transcription start, i.e., nucleotides G located at 116 bp upstream of $bla_{CTX-M-55}$ (Figure 5), which corresponded to the P1 promoter shared by $bla_{CTX-M-55}^{p1081-CTXM}$ (Qu et al., 2014) and $bla_{CTX-M-55}^{p628-CTXM}$ (Figure 7). Compared with the 127 bp spacer, the 45 or 48 bp spacer for $bla_{CTX-M-55}^{p1081-CTXM}$ or $bla_{CTX-M-55}^{p628-CTXM}$ is a truncated form due to absence of a 82 or 79 bp region respectively. The deletion event impairs the $-35$ element of P2, most likely making the P2 activity undetectable for $bla_{CTX-M-55}^{p1081-CTXM}$ and $bla_{CTX-M-55}^{p628-CTXM}$ (Figure 7).

In addition, the primer extension assay showed that addition of increasing amounts of imipenem or ampicillin during cultivation of indicated strains 628, 628-KPC-EC600 and 628-CTXM-EC600 had no effect on activity of all the above promoters detected for $bla_{KPC-2}$ or $bla_{CTX-M-55}$, denoting constitutive expression of the above two resistance genes (Figure 5).

Concluding Remarks

KPC-2 and CTX-M-55 enzymes are produced by two different conjugative plasmids, p628-KPC and p628-CTXM respectively, in K. pneumoniae strain 628, and the sequences of these two plasmids are >98% identical to other relevant plasmids carrying the same resistance determinants previously sequenced. The detected $bla_{KPC-2}$ gene is captured by a Tn1722-based unit transposon carried by an IncFII$_K$-type multi-drug-resistant plasmid p628-KPC, and this gene has two different promoters, the intrinsic P1 and the ISKpn27/Tn3-provided P2, both characteristic of constitutive expression. The detected $bla_{CTX-M-55}$ gene, being the sole drug-resistant determinant in the plasmid, is mobilized in an $ISEcp1$-based transposition unit carried by an IncI1 plasmid p628-CTXM, and this gene has a single $ISEcp1$-provided promoter driving $bla_{CTX-M-55}$ expression in a constitutive manner. Coexistence of $bla_{KPC}$ and $bla_{CTX-M}$ in K. pneumoniae has been reported many times, but this is the first report to gain deep insights into genetic platforms, promoters, and expression of the two coexisted $bla$ genes. The IncFII$_K$ and IncI1 plasmids have been frequently identified to carry horizontally acquired drug-resistant gene modules and could be transmitted across a number of bacterial species (Woodford et al., 2009; Jiang et al., 2010; Sampei et al., 2010; Holt et al., 2013), and increased surveillance of these drug-resistant plasmids is needed.

Funding

This work is funded by National High-Tech Research and Development Program (2014AA021402), National Key Program for Infectious Disease of China (2013ZX10004216), and National Natural Science Foundation of China (31471184 and 31170127).

Supplementary Material

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2015.00838
References

Kryzhanovsky, S. V., van Belkum, A. J., Thapa, I., Bastola, D., Williams, L. O., Safranek, T. J., et al. (2013). KPC-4 Is encoded within a truncated Tn4001 in an IncI1M plasmid, pNE1280, isolated from Enterobacter cloacae and Serratia marcescens. Antimicrob. Agents Chemother. 57, 37–41. doi: 10.1128/AAC.01062-12

Cai, J. C., Zhou, W. L., Zhang, R., and Chen, G. X. (2008). Emergence of Serratia marcescens, Klebsiella pneumoniae, and Escherichia coli isolates possessing the plasmid-mediated carbapenem-hydrolyzing beta-lactamase KPC-2 in intensive care units of a Chinese hospital. Antimicrob. Agents Chemother. 52, 2014–2018. doi: 10.1128/AAC.01539-07

