

# Genetic Characterization of Four Groups of Chromosome-Borne Accessory Genetic Elements Carrying Drug Resistance Genes in *Providencia*

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**Purpose:** The aim of this study was to gain a deeper genomics and bioinformatics understanding of diversification of accessory genetic elements (AGEs) in *Providencia*.

**Methods:** Herein, the complete genome sequences of five *Providencia* isolates from China were determined, and seven AGEs were identified from the chromosomes. Detailed genetic dissection and sequence comparison were applied to these seven AGEs, together with additional 10 chromosomal ones from GenBank (nine of them came from *Providencia*).

**Results:** These 17 AGEs were divided into four groups: Tn6512 and its six derivatives, Tn6872 and its two derivatives, Tn6875 and its one derivative, and Tn7 and its four derivatives. These AGEs display high-level diversification in modular structures that had complex mosaic natures, and particularly different multidrug resistance (MDR) regions were presented in these AGEs. At least 52 drug resistance genes, involved in resistance to 15 different categories of antimicrobials and heavy metal, were found in 15 of these 17 AGEs.

**Conclusion:** Integration of these AGEs into the *Providencia* chromosomes would contribute to the accumulation and distribution of drug resistance genes and enhance the ability of *Providencia* isolates to survive under drug selection pressure.

**Keywords:** *Providencia*, integrative and conjugative elements, integrative and mobilizable elements, unit transposons, multidrug resistance

## Introduction

*Providencia* species, mostly frequently identified as *Providencia rettgeri* and *Providencia stuartii*, are opportunistic pathogens causing urinary tract infections, diarrhea, and bacteremia in immunocompromised patients.<sup>1</sup> *Providencia* is intrinsically resistant to penicillins and the first-generation cephalosporins due to inducible expression of AmpC  $\beta$ -lactamases,<sup>2</sup> aminoglycosides by the reason of inducible expression of AAC(2')-Ia,<sup>3</sup> tetracyclines because of constitutive expression of a multidrug efflux pump AcrAB,<sup>4</sup> and polymyxins resulting from the presence of a cell envelop that inhibits colistin to combine with the susceptible lipid target sites or the lipid A modification to reduce binding.<sup>5</sup> In recent years, the wide use of aminoglycosides,  $\beta$ -lactams, and fluoroquinolones for antimicrobial therapy aggravates the acquisition and dissemination of diverse antimicrobial resistance genes in *Providencia*.<sup>6</sup> Acquired antimicrobial

resistance genes in *Providencia* are commonly captured and horizontally transferred by accessory genetic elements (AGEs), such as integrative and conjugative elements (ICEs), integrative and mobilizable elements (IMEs), and unit transposons.

ICEs have the ability to transfer from one cell to another cell through conjugation and are autonomous in conjugation by mainly utilizing a type IV secretion system (T4SS) and a coupling protein.<sup>7</sup> Up to now, three distinct families of ICEs, namely Tn6512 (R391),<sup>8</sup> ICEEc2,<sup>9</sup> and ICEPm1<sup>10</sup> have been reported in *Providencia*. Tn6512 is initially found in *Providencia rettgeri* in 1972,<sup>8</sup> and since then a wealth of Tn6512-related ICEs have been identified in not only *Providencia*,<sup>8,11</sup> but also Enterobacteriaceae,<sup>12</sup> Vibrionaceae,<sup>13</sup> *Shewanella*,<sup>14</sup> *Actinobacillus*,<sup>15</sup> *Alteromonas*,<sup>16</sup> *Pseudoalteromonas*.<sup>17</sup> Tn6512-related ICEs carry at least five hotspots (located within the intragenic or intergenic sites of their backbone regions) for integration of foreign resistance genes,<sup>18</sup> which causes these ICEs being mosaic and diversified with respect to their modular structures.

IMEs do not encode the T4SS machinery and the coupling protein and thus is nonautonomous in conjugation.<sup>19</sup> The conjugal transfer of an IME from a donor cell into a recipient cell relies on the T4SS gene sets of a helper conjugative element such as an IncA/C conjugative plasmid.<sup>20</sup> So far, three distinct families of IMEs, namely Tn6523 (SG11),<sup>21</sup> Tn6588,<sup>22</sup> and Tn6591<sup>22</sup> have been reported in *Providencia*.

ICEs and IMEs can be transferred in an intercellular manner, while the Tn7-family unit transposons use a “cut-and-paste” transposition mechanism to transfer intracellularly, which is typically promoted by the core transposition determinants TnsA (endonuclease), TnsB (transposase), TnsC (transposition regulator), and TnsD plus TnsE (target-site selection proteins).<sup>23</sup> Tn7-family transposons have widely been found as the vectors of diverse antimicrobial resistance genes in Morganellaceae including *Providencia rettgeri*,<sup>22</sup> Enterobacteriaceae,<sup>24</sup> Pseudomonadaceae,<sup>25</sup> Burkholderiaceae,<sup>26</sup> *Acinetobacter*,<sup>27</sup> *Shewanella*,<sup>24</sup> *Hahella*,<sup>24</sup> *Pelobacter*,<sup>24</sup> *Idiomarina*,<sup>24</sup> *Acidithiobacillus*,<sup>24</sup> *Neisseria* (eg *N. brasiliensis* with an accession number CP046027), *Nitrosomonas* (eg *N. stercoris* with an accession number AP019755), and *Bacillus*.<sup>24</sup>

This study presented the complete sequences of seven chromosome-borne AGEs in five sequenced *Providencia* isolates from China. Detailed genetic dissection and comparison were applied to these seven AGEs, together with additional 10 chromosome-borne ones from GenBank (nine of them came from *Providencia*). These 17 AGEs could be classified into ICEs, IMEs, and Tn7 derivatives. Data presented here provided a deeper genomics and bioinformatics understanding of diversification of AGEs in *Providencia*.

