Genetic Characterization of a \textit{bla}_{\text{VIM–24}}-Carrying IncP-7β Plasmid \textit{p1160-VIM} and a \textit{bla}_{\text{VIM–4}}-Harboring Integrative and Conjugative Element \textit{Tn6413} From Clinical \textit{Pseudomonas aeruginosa}

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This study presents three novel integrons In1394, In1395, and In1443, three novel unit transposons Tn6392, Tn6393, and Tn6403, one novel conjugative element (ICE) Tn6413, and the first sequenced IncP-7 resistance plasmid \textit{p1160-VIM} from clinical \textit{P. aeruginosa}. Detailed sequence comparison of \textit{p1160-VIM} (carrying \textit{bla}_{\text{VIM–24}}) and \textit{Tn6413} (carrying \textit{bla}_{\text{VIM–4}}) with related elements were performed. Tn6392, Tn6393, and Tn6403 were generated from integration of In1394 (carrying \textit{bla}_{\text{VIM–24}}), In1395 and In1443 (carrying \textit{bla}_{\text{VIM–4}}) into prototype Tn3-family unit transposons Tn5563, Tn1403, and Tn6346, respectively. To the best of our knowledge, this is the first report of a \textit{bla}_{\text{VIM–24}}-carrying \textit{P. aeruginosa} isolate.

Keywords: IncP-7 plasmid, unit transposon, integrative and conjugative element, \textit{bla}_{\text{VIM}}, \textit{Pseudomonas aeruginosa}

INTRODUCTION

Plasmids of thirteen incompatibility groups in \textit{Pseudomonas} (IncP-1 to IncP-7 and IncP-9 to IncP-14) have been recognized, varying in genetic structure, size and host range. IncP-7 plasmids, with a narrow host range, are of particular interest in environmental biodegradative potentials. Most sequenced members of this group, such as \textit{pCAR1} (Maeda et al., 2003), \textit{pND6}_1 (Li et al., 2004), \textit{pWW53} (Pickup and Williams, 1985), \textit{pDK1} (Kunz and Chapman, 1981), and \textit{pHE24} (Supplementary Table S1), belong to toluene catabolic or degradation plasmids (D-plasmids) rather than resistance plasmids (R-plasmids).

Integrative and conjugative elements (ICEs), also known as conjugative transposons, are typically found integrated into host bacterial chromosomes and encode integrase (Int), excisionase (Xis) and type IV secretion system responsible for integration, excision, interbacterial transfer, respectively. ICEs confer antibiotic resistance (such as Tn916) (Franke and Clewell, 1981), heavy
metal resistance (such as R391) (Peters et al., 1991), and carbon utilization (such as ICEecl) (Gaillard et al., 2006).

Verona integron-encoded metallo-β-lactamase (VIM) is one of the most predominant families among class B carbapenemases and can hydrolyze nearly all β-lactams including carbapenems, except aztreonam (Queenan and Bush, 2007). This study dealt with a detailed genetic characterization of a novel blaVIM-24-carrying IncP-7β plasmid p1160-VIM and a novel blaVIM-4-carrying ICE Tn6413 recovered from two different clinical P. aeruginosa isolates.

MATERIALS AND METHODS

Bacterial Isolates
Pseudomonas aeruginosa 1160 was isolated in 2015 from a sputum specimen of an elderly patient in a teaching hospital in Hebei Province, China. P. aeruginosa 6762 was recovered in 2016 from a sputum specimen of an elderly patient in a public hospital in Lanzhou Province, China. Bacterial species was identified by 16S rRNA gene sequencing and PCR detection of P. aeruginosa-specific oafA gene (Choi et al., 2013).

Conjugal Transfer
Conjugal transfer experiments were carried out with rifampin-resistant P. aeruginosa PA01 used as recipients and each of the blaVIM-positive 1160 or 6762 isolate as 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 30°C for 12 to 18 h. Bacteria were washed from filter membrane and spread on Muller-Hinton (MH) agar (BD Biosciences) plates containing 1000 µg/ml rifampin together with 2 µg/ml meropenem for selecting an P. aeruginosa transconjugant carrying blaVIM.