Chen, L., Hu, H., Chavda, K. D., Zhao, S., Liu, R., Liang, H., et al. (2014a). Complete sequence of a KPC-producing IncN multidrug-resistant plasmid from an epidemic Escherichia coli sequence type 131 strain in China. Antimicrob. Agents Chemother. 58, 2422–2425. doi: 10.1128/AAC.02587-13

Chen, Y. T., Lin, J. C., Fung, C. P., Lu, P. L., Chuang, Y. C., Wu, T. L., et al. (2014b). Partial excision of blaKPC from Tn4401 in Carbapenem-resistant Klebsiella pneumoniae. Antimicrob. Agents Chemother. 56, 1635–1637. doi: 10.1128/AAC.00188-14

Chen, L., Chavda, K. D., Mediavilla, J. R., Jacobs, M. R., Levi, M. H., Bonomo, R. A., et al. (2012). Partial excision of blacPC from Tn4401 in Carbapenem-resistant Klebsiella pneumoniae. Antimicrob. Agents Chemother. 56, 1635–1637. doi: 10.1128/AAC.00188-14

Chen, Y. T., Lin, J. C., Fung, C. P., Lu, P. L., Chuang, Y. C., Wu, T. L., et al. (2014b). KPC-2-encoding plasmids from Escherichia coli and Klebsiella pneumoniae in Taiwan. J. Antimicrob. Chemother. 69, 628–651. doi: 10.1093/jac/dkt049

Chen, Z., Li, H., Feng, J., Li, Y., Chen, X., Guo, X., et al. (2015). NDM-1 encoded by a PNDM-B01-like plasmid pSIP-NDM in clinical Enterobacter aerogenes. Front. Microbiol. 6:294. doi: 10.3984/fmicb.2015.00294

Chelnokov, I., Shklyar, M., Leavitt, A., Sadovsky, E., Navon-Venezia, S., Ben-Frnc, J. A., Reich, C. I., Sharma, S., Weisbaum, J. S., Wilson, B. A., and Olsen, G. (2012). Partial excision of blaKPC from Tn4401 in Carbapenem-resistant Klebsiella pneumoniae isolates in the United States: clonal expansion of multilocus sequence type 258. Antimicrob. Agents Chemother. 56, 3002–3029. doi: 10.1128/AAC.00299-07

Carmeli, Y., et al. (2005). Emergence of KPC-2 and KPC-3 in carbapenem-resistant Klebsiella pneumoniae strains in an Israeli hospital. Antimicrob. Agents Chemother. 51, 3026–3029. doi: 10.1128/AAC.00299-07

Leavitt, A., Navon-Venezia, S., Chmelnitsky, I., Schwerba, M. J., and Carmeli, Y. (2007). Emergence of KPC-2 and KPC-3 in carbapenem-resistant Klebsiella pneumoniae strains in an Israeli hospital. Antimicrob. Agents Chemother. 51, 3026–3029. doi: 10.1128/AAC.00299-07

Naas, T., Bonnin, R. A., Cuzon, G., Villegas, M. V., and Nordmann, P. (2013). Complete sequence of two KPC-harbouring plasmids from Pseudomonas aeruginosa. J. Antimicrob. Chemother. 68, 1757–1762. doi: 10.1093/jac/dkt094

Naas, T., Cuzon, G., Truong, H. V., and Nordmann, P. (2012). Role of ISKpn7 and deletions in blaKPC gene expression. Antimicrob. Agents Chemother. 56, 4753–4759. doi: 10.1128/AAC.00334-12

Naas, T., Cuzon, G., Villegas, M. V., Lartigue, M. F., Quinn, J. P., and Nordmann, P. (2008). Genetic structures at the origin of acquisition of the beta-lactamase bla KPC gene. Antimicrob. Agents Chemother. 52, 1257–1263. doi: 10.1128/AAC.01451-07

Peirano, G., Siki, L. M., Val Passos, V. L., Pinto, M. C., Guerra, L. R., and Asemi, M. D. (2009). Carbapenem-hydrolysing beta-lactamase KPC-2 in Klebsiella pneumoniae isolates in Rio de Janeiro, Brazil. J. Antimicrob. Chemother. 63, 265–268. doi: 10.1093/jac/dkn484