## Materials and Methods

*Providencia rettgeri* PROV275, PROV002, and PROV087 (Table S1) causing nosocomial infections were recovered from three different Chinese public hospitals in 2014, 2013, and 2016, respectively. *Providencia alcalifaciens* PROV023 and PROV013 (Table S1) were collected from two different China livestock farms in 2017. Whole-genome sequencing of these five *Providencia* isolates were conducted with a sheared DNA library with average size of 15 kb (ranged from 10 kb to 20 kb) on a PacBio RSII sequencer (Pacific Biosciences, CA, USA) and further sequence data mining was performed as described previously.<sup>28</sup> The sequencing data was conducted using NanoPack<sup>29</sup> and FastQC (<https://www.bioinformatics.babraham.ac.uk/projects/fastqc>) (Table S2). Conjugal transfer of Tn6862 from the wild-type PROV023 isolate into the rifampin-resistant recipient strain *Escherichia coli* EC600 was performed as described previously.<sup>30</sup> Bacterial antimicrobial susceptibility was tested by BioMérieux VITEK 2, and interpreted as per the 2020 Clinical and Laboratory Standards Institute (CLSI) guidelines.<sup>31</sup> The antimicrobial drug susceptibility profiles of the five *Providencia* isolates, EC600, and the transconjugant was shown in Table S3. All the wild-type and transconjugant strains were subjected to PCR amplification followed by amplicon sequencing, for determining the sequences of bacterial 16S rDNA, the presence of key markers such as *aphA1*, *strA*, *strB*, *int*, and *cpl*, and also the location/boundary of AGEs such as Tn6860, Tn6861, Tn6862, Tn6873, and Tn6876 (data not shown). The complete chromosome sequences of the PROV275, PROV002, PROV023, PROV013, and PROV087 isolates were submitted to GenBank under accession numbers CP059298, CP059345, CP059348, CP059346, and CP059347, respectively. The GenBank accession numbers of all the six plasmids of these five isolates were listed in Table S1.

## Results

### Four Groups of 17 Chromosome-Borne AGEs

In this study, the complete genome sequences from *Providencia rettgeri* PROV275, PROV002, and PROV087 and *Providencia alcalifaciens* PROV023 and PROV013 were determined, and then a total of seven chromosome-borne AGEs were identified: i) Tn6860, Tn6861, and a 102.1-kb Tn7-related element T7RE<sub>PROV087</sub> from strains PROV275, PROV002, and PROV087, respectively; ii) Tn6862 and a 35.9-kb T7RE<sub>PROV023</sub> from strain PROV023; and iii) Tn6873 and Tn6876 from strain PROV013. Then, a detailed sequence comparison was applied to a collection of four groups of 17 chromosome-borne AGEs (16 of them from *Providencia*): i) seven related ICEs Tn6512,<sup>8</sup> Tn6860, Tn6861, Tn6862, Tn6863,<sup>18</sup> Tn6864,<sup>11</sup> and Tn6865; ii) three related IMEs Tn6872, Tn6873, and Tn6874; iii) two related IMEs Tn6875 and Tn6876; and iv) Tn7 and its four derivatives T7RE<sub>PROV087</sub>, T7RE<sub>PROV023</sub>, a 58.0-kb T7RE<sub>MF1</sub>, and a 40.7-kb T7RE<sub>Pr-15-2-50</sub> (Table 1). At least 52 drug resistance genes, involved in resistance to 15 different categories of antimicrobials and heavy metal, were identified in 15 (except for Tn6872 and Tn6875) of these 17 AGEs (Figure 1 and Table S4). All of these T7RE, T1696RE (see below), and T21RE (see below) elements could not be recognized as intact transposons due to truncation of relevant core transposition modules.

### Six Tn6512-Related ICEs Tn6860, Tn6861, Tn6862, Tn6863, Tn6864, and Tn6865

All these seven ICEs including Tn6512 were integrated into the same 17-bp target site located within the 5' end of the chromosomal gene *prfC* (peptide chain release factor 3).<sup>32</sup> These seven ICEs (Figure S1) had similar conserved backbones,<sup>33</sup> containing *int* (integrase), *xis* (excisionase), *rlx* (relaxase), *oriT* (origin of conjugative replication), *cpl* (coupling protein), a TivF-type T4SS gene set, and *attL/R* (attachment sites at the left/right end). A toxin-antitoxin system was encoded by each of the five ICEs (*hipBA* in Tn6512, and *abiEi-ii* in Tn6860, Tn6861, Tn6863, and Tn6864) except for Tn6862 and Tn6865. Type I restriction-modification system *hsdMSR* was carried by five of them except for Tn6512 and Tn6865.

The major modular differences were recognized at least 10 regions/sites (Figure 2 and Table S5) across the whole genomes of these seven ICEs. Firstly, a total of 23 events of acquisition of exogenous DNA region occurred at nine regions/sites within these seven ICEs. Secondly, a total of 12 events of backbone region deletion (10 of them were resulted from acquisition of exogenous DNA region) occurred at five regions/sites within these seven ICEs. Finally, totally 21 events of substitution of backbone region occurred at four regions/sites within six ICEs.

Each of these seven ICEs carried two to five accessory modules (Figure S1), including multidrug resistance (MDR) regions, composite transposons, IME, integron, insertion sequence (IS) elements, and so called “inserted regions”. Firstly, the 33.5-kb, 62.0-kb, 29.8-kb, 14.2-kb, 18.3-kb, and 33.0-kb MDR regions were integrated at the same site within Tn6860, Tn6861, Tn6862, Tn6863, Tn6864, and Tn6865, respectively. Secondly, an IS15DI (a minor variant of IS26)-composite transposon Tn6578 (containing *aphA1*), an IME Tn6912 (containing *bla*<sub>HMS-1</sub>), and an IS*Ppu12*-composite transposon Tn6896 were inserted into the different sites within Tn6512, Tn6860, and Tn6864, respectively. Tn6896 (Figure 3) was bracketed by 8-bp direct repeats (DRs; target site duplication signals for transposition), and carried *tetA* (C)-carrying ΔTn6309, and a concise class 1 integron In525 with the gene cassette array (GCA) *aadA2-ereA-dfrA32*. Thirdly, a class 9 integron In9-1 (containing *dfrA1*) was inserted within Tn6863. Finally, multiple IS elements and inserted regions were found at the different sites within these ICEs.