Sequencing and Annotation
The genomic DNA of strain 6762 or the plasmid DNA of strain 1160 was isolated using an UltraClean Microbial Kit or a Large Construct Kit (Qiagen, NW, Germany), respectively, and then sequenced from a mate-pair library with average insert size of 5 kb (ranged from 2 to 10 kb) using a MiSeq sequencer (Illumina, CA, United States). DNA contigs were assembled based on their contig coverages using Newbler 2.6 (Nederbragt, 2014). Open reading frames and pseudogenes were predicted using RAST 2.0 (Brettin et al., 2015) combined with BLASTP/BLASTN (Borutyn et al., 2013) searches against the UniProtKB/Swiss-Prot database (Boutet et al., 2016) and the RefSeq database (O’Leary et al., 2016). Annotation of resistance genes, mobile elements, and other features was carried out using the online databases including CARD (Liang et al., 2017), ResFinder (Zankari et al., 2012), ISfinder (Siguer et al., 2006), INTEGRALL (Moura et al., 2009), and the 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 0.48.1.

Phylogenetic Analysis
The nucleotide sequences of repA coding regions of indicative plasmids were aligned using MUSCLE 3.8.31 (Edgar, 2004). The unrooted neighbor-joining trees were generated from the aligned repA sequences using MEGA7 (Kumar et al., 2016), and evolutionary distances were estimated using the maximum composite likelihood method, with a bootstrap iteration of 1000.

Phenotypic Assays
Activity of Ambler class A/B/D carbapenemases in bacterial cell extracts was determined by a modified CarbaNP test (Wei et al., 2016). Bacterial antimicrobial susceptibility was tested by BioMérieux VITEK 2 and interpreted as per the 2017 Clinical and Laboratory Standards Institute (CLSI) guidelines (Wayne, 2017).

Nucleotide Sequence Accession Numbers
The sequence of p1160-VIM and that of the 6762 chromosome were submitted to GenBank under accession numbers MF144194 and CP030075, respectively.

RESULTS AND DISCUSSION
Overview of Sequenced p1160-VIM and Tn6413
Two blaVIM-positive P. aeruginosa isolates, designated 1160 and 6762, were subjected to high-throughput genome sequencing. The 1160 isolate harbored a blaVIM-24-carrying plasmid p1160-VIM, which had a circular DNA sequence of 205.4 kb in length, with an average G+C content of 56.3%. p1160-VIM belonged to the IncP-7 group because it had a IncP-7 repA gene responsible for plasmid replication initiation.

A 114.1-kb blaVIM-4-harboring ICE Tn6413 was found to integrate into tRNA^Glu gene in the 6762 chromosome. The modular structure of each of p1160-VIM and Tn6413 was divided into the backbone (responsible for replication, maintenance and conjugal transfer) and separate accessory modules (defined as acquired DNA regions associated with mobile elements) integrated at different sites of the backbone (Supplementary Figures S1, S2 and Table 1).

p1160-VIM could be transferred from the 1160 isolate into P. aeruginosa PA01 through conjugation, generating the transconjugant 1160-VIM-PA01. The self-transmissible nature of p1160-VIM was consistent with the presence of complete conjugal transfer regions in this plasmid. Strains 1160 and 1160-VIM-PA01 had class B carbapenemase activity, and they were resistant to cefuroxime, cefazidime, ceftriaxone and cephalase (with minimal inhibitory concentration values ≥ 64), and imipenem and meropenem (with minimal inhibitory concentration values ≥ 4), which were resulted from production

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1https://inkscape.org/en/
of VIM enzymes in these strains. Repeated conjugation attempts failed to transfer Tn6413 from the 6762 isolate to PAO1.

**Subgrouping of IncP-7 Plasmids Including p1160-VIM**

A group of ten completely or partially sequenced plasmids (Supplementary Table S1; including p1160-VIM) with IncP-7 repA genes (≥ 95% nucleotide identity to that of p1160-VIM), were collected, and two phylogenetic trees (Figure 1) were constructed based on repA nucleotide and amino acid sequences, respectively. These ten plasmids could be divided into two separately clustering subgroups designated IncP-7α and IncP-7β. As shown by pairwise comparison of repA nucleotide sequences, plasmids within each of these two subgroups showed ≥ 99% nucleotide identity, while plasmids from these two different subgroups displayed ≤ 96% nucleotide identity (Supplementary Table S2a). Considerable genetic diversity was found between the repA genes of IncP-7α and IncP-7β, representing two separated lineages.