Pérez-Chaparro, P. J., Cerdeira, L. T., Queiroz, M. G., De Lima, C. P., Levy, C. V., Pavez, M., et al. (2014). Complete nucleotide sequences of two blaKPC-2-bearing IncN Plasmids isolated from sequence type 442 Klebsiella pneumoniae clinical strains four years apart. Antimicrob. Agents Chemother. 58, 2958–2960. doi: 10.1128/AAC.02341-13

Qu, F., Ying, Z., Zhang, C., Chen, Z., Chen, S., Cui, E., et al. (2014). Carbapenem-encoding extended-spectrum beta-lactamase CTX-M-55 in a clinical Shigella sonnei strain, China. Future Microbiol. 9, 1143–1150. doi: 10.2217/fmb.14.53

Roberts, A. P., Chandler, M., Courvalin, P., Guédon, G., Mullany, P., Pembroke, T., et al. (2008). Revised nomenclature for transposable genetic elements. Plasmid 60, 167–173. doi: 10.1016/j.plasmid.2008.08.001

Sampei, G., Furuya, N., Tachibana, K., Saitou, T., Suzuki, T., Mizobuchi, K., et al. (2010). Complete genome sequence of the incompatibility group II plasmid R64. Plasmid 64, 92–103. doi: 10.1016/j.plasmid.2010.05.003

Shen, P., Wei, Z., Jiang, Y., Du, X., Ji, S., Yu, Y., et al. (2009). Novel genetic environment of the carbapenem-hydrolyzing beta-lactamase KPC-2 among Enterobacteriaceae in China. Antimicrob. Agents Chemother. 53, 4333–4338. doi: 10.1128/AAC.00260-09

Siguier, P., Perochon, J., Lestrade, L., Mahillon, J., and Chandler, M. (2006). ISfinder: the reference centre for bacterial insertion sequences. Nucleic Acids Res. 34, D32–D36. doi: 10.1093/nar/gkj014

Souli, M., Galani, I., Antoniadou, A., Papadomichelakis, E., Poulakou, G., Panagea, T., et al. (2010). An outbreak of infection due to beta-lactamase Klebsiella pneumoniae Carbanapenemase 2-producing K. pneumoniae in a Greek University Hospital: molecular characterization, epidemiology, and outcomes. Clin. Infect. Dis. 50, 364–373. doi: 10.1086/649865

Zouveilekis, L. S., Markogiannakis, A., Psichogiou, M., Tassios, P. T., and Daikos, G. L. (2012). Carbanapenemases in Klebsiella pneumoniae and other
Enterobacteriaceae: an evolving crisis of global dimensions. *Clin. Microbiol. Rev.* 25, 682–707. doi: 10.1128/CMR.05035-11

Woodford, N., Carattoli, A., Karisik, E., Underwood, A., Ellington, M. J., and Livermore, D. M. (2009). Complete nucleotide sequences of plasmids pEK204, pEK499, and pEK516, encoding CTX-M enzymes in three major *Escherichia coli* lineages from the United Kingdom, all belonging to the international O25:H4-ST131 clone. *Antimicrob. Agents Chemother.* 53, 4472–4482. doi: 10.1128/AAC.00688-09

Zhang, Y., Gao, H., Wang, L., Xiao, X., Tan, Y., Guo, Z., et al. (2011). Molecular characterization of transcriptional regulation of rovA by PhoP and RovA in *Yersinia pestis*. *PLoS ONE* 6:e25484. doi: 10.1371/journal.pone.0025484

Zong, Z., Partridge, S. R., and Iredell, J. R. (2010). ISEcp1-mediated transposition and homologous recombination can explain the context of bla(CTX-M-62) linked to qnrB2. *Antimicrob. Agents Chemother.* 54, 3039–3042. doi: 10.1128/AAC.00041-10

**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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