These six MDR regions (Figure 4) shared only a 0.5-kb Tn3-family Tn remnant together with an IS*Shf9* element, and they carried dramatically different profiles of resistance loci. The 33.5-kb MDR region carried three resistance loci: a truncated ISCR2-*floR* unit, a 12.6-kb region (containing *bla*<sub>CTX-M-3</sub>-carrying ΔTn6502a) from IncM plasmid R69,<sup>34</sup> and IS*Ec29-mph*(E)-IS15DI unit. The 62.0-kb MDR region was composed of five resistance loci: a 23.4-kb Tn1696-related element T1696RE<sub>PROV002</sub> (see below), *tetA*(C)-carrying Tn6309, a concise class 1 integron In1793 (GCA: *gacG2-aadA6-gacG2*), a truncated IS26-*mph*(A)-IS6100 unit, and *mer* region. The 29.8-kb, 14.2-kb, and 18.3-kb MDR regions shared a truncated ISCR2-*sul2* unit (containing *strAB*-carrying ΔTn5393c), but each of them integrated one or two additional resistance loci: i) an interrupted ISCR2-*floR* unit and an IS1006-composite transposon Tn6976 (which was bracketed by 5-bp DRs and contained two resistance loci: *aphA1*-carrying ΔTn4352 and IS26-*aacC4-aph*

**Table I** Major Features of the 17 AGEs Characterized in This Work

Group	Accessory Genetic Element	Accession Number	Chromosomal Nucleotide Position	Length (bp)	Host Bacterium	Reference
Tn6512-related ICEs	Tn6512	AY090559	Not applicable	88,549	<i>Providencia rettgeri</i> 107	8
	Tn6860	CP059298	575696.691964	116,269	<i>Providencia rettgeri</i> PROV275	This study
	Tn6861	CP059345	727272.869956	142,685	<i>Providencia rettgeri</i> PROV002	This study
	Tn6862	CP059348	704853.810761	105,909	<i>Providencia alcalifaciens</i> PROV023	This study
	Tn6863	GQ463139	Not applicable	96,586	<i>Providencia alcalifaciens</i> Ban I	18
	Tn6864	MT219827	Not applicable	107,207	<i>Providencia rettgeri</i> RF14-2	11
	Tn6865	CP031123	751328.820050	68,723	<i>Providencia huaxiensis</i> WCHPr000369	Not applicable
Tn6872-related IMEs	Tn6872	LR134189	025117.3045606	20,490	<i>Providencia rustigianii</i> NCTC6933	Not applicable
	Tn6873	CP059346	2973270.3043055	69,786	<i>Providencia alcalifaciens</i> PROV013	This study
	Tn6874	AP022371	3358722.3420122	61,401	<i>Providencia rettgeri</i> BML2496	Not applicable
Tn6875-related IMEs	Tn6875	CP031508	1032159.1038038	5880	<i>Providencia stuartii</i> FDAARGOS_87	Not applicable
	Tn6876	CP059346	2583916.2605820	21,905	<i>Providencia alcalifaciens</i> PROV013	This study
Tn7-related elements	Tn7	KX117211	Not applicable	14,067	<i>Escherichia coli</i> 3.5-R3	47
	102.1-kb T7RE <sub>PROV087</sub>	CP059347	33162.135304	102,143	<i>Providencia rettgeri</i> PROV087	This study
	35.9-kb T7RE <sub>PROV023</sub>	CP059348	47906.83831	35,926	<i>Providencia alcalifaciens</i> PROV023	This study
	58.0-kb T7RE <sub>MFI</sub>	CP048621	1077700.1135740	58,041	<i>Providencia stuartii</i> MFI	Not applicable
	40.7-kb T7RE <sub>Pr-15-2-50</sub>	CP039844	31494.72272	40,779	<i>Providencia rettgeri</i> Pr-15-2-50	Not applicable

(4)-*Ia*-*ISec59* unit) in 29.8-kb MDR region; ii) *ISCR2*-*floR* unit in 14.2-kb MDR region; and iii) *ISCR2*-*erm42* unit and a truncated *ISCR2*-*tet*(X6) unit in 18.3-kb MDR region. The 33.0-kb MDR region had the sole resistance locus In1571, whose 5'-conserved segment (5'-CS: *intI*-*attII*) was interrupted by IS26. In1571 was a complex class 1 integron, which contained two resistance loci: GCA (also called VR1: variable region 1) *fosC2*-*aacA4*'-8-*ereA1e*-*bla*<sub>OXA-21</sub>-*dfrA1r*, and VR2 composed of *ISCR1*-*qnrVC1* unit and a truncated *ISCR1*-*bla*<sub>PER-4</sub> unit.





**Figure 1** Heatmap of prevalence of drug resistance genes.

**Notes:** Original data are shown in [Table S4](#).

### Three Related IMEs Tn6872, Tn6873, and Tn6874

All these three IMEs were integrated 102-bp upstream the chromosomal gene *mutS* (DNA mismatch repair protein). The prototype IME Tn6872 was initially found in *Providencia rustigianii* NCTC6933 (accession number LR134189). These three IMEs ([Figure 5](#)) shared the core IME backbone markers *int*, *oriT* and *attL/R*. Tn6872 harbored its three unique backbone regions *orf201*–to–*orf207*, *orf627*–to–*orf1068*, and *orf1338*–to–*uvrD*; correspondingly, Tn6873/Tn6874 carried their unique backbone regions *hsdMSR* (type I restriction-modification system)–*mrr*–*orf411*, *orf597*–to–*orf1107*, and *orf1311*–to–*hnhc*, instead of the above three Tn6872-unique regions, respectively. No accessory modules were found in Tn6872, but a 47.0-kb MDR region and a 38.5-kb MDR region were integrated at the same site within Tn6873 and Tn6874, respectively.

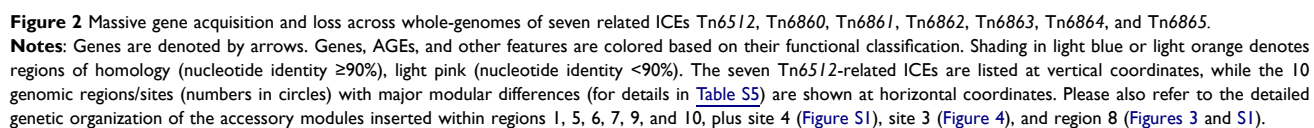
The 47.0-kb MDR region ([Figure 6](#)) contained six resistance loci: a concise class 1 integron In27 (GCA: *dfrA12*–*gcuF*–*aadA2*),  $\Delta$ Tn4352 containing *aphA1*, IS26–*aph(4)*–*Ia*–*aacC4*–ISEc59 unit, ISCR2–*sul2* unit, a truncated ISCR2–*floR* unit, and a Tn21-related transposon Tn6974 (see below). The 38.5-kb MDR region carried an IME Tn6977, and a Tn1696-related transposon Tn6910 (see below).