Predicted iterons (RepA-binding sites) were found within the oriV region downstream of repA, and plasmids from both subgroups shared a conserved iteron motif and an identical iteron copy number (Figure 1 and Supplementary Table S1).

pCAR1 (Maeda et al., 2003) and pDK1 (Kunz and Chapman, 1981) were identified as IncP-7α and IncP-7β reference plasmids, respectively, because they were the first sequenced plasmids harboring complete conjugal transfer regions. In the phylogenetic

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**TABLE 1 | Major features of plasmids and ICEs analyzed.**

| Category | Plasmids | Chromosomally integrated ICEs |
|----------|----------|------------------------------|
| Accession number | pCAR1 AB088420 | Tn6413 CP030075 |
| Group | pDK1 AB434906 | Tn6533 AP014651 |
| Reference of the relevant group | p1160-VIM MF144194 | Tn6534 KX196168 |
| Total length (bp) | Tn6417 Cp013993 |
| Total number of ORFs | 157 | Tn6417 Yes |
| Mean G+C content, % | 60.5 | Tn6533 Yes |
| Length of the backbone (bp) | 61.3 | Tn6534 85.992 |
| Accessory modules | 115,716 84,038 | Tn6417 108,186 |
| Host bacterium | 76,947 84,181 | Tn6533 104 |
| Nucleotide positions in the chromosome | 135,455 83,215 | Tn6534 107 |
| p1160-VIM and Tn6413 were sequenced this work, and all the other elements analyzed were derived from GenBank. $, carrying resistance genes.
Comparison of p1160-VIM With pCAR1 and pDK1

pCAR1, pDK1, and p1160-VIM were included in a genomic comparison. These three plasmids had > 92% nucleotide identity across > 52% of their backbone sequences (Supplementary Table S2b), and their conserved backbone was composed of gene or gene loci responsible for replication initiation (repA), partitioning (parABCW), and conjugal transfer (rlx, cpl, tivF3, and tivF6). There were three major modular differences within their backbones (Figure 2): (i) a terABC region could be found in p1160-VIM and pCAR1 rather than pDK1; (ii) a 23.9-kb orf324–to–orf891 region was found in only p1160-VIM and pDK1. All these modular differences were resulted from integration of relevant accessory modules.

pCAR1, pDK1, and p1160-VIM carried totally different profiles of accessory modules (Table 1), which were composed of 10 distinct IS elements (ISPre2, ISPre3, ISPre4, IS1162, ISpa73, ISpa75, ISpa79, ISpa80, and ISpa83), 5 different intact Tn3-family unit transposons (Tn4676 from pCAR1, Tn4662, and Tn4663 from pDK1, and Tn6392 and Tn6393 from p1160-VIM; a typical unit transposon encodes a transposase and a site-specific recombinase or resolvase as core transposition determinants, and also carries one or several accessory genes), and one Tn3-family transposon remnant. Only Tn6392 and Tn6393 of the above accessory modules (Table 2). Tn4676 (Supplementary Figure S4a) carried core transposition genes (tnpAC and tnpST) genetically related to Tn4651 (Maeda et al., 2003), and also
an ant (two-component anthranilate 1,2-dioxygenase) operon (Urata et al., 2004) interrupted by insertion of ISPre1 and a car (carbazole/dioxin degradation) operon (Nojiri et al., 2001). Tn4662 encoded a RelBE toxin-antitoxin system involved in plasmid maintenance. Tn4663 (Supplementary Figure S4b) was derived from Tn4659 (Yano et al., 2007) and harbored a toluene-catabolic xyl gene cluster (Yano et al., 2010).

Comparison of Tn6392 With Tn5563
Tn6392 (Figure 3) from p1160-VIM was a novel derivative of Tn5563, which was originally characterized in P. alcaligenes and had the structure IRL (inverted repeat left)–tnpR (resolvase)–res (resolution site)–orf2 (hypothetical protein)–pliT (pliT domain-containing protein)–tnpA (transposase)–mer (mercuric resistance gene locus)–IRR (inverted repeat right), bracketed by 5-bp or 7-bp direct repeats (DRs; target site duplication signals) at both ends (Yeo et al., 1998). Tn6392 differed from Tn5563 by insertion of a novel class 1 integron In1394 into res. The prototype Tn402-associated class 1 integron was typically organized as IRi (inverted repeat at the integrase end), 5′-CS [5′-conserved segment: intI1 (integrase)-attI1 (a specific recombination site)], GCA (gene cassette array), 3′-CS [3′-conserved segment: qacED1–sul1–orf5–orf6], a Tn402 tni module [tniA (transposase)–tniB (ATP-binding protein)–tniQ (transposition auxiliary protein)–res–tniR (serine resolvase)], and IRT (inverted repeat at the tni end) (Gillings et al., 2008). In1394, bracketed by 5 bp DRs at both ends, contained all the above core integron structures except 3′-CS. The GCA of In1394 consisted of a blaVIM–24 gene and two copies of aacA4.