### Two Related IMEs Tn6875 and Tn6876

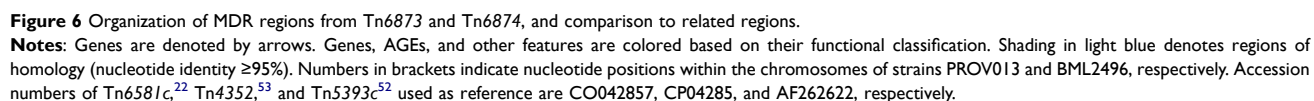
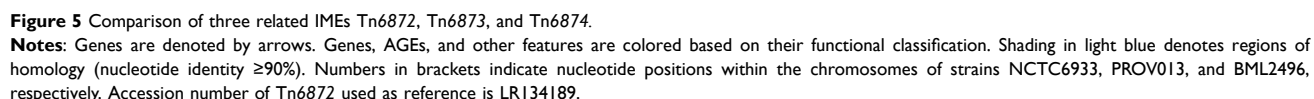
Tn6875 and Tn6876 were integrated 494-bp downstream the chromosomal gene *smpB* (SsrA-binding protein). The prototype IME Tn6875 was initially found in *Providencia stuartii* FDAARGOS\_87 (accession number CP031508). Tn6875 and Tn6876 ([Figure 7](#)) shared the core IME backbone markers *int* and *attL/R*, but they displayed two major modular variations: i) Tn6875 contained its unique backbone region *rimI*–to–*orf567*, while Tn6876 harbored its unique backbone region containing *orf288*; and ii) Tn6875 carried no accessory modules, but Tn6876 acquired a 20.0-kb MDR region composed of *tetA(B)*–containing Tn10 and 10.4-kb T1696RE<sub>PROV013</sub> (see below).

### Four Tn7 Derivatives T7RE<sub>PROV087</sub>, T7RE<sub>PROV023</sub>, T7RE<sub>MF1</sub>, and T7RE<sub>Pr-15-2-50</sub>

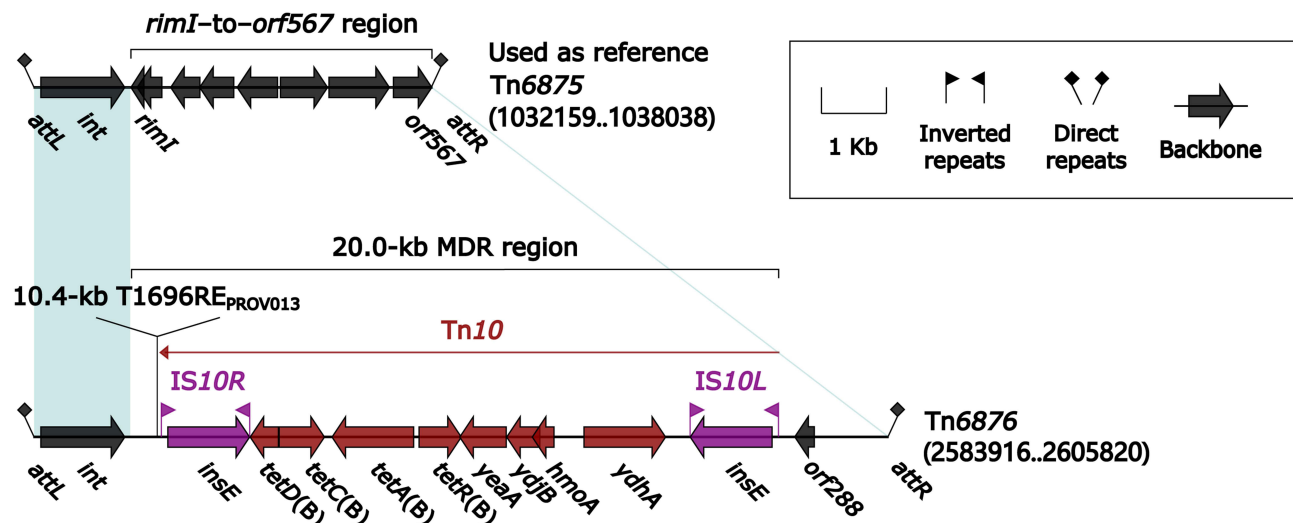
Tn7 and its three derivatives T7RE<sub>PROV087</sub>, T7RE<sub>PROV023</sub>, and T7RE<sub>Pr-15-2-50</sub> were inserted 25-bp downstream of the chromosomal gene *glmS* (glutamine-fructose-6-phosphate aminotransferase), while the remaining one T7RE<sub>MF1</sub> was inserted within the chromosomal gene *orf1389* (carbohydrate porin). T7RE<sub>PROV087</sub>, T7RE<sub>PROV023</sub>, T7RE<sub>MF1</sub>, and







These four MDR regions (Figure 9) shared only a 1.7-kb Tn/722 remnant together with an IS26 element, and they displayed considerable modular diversification: i) a concise class 2 integron In2-16 with GCA *lnu(F)Ib-dfrA1-aadA1a* in the MDR regions from T7RE<sub>PROV087</sub>, T7RE<sub>PROV023</sub>, and T7RE<sub>MEF1</sub>, while a complex In2-16 carrying VR1/GCA as



**Figure 7** Comparison of two related IMEs Tn6875 and Tn6876.

**Notes:** Genes are denoted by arrows. Genes, AGEs, and other features are colored based on their functional classification. Shading in light blue denotes regions of homology (nucleotide identity  $\geq 95\%$ ). Numbers in brackets indicate nucleotide positions within the chromosomes of strains FDAARGOS\_87 and PROV013, respectively. Accession number of Tn6875 used as reference is CP031508.

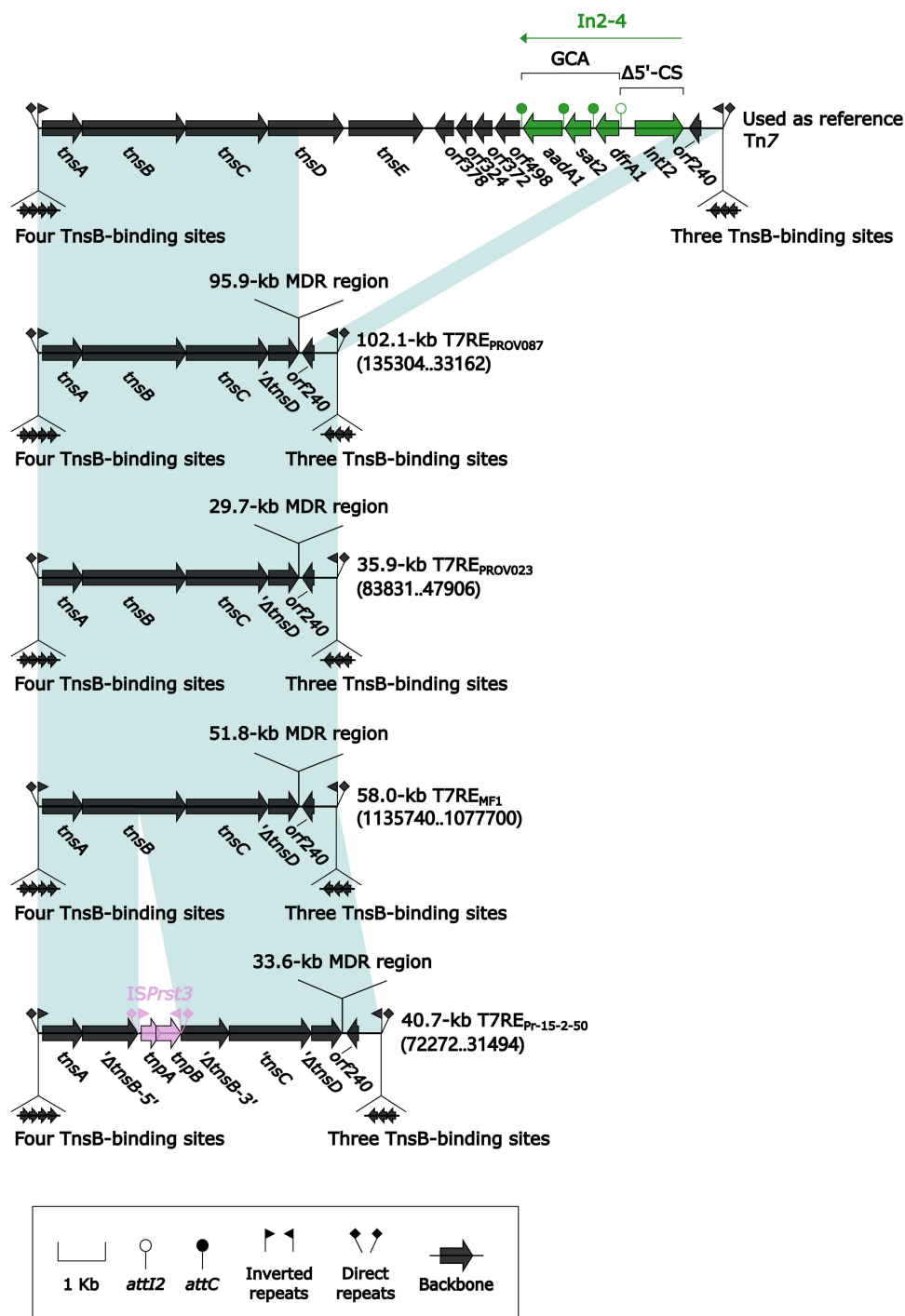
above and additionally two copies of VR2 (*ISCR1*–*ble*<sub>MBL</sub>–*bla*<sub>NDM-1</sub> unit) in the MDR region from T7RE<sub>Pr-15-2-50</sub>; ii) a truncated *ISCR2*–*floR* unit, *ISCR2*–*sul2* unit, *IS26*–*aacC4*–*aph(4)*–*Ia*–*ISec59* unit, and a truncated *chrA*–*orf98* unit shared by the MDR regions from T7RE<sub>PROV087</sub> and T7RE<sub>MF1</sub>; iii) a 71.2-kb Tn21-related element T21RE<sub>PROV087</sub> (see below) and a 21.2-kb T21RE<sub>MF1</sub> (see below) in the MDR regions from T7RE<sub>PROV087</sub> and T7RE<sub>MF1</sub>, respectively; iv) *IS26*–*mph(E)*–*IS26* unit acquired by the MDR regions from T7RE<sub>MF1</sub> and T7RE<sub>PROV023</sub>; v) intact or truncated Tn4352 (containing *aphA1*) in the MDR regions from T7RE<sub>MF1</sub> and T7RE<sub>Pr-15-2-50</sub>; vi) a complex class 1 integron In37 with four resistance loci: VR1/GCA (*aacA4cr*–*bla*<sub>OXA-1</sub>–*catB3*–*arr-3*), a disrupted Tn2 (containing *bla*<sub>TEM-1</sub>),  $\Delta$ Tn6502a (containing *bla*<sub>CTX-M-3</sub>), and VR2 (*ISCR1*–*rmtB* unit) in the MDR region from T7RE<sub>PROV023</sub>; and vii)  $\Delta$ Tn1548 carrying *ISCR1*–*armA* unit and an interrupted *ISec29*–*mph(E)*–*IS15DI* unit in the MDR region from T7RE<sub>Pr-15-2-50</sub>.

### Three Tn1696 Derivatives Tn6910, T1696RE<sub>PROV002</sub>, and T1696RE<sub>PROV013</sub>

Herein, a detailed sequence comparison (Figure 10) was applied to the three Tn1696 derivatives Tn6910, T1696RE<sub>PROV002</sub>, and T1696RE<sub>PROV013</sub> (identified as the inner components of Tn6874, Tn6861, and Tn6876, respectively; see above), together with Tn1696,<sup>35</sup> Tn6338,<sup>36</sup> and Tn6909<sup>22</sup> derived from GenBank. Tn1696 was one of the Tn3-family prototype unit transposons, and it was originally found in plasmid R1003 from *Pseudomonas aeruginosa* and contained a core backbone structure: IRL (inverted repeat left)–*tnpA*–*tnpR* (resolvase)–*res* (resolution site)–*mer*–IRR (inverted repeat right).<sup>35</sup> These above five Tn1696 derivatives exhibited three major modular differences cross the core backbones: i) an intact version of *tnpAR*–*res* in Tn6338, Tn6909, and Tn6910, while a truncated version in T1696RE<sub>PROV002</sub> and T1696RE<sub>PROV013</sub>; ii) a Tn1696 *mer* locus in Tn6338 and Tn6910, and a Tn21 *mer* locus instead in Tn6909; none of *mer* locus in T1696RE<sub>PROV002</sub> and T1696RE<sub>PROV013</sub>; and iii) intact IRL/R in Tn6910, and IRL/R interrupted by insertion of *IS4321* in Tn6909; only IRL in T1696RE<sub>PROV013</sub>, and IRL interrupted by insertion of *IS5075* in Tn6338; only IRR in Tn6338; none of IRL/R in T1696RE<sub>PROV002</sub>.