Comparison of Tn6393 With Tn1403
Tn6393 (Figure 4) was a novel derivative from Tn1403 after insertion of a novel class 1 integron In1395 instead of In28 at the same position within res. Tn1403 was initially identified in P. aeruginosa and displayed a backbone structure IRL–tnpAR–res–sup–uspA–dksA–yjiK–IRR, with integration of accessory modules In28 and Tn5393c into res and dksA, respectively (Stokes et al., 2007). In1395 belonged to complex class 1 integron, which was typically organized as IRI–5′-CS–VR1 (variable region 1)–3′-CS1 (the first copy of 3′-CS1: qacED1–sul1)–ISCR1 (comment region)–VR2 (variable region 2)–3′-CS2 (a second 3′-CS: qacED1–sul1–orf5–orf6)–tni–IRT. In1395, bracketed by 5-bp DRs at both ends, was composed
of IRi, 5′-CS, VR1 [GCA: gcu104–aacA1–catB3q1Spa62–gcu161–ereA1c:ISpa62], 3′-CS1, ISCR1 (further interrupted by ΔTn4662b–ΔIs6505–msr(E)–mph(E)–Tn4662b), VR2 [containing qnr, ΔISCR1, folA and other genes], 3′-CS2, IS6100 (replacing tni) and IRt.

Comparison of Tn6413 With Tn6534, Tn6533, and Tn6417

Tn6413 (Supplementary Figure S2) was a novel ICE that could be divided into a single 30-kb accessory module Tn6403 (Figure 6) and the remaining backbone regions. Tn6413 belonged to a collection of 31 ICE or ICE-like sequences (Supplementary Table S3, including Tn6417, Tn6534, and Tn6533) with > 95% nucleotide identity across > 59% of Tn6413 backbone. Tn6417 was the first sequenced one and identified as the reference of these 31 Tn6417-family ICE sequences. A genomic comparison (Figure 5) was subjected to Tn6413, Tn6534, Tn6533, and Tn6417 because they shared mostly highly similar backbones with 99% nucleotide identity and > 94% query coverage. These four Tn6417-family ICEs, which genetically differed from the two existing ICE families in P. aeruginosa (Kung et al., 2010) shared conserved DNA processing and conjugation genes. Three major modular differences were found within the backbones of these four ICEs: (i) presence of orf348 in only Tn6417; (ii) presence of orf645 in only Tn6417; and (iii) 3′-terminal regions (orf432–orf1188, orf693–orf468, and orf693–orf1068 from Tn6413, Tn6534, Tn6533 and Tn6417, respectively) differed from one another.

Each of these four Tn6417-family ICEs carried a single accessory module: Tn6403, Tn6531, Tn6530, and Tn6532 (Figure 6) from Tn6413, Tn6533, Tn6534, and Tn6417, respectively; all these accessory modules were integrated at the same site of the ICE backbones and identified as Tn6346 derivatives. The Tn3-family unit transposon Tn6346, originally found in heavy metal-tolerant Achromobacter spp., was a hybrid of the core transposition module tnpAR–res of Tn5051 and the mer region of Tn501 (Ng et al., 2009). Tn6403, Tn6531, Tn6530, and Tn6532 differed from Tn6346 by (i) interruption of original tnpAR of Tn6346 due to insertion of IS1071, and (ii) insertion of four different class 1 integrons at the same position within the urf2 gene of mer. Tn6403, Tn6530 and Tn6532, rather than Tn6531, were bracketed by 5-bp DRs.

In127, In1328, In1155, and In159 found in Tn6403, Tn6531, Tn6530, and Tn6532, respectively, were intact integrons because all of them had paired terminal 25-bp repeats. Except
In1155, all the other three were bracketed by 5-bp DRs. Notably, these integrons captured additional elements beside GCAs: IS26–msr(E)–mph(E)–IS26 unit and a novel bla\textit{VIM}–4-carrying class 1 integron In1443, \textit{cmi}A9–tetRA(G)–Tn5393c–ISCR3 and \textit{cmx}–carrying In0, \textit{cmi}A9–tetRA(G)–ISCR3 and empty In0, and empty In0 in In127, In1328, In1155, and In159, respectively. In1443 was organized as IRi–5'–CS (interrupted by insertion of IS\textit{Pa}82)–GCA (bla\textit{VIM}–4–\textit{aad}A7–\textit{aad}A4)–\Delta 3'–CS–IS6100 (replacing \textit{tni})–IRt.