Each of these five Tn1696 derivatives acquired one or more integrons into *res* site, instead of In4 (GCA: *aacC1*–*gcuE*–*aadA2*–*cmlA1*) in Tn1696: a complex class 1 integron In469 in Tn6338, a different complex In469 in T1696RE<sub>PROV002</sub>, a concise class 1 integron In27 (GCA: *dfrA12*–*gcuF*–*aadA2*) in T1696RE<sub>PROV013</sub>, a distinct complex In27 in Tn6909, and the four tandem concise class 1 integrons including In1849 (GCA: *dfrA1t*–*aacA4*–*bla*<sub>OXA-1</sub>), two copies of In994 (GCA: *bla*<sub>IMP-1</sub>), and In151 [GCA: *catB8*–*lnu(F)3*] in Tn6910. These two complex In469 harbored the same VR1/GCA (*arr-3*–*dfrA27*–*aadA16*), but differed from each other by capturing different additional VR modules: VR2 (*ISCR1*–*qnrB4*–*bla*<sub>DHA-1</sub> unit) in In469 from Tn6338, and VR2 (*ISCR1*–*bla*<sub>PER-1</sub> unit) together with VR3 (a truncated *ISCR1*–*aphA6* unit,  $\Delta$ ISSod18,



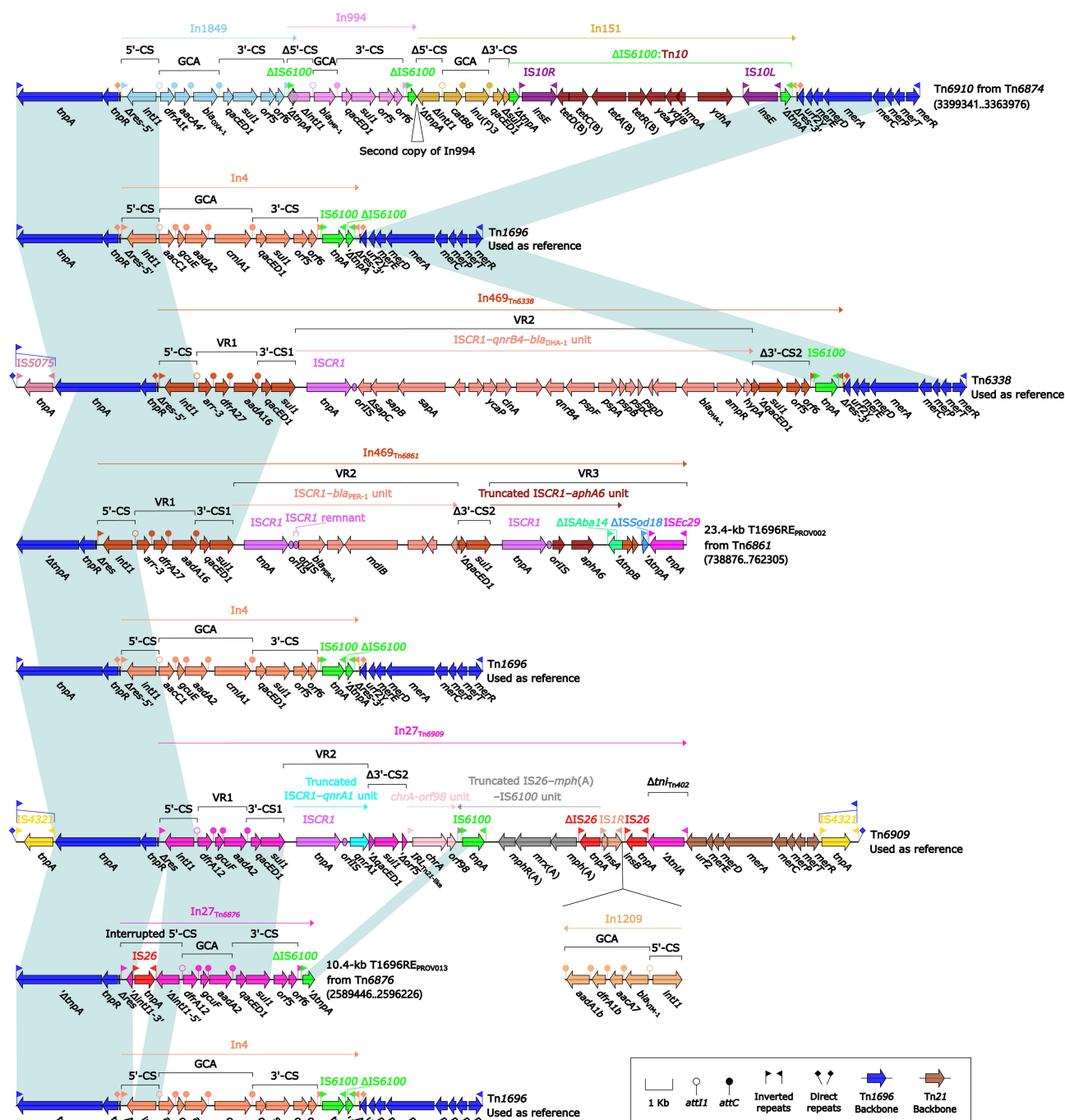


**Figure 8** Comparison of Tn7 and its four derivatives.

**Notes:** Genes are denoted by arrows. Genes, AGEs, and other features are colored based on their functional classification. Shading in light blue denotes regions of homology (nucleotide identity  $\geq 95\%$ ). Numbers in brackets indicate nucleotide positions within the chromosomes of strains PROV087, PROV023, MF1, and Pr-15-2-50, respectively. Accession number of Tn7<sup>47</sup> used as reference is KX117211.

and ISEc29) in In469 from T1696RE<sub>PROV002</sub>. The complex In27 from Tn6909 carried GCA (VR1) as above and still captured four additional resistance modules: a truncated ISCR1-*qnrA1* unit (VR2), *chrA-orf98* unit, a truncated IS26-*mph* (A)-IS6100 unit, and a concise class 1 integron In1209 (GCA: *aadA1b-dfrA1b-aacA7-bla<sub>VIM-1</sub>*).



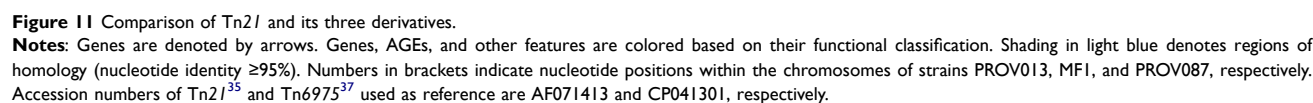


**Figure 10** Comparison of Tn1696 and its three derivatives.

**Notes:** Genes are denoted by arrows. Genes, AGEs, and other features are colored based on their functional classification. Shading in light blue denotes regions of homology (nucleotide identity  $\geq 95\%$ ). Numbers in brackets indicate nucleotide positions within the chromosomes of strains BML2496, PROV002, and PROV013, respectively. Accession numbers of Tn1696,<sup>35</sup> Tn6338,<sup>36</sup> and Tn6909<sup>22</sup> used as reference are U12338, KY270853, and CP032168, respectively.

differences: i) a Tn21 *mer* locus in Tn6975, and a Tn1696 *mer* locus instead in Tn6974; none of *mer* locus in T21RE<sub>MF1</sub> and T21RE<sub>PROV087</sub>; and ii) IRL/R in Tn6974 and Tn6975, while only IRL in T21RE<sub>PROV087</sub> and T21RE<sub>MF1</sub>.

Each of these four Tn21 derivatives acquired an integron instead of In2 (GCA: *aadA1a*) in Tn21: a concise class 1 integron In1808 (GCA: *aacA4cr*–*bla<sub>OXA-1</sub>*–*ΔcatB3*–*arr-3*–*dfrA27*–*aadA16*) in Tn6974, a concise class 1 integron In27 in Tn6975, a distinct complex In27 in T21RE<sub>MF1</sub>, and a complex class 1 integron In263 in T21RE<sub>PROV087</sub>. The concise In27 contained three resistance loci: *dfrA12*–*gcuF*–*aadA2* (GCA), *chrA*–*orf98* unit, and IS26–*mph*(A)–IS6100 unit. The



complex In27 possessed a VR1/GCA as above and further acquired a VR2 (*ISCR1*–*bla*<sub>DHA-1</sub> unit). The complex In263 had a VR1/GCA (*aacA4cr*–*arr-3*) and additionally nine copies of VR2 (truncated *ISCR1*–*bla*<sub>DHA-1</sub> unit).

## Transferability and Antimicrobial Susceptibility

Tn6862, which was selected to represent Tn6512-related ICEs, could be transferred from the wild-type PROV023 isolate into *E. coli* EC600 through conjugation, generating the transconjugant EC600/Tn6862. Both PROV023 and EC600/Tn6862 were highly resistant to amikacin with a minimum inhibitory concentration (MIC) value  $\geq 64$   $\mu\text{g/mL}$  (Table S3) owing to production of aminoglycoside 3'-phosphotransferase.

## Plasmids in the Five Isolates Sequenced in This Study

*Providencia rettgeri* PROV087 and *Providencia alcalifaciens* PROV023 carried no plasmids. Identified were an IncC plasmid pPROV275-1NDM (carrying *bla*<sub>NDM-1</sub>, *bla*<sub>CMY-6</sub>, *aacA4*, *rmtC*, *sul1*, and *qacED1*), an Inc<sub>pGZH766</sub>-NDM plasmid pPROV275-2NDM (having *bla*<sub>NDM-1</sub>, *bla*<sub>PER-4</sub>, *bla*<sub>OXA-10</sub>, *aadA1*, *aadB*, *aphA6*, *catA1*, *catB8*, *sul1*, and *qacED1*) together with a Col3M plasmid pPROV275-qnrD (containing *qnrD1*) in *Providencia rettgeri* PROV275, an IncW plasmid pPROV002-IMP (carrying *bla*<sub>IMP-4</sub>, *bla*<sub>NDM-1</sub>, *aacA4*, *catB3*, *sul1*, and *qacED1*) in *Providencia rettgeri* PROV002, and an Inc<sub>pGZH766</sub>-NDM plasmid pPROV013-PER [containing *bla*<sub>PER-4</sub>, *bla*<sub>OXA-1</sub>, *aacA4*, *mph*(E), *msr*(E), *arr-3*, *catB3*, *lnu*(G), *sul1*, and *qacED1*] together with an IncFII plasmid pPROV013-NR (harboring no resistance genes) in *Providencia alcalifaciens* PROV013.

## Discussion

This work presents the complete sequences of seven chromosomal AGEs in *Providencia*, and they can be further divided into four groups: three Tn6512-related ICEs Tn6860, Tn6861, and Tn6862; one Tn6872-related IME Tn6873; one Tn6875-related IME Tn6876; and two Tn7-related elements T7RE<sub>PROV087</sub> and T7RE<sub>PROV023</sub>. Newly identified are five (except for T7RE<sub>PROV087</sub> and T7RE<sub>PROV023</sub>) of the above seven AGEs, together with one unit transposon Tn6974, one composite transposon Tn6976, and two integrons In1793 and In1808, all of which are located within these five AGEs. There are additional 10 newly designated (firstly designated in this study, but with previously determined sequences) AGEs: i) one IME Tn6912 located within Tn6860 sequenced in this study; ii) one ICEs Tn6865, four IMEs Tn6872, Tn6874, Tn6875, and Tn6977, two unit transposons Tn6910 and Tn6975, one composite transposon Tn6896, and one IS element *ISPrre11* which come from GenBank and are included in the sequence comparison herein. This is also the first report of identifying Tn6872 and Tn6875 in *Providencia*. The two previously designated ICEs ICE<sub>PalBan1</sub> and ICE<sub>PreChnRF14-2</sub> were renamed herein as standard Tn designations Tn6863 and Tn6864, respectively.

The transposition of Tn7-related unit transposons, generating bracketed 5-bp DRs, is generally site-specific at 25-bp downstream of *glmS*, and these target sites with a consensus CCgcGtAAccTgGCaAAatcgGTtACgGTtGAgTaa<sup>38</sup> together with Tn7-related elements are presented in a wide range of bacteria as mentioned above. Three of the four Tn7-related elements (all bracketed by 5-bp DRs) characterized herein, as expected, are located downstream of *glmS*, but T7RE<sub>MF1</sub> is presented within *orf1389*. The presence of bracketed 5-bp DRs denotes that T7RE<sub>MF1</sub> is most likely transposed directly into *orf1389* that does not contain the above consensus target site; similarly, additional target sites, not resembling that downstream of *glmS*, have been proposed previously for integration of Tn7-related unit transposons.<sup>25</sup> Tn6512-related ICEs have 17-bp highly conserved *attP* (attachment site on the ICE) sequences with a consensus ATcATcTcTcACCCtGA. Integration of these ICEs needs the 17-bp relatively non-conserved *attB* (attachment site on the chromosome) target sites within the 5' end of *prfC*,<sup>32</sup> and can occur with up to 23.5% sequence mismatch between *attB* and *attP*.<sup>39</sup> These *attB* sites together with Tn6512-related ICEs are widely distributed in Gram-negative bacteria as mentioned above. Taken together, Tn7-related unit transposons and Tn6512-related ICEs have a wide host range.