CONCLUSION

IncP-7 R-plasmids are not commonly found in natural isolates, and p1160-VIM represents the first fully sequenced IncP-7 R-plasmid. Based on \textit{rep}A sequences, IncP-7 plasmids can be further divided into two separately clustering subgroups IncP-7\textit{a} and IncP-7\textit{b}. The two novel bla\textit{VIM}-carrying transposons Tn6392 and Tn6413, which are integrated into the IncP-7\textit{b} plasmid p1160-VIM and the \textit{P. aeruginosa} chromosome, respectively, represent two different categories of transposons: Tn3-family unit transposon and Tn6417-family ICE. Tn6392 and Tn6413 contain novel class 1 integrons In1394 and In1443, which harbor the two GCAs \textit{aac}A4–bla\textit{VIM}–24–\textit{aac}A4 and \textit{bla\textit{VIM}–4–\textit{aad}A7–\textit{aad}A4, respectively. The \textit{bla\textit{VIM}–24} gene was initially discovered from a \textit{Klebsiella pneumoniae} isolate in Colombia in 2011 (Montealegre et al., 2011). This study presents the first report of a \textit{bla\textit{VIM}–24}-carrying \textit{P. aeruginosa} isolate and a \textit{bla\textit{VIM}–4}-carrying IncP-7 plasmid. Both p1160-VIM and Tn6413 are conjugative (self-transmissible) mobile elements, promoting horizontal transfer of resistance genes carried. Presence of IRi/IRt and a complete \textit{tni} module would ensure In1394 self-transferable, while replacement of \textit{tni} by IS6100 would impair mobility of In1443. Class 1 integrons (e.g., In1394 and In1443)
could be integrated into a transposon (e.g., Tn6392 and Tn6413) to restore or enhance their mobility.

ETHICS STATEMENT

The use of human specimens and all related experimental protocols were approved by the Committee on Human Research of the First Affiliated Hospital of Hebei North University and that of the General Hospital of Xinjiang Military Region, and carried out in accordance with the approved guidelines. 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 ZY conceived the study and designed experimental procedures. LZ, ZZ, LH, XJ, and YJZ performed the experiments. LZ, ZZ, YJZ, JE, BG, YEZ, and WY analyzed the data. LZ, ZZ, and HY contributed reagents and materials. LZ, ZZ, YJZ, JF, BG, YEZ, and WY wrote this manuscript.

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**SUPPLEMENTARY MATERIAL**

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb. 2019.00213/full#supplementary-material

**FIGURE S1** | Plasmid schematic maps. Three plasmids pCAR1, pDK1 and p1160-VIM are included. Genes are denoted by arrows, and the backbone and accessory module regions are highlighted in black and color, respectively. Innermost circle presents GC-skew [gG−cG]+[gC−cG], with a window size of 500 bp and a step size of 20 bp. Next-to-innermost circle presents GC content.

**FIGURE S2** | ICE schematic maps. Four ICEs Tn6413, Tn6553, Tn6533, and Tn6417 are included. Genes are denoted by arrows, and the backbone and accessory module regions are highlighted in black and color, respectively. Innermost circle presents GC-skew [gG−cG]+[gC−cG], with a window size of 500 bp and a step size of 20 bp. Next-to-innermost circle presents GC content.

**FIGURE S3** | Alignment of repA nucleotide sequences. Red-labeled nucleotides indicate SNP sites. Sequence of IncP-7*β* reference plasmid pDK1 was bolded.

**FIGURE S4** | Organization of Tn4676 or Tn4663 comparison with related regions. Genes are denoted by arrows. Genes, mobile elements and other features are colored based on their functional classification. Blue shading denotes regions of homology (nucleotide identity > 95%), and pink shading denotes regions of homology (average nucleotide identity 82%). Numbers in brackets indicate nucleotide positions within corresponding plasmid sequences. Accession number of Tn6551 for reference is AJ544088.

**TABLE S1** | Collection of repA and iteron sequences of IncP-7 plasmids.

**TABLE S2** | Pairwise comparison of repA and backbone sequences.

**TABLE S3** | List of the Tn6417-related sequences.
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