Conjugal transfer experiments confirm that Tn6512-related ICEs with the complete gene sets for conjugal transfer have the ability to transfer from the wild-type isolates of *Proteus*,<sup>40</sup> *Vibrio*,<sup>41</sup> *Shewanella*,<sup>42</sup> and *Providencia* (this study) to the recipient bacteria *E. coli*, and that a circular extrachromosomal Tn6512 has the ability to transfer into the recipient bacteria *Salmonella*,<sup>39</sup> *Enterobacter*,<sup>39</sup> and *Serratia*,<sup>39</sup> indicating that this group of ICEs have a very broad host range.



Integration of Tn6872-related IMEs needs 24-bp highly conserved *attB* sequences (with a consensus AACCTAcAaTTtAACCTACACTTA) located 102-bp upstream of *mutS*. These *attB* sequences together with Tn6872-related elements are mainly found in Morganellaceae including *Providencia* (this study), *Proteus* (eg *Proteus mirabilis* with an accession number CP053894), and *Morganella* (eg *M. morganii* with an accession number CP064830). Integration of Tn6875-related IMEs recognizes 11-bp highly conserved *attB* sequences (with a consensus CcTatTTTATC) located 494-bp downstream of *smpB*. These *attB* sites together with Tn6875-related elements are mainly distributed in Morganellaceae including *Providencia* (this study), *Proteus* (eg *Proteus mirabilis* with an accession number CP042857), and *Morganella* (eg *M. morganii* with an accession number CP068145), as well as Enterobacteriaceae including *Salmonella* (eg *S. enterica* with an accession number CP053319) and *Escherichia* (eg *E. coli* with an accession number CP056565). It seems that these two groups of IMEs spread mainly in Morganellaceae and/or Enterobacteriaceae.

Five (except for Tn6862 and Tn6865) of the seven Tn6512-related ICEs encode the toxin-antitoxin systems: HipBA in Tn6512, and AbiEi-ii in Tn6860, Tn6861, Tn6863, and Tn6864. These toxin-antitoxin systems have been shown to stabilize Tn6512-related ICEs by preventing their loss when they are extrachromosomal.<sup>43,44</sup> The loss of the AbiEi-ii toxin-antitoxin system from Tn6512 and Tn6862 is due to the substitution of ICE backbone region 7 as shown in Figure 2, while that from Tn6865 is caused by the insertion of the MDR region within the ICE backbone gene *umuC* (site 3 in Figure 2). Five (except for Tn6512 and Tn6865) of the seven Tn6512-related ICEs and two (except for Tn6872) of the three Tn6872-related IMEs carry a type I restriction-modification system HsdMSR. This system can degrade the unmethylated incoming DNA to stabilize AGEs against challenges by competitor elements.<sup>45</sup> Tn6512 lose the HsdMSR system because of the substitution of backbones region 5 (Figure 2). The loss of this system from Tn6865 is on account of the insertion of the MDR region. Tn6872 do not harbor this system due the substitution of *orf201*–to–*orf207* region (Figure 5). It is speculated herein that the loss of toxin-antitoxin or restriction-modification systems might lead to the instability of relevant ICEs and IMEs after they are excised from the chromosomes.<sup>46</sup>

Tn7-related unit transposons accomplish transposition using the core transposition module *tnsABCDE*.<sup>23</sup> All the four Tn7-related elements analyzed herein have undergone the truncation of *tnsABCDE*, which results from the integration of MDR regions. The lesion of *tnsABCDE* in these four Tn7-related elements would lose their ability of intracellular transfer.

Each of 13 of the totally characterized 17 AGEs carries a MDR region, and notably the resulting 13 MDR regions carry at least 49 drug resistance genes, which can be grouped into 15 different categories of antimicrobials and heavy metal, including  $\beta$ -lactam, aminoglycoside, macrolide, tetracycline, trimethoprim, phenicol, sulphonamide, lincosamide, rifampicin, quinolone, fosfomycin, bleomycin, chromate, quaternary ammonium compound, and mercury. Among these 49 drug resistance genes, predominantly found are those resistant to aminoglycosides (n=14) and  $\beta$ -lactams (n=9), which might be due to the wide clinical use of aminoglycosides and  $\beta$ -lactams against *Providencia*-induced infections. Intact or truncated versions of Tn21- and Tn1696-related transposons, Tn4352, Tn5393c, In27, ISCR2–*floR* unit, ISCR2–*sul2* unit, ISEc29–*mph*(E)–IS15DI unit, and IS26–*aacC4*–*aph*(4)–*Ia*–ISEc59 unit are frequently found within these 13 MDR regions, indicating the assembly of these MDR regions from various collections of AGEs and associated resistance genes via complex transposition and homologous recombination. In addition, there are so called “concise” accessory modules (Tn6912, Tn6578, Tn6896, In2-4, In9-1, and *mer* region) that are presented in six AGEs, including four harboring MDR regions and two not. These “concise” accessory modules harbor at least 10 drug resistance genes, which mediate the resistance to six different categories of antimicrobials and heavy metal, including aminoglycoside,  $\beta$ -lactam, tetracycline, trimethoprim, macrolide, and mercury.

On the one hand, these four groups of AGEs have a wide range of hosts including *Providencia*, and they are stable in the host bacteria and further able to transfer across different bacterial species. On the other hand, these four groups of AGEs display high-level diversification in modular structures, which have complex mosaic natures and carry a large number of drug resistance genes, and particularly different MDR regions are presented in these AGEs. Integration of these AGEs into the *Providencia* chromosomes contributes to the accumulation and distribution of drug resistance genes and enhances the ability of *Providencia* isolates to survive under drug selection pressure.

## Data Sharing Statement

The complete chromosome sequences of the PROV275, PROV002, PROV023, PROV013, and PROV087 isolates were submitted to GenBank under accession numbers CP059298, CP059345, CP059348, CP059346, and CP059347, respectively. The GenBank accession numbers of all the six plasmids of these five isolates were listed in [Table S1](#).

## Ethics Statement

This study used the bacterial isolates obtained from the Chinese public hospitals as listed in [Table S1](#). The bacterial isolation was part of the routine hospital laboratory procedures and the bacterial isolates involved in this study were not isolated from patients directly. The genetic analysis of accessory genetic elements in this study had no experimentation involving humans or animals. According to relevant legislation/policy of China (<http://www.nhc.gov.cn/fzs/s3576/201808/14ee8ab2388440c4a44ecce0f24e064c.shtml>), this study was not subject to ethical review. The research involving biohazards and all related procedures were approved by the Biosafety Committee of the Beijing Institute of Microbiology and Epidemiology.

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## Disclosure

The authors report no conflicts of interest in this work.

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