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Genome and Plasmid Analysis of bla_{IMP-4}-Carrying *Citrobacter freundii* B38

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Sequencing of the bla_{IMP-4}-carrying *C. freundii* B38 using the PacBio SMRT technique revealed that the genome contained a chromosome of 5,134,500 bp and three plasmids, pOZ172 (127,005 bp), pOZ181 (277,592 bp), and pOZ182 (18,467 bp). Plasmid pOZ172 was identified as IncFIIY, like pP10164-NDM and pNDM-EcGN174. It carries a class 1 integron with four cassettes (bla_{IMP-4}-*qacG2-aacA4-aphA15*) and a complete hybrid *tni* module (*tniR-tniQ-tniB-tniA*). The recombination of *tniR* from Tn*402* (identical) with *tniQBA* from Tn*5053* (99%) occurred within the *res* site of Tn*402*/*5053*. The Tn*402*/*5053*-like integron, named Tn*6017*, was inserted into Tn*1722* at the *res* II site. The replication, partitioning, and transfer systems of pOZ181 were similar to those of IncHI2 plasmids (e.g., R478) and contained a *sul1*-type class 1 integron with the cassette array *orf-dfrA1-orf-gcu37-aadA5* linked to an upstream Tn*1696 tnpA-tnpR* and to a downstream 3′ conserved sequence (3′-CS) and IS*CR1*. A Tn*2* transposon encoding a bla_{TEM-1} β-lactamase was identified on pOZ182. Other interesting resistance determinants encoded on the B38 chromosome included multidrug resistance (MDR) efflux pumps, an AmpC β-lactamase, and resistances to Cu, Ag, As, and Zn. This is the first report of a complete *tni* module linked to a bla_{IMP-4}-carrying class 1 integron, which, together with other recently reported non-*sul1* integrons, represents the emergence of a distinct evolutionary lineage of class 1 integrons lacking a 3′-CS (*qacEΔ1-sul1*). The unique cassette array, complete *tni* module of Tn*6017*, and incompatibility group of pOZ172 suggest a bla_{IMP-4} evolutionary pathway in *C. freundii* B38 different from that for other bla_{IMP-4} genes found in Gram-negative bacteria in the Western Pacific region.

Since the1990s there have been increasing reports of Gram-negative bacteria producing class B metallo-β-lactamases that confer resistance to carbapenems, usually encoded by $bla_{IMP/VIM/GIM/SIM/NDM}$ genes. These genes (except for bla_{NDM}) have been found to be carried by class 1 integrons, except for some bla_{IMP-1} genes reported in *S. marcescens* from Japan, which were carried by class 3 integrons (accession numbers AB070224 [1] and AF416297 [2]). Most class 1 integrons containing $bla_{IMP/VIM/GIM/SIM}$ (but not bla_{NDM}) are of the *sul1* type, containing a 3′ conserved sequence (3′-CS) downstream. However, bla_{IMP-9}, bla_{VIM-2}, and bla_{IMP-34} have recently been found on *tniABQR*-type class 1 integrons (3–6).

The IMP-4 metallo-β-lactamase was first found in *Acinetobacter* spp. that caused outbreaks in the intensive care unit (ICU) wards of a university hospital in Hong Kong (7, 8) and in *Citrobacter youngae* (now identified as *Citrobacter freundii* in this study) from a patient with a leg ulcer in Guangzhou, China (9). bla_{IMP-4}-mediated carbapenem resistance has now spread to many parts of the world, particularly Australia, where it has caused serious nosocomial outbreaks by different Gram-negative bacteria and has appeared in various genetic contexts. The most prevalent context was a Sydney multiresistance region (MRR) flanked by IS*26* that contains a class 1 integron carrying resistance cassettes bla_{IMP-4}-*qacG2-aacA4-catB3* (10). Interestingly, this is the same cassette array first described in a bla_{IMP-4}-carrying class 1 integron in *Acinetobacter* spp. from Hong Kong (8) and from Singapore (11) The determinant bla_{IMP-4} has been reported to be found in a wide range of Gram-negative species and to be the cause of a series of nosocomial outbreaks in the Western Pacific region. The bla_{IMP-4}-carrying class 1 integron on plasmid pOZ172 (156 kb) in *C. freundii* B38 and its *Escherichia coli* transconjugant B38T, first described by Hawkey et al. in 2001 (9), was further characterized by Xiong et al. (12) as carrying a class 1 integron with a slightly different cassette array, bla_{IMP-4}-*qacG2-aacA4-aphA15*, and a hybrid Tn*402*-like *tniABQR* module. A second, *sul1*-type integron was identified in B38 but not in the transconjugant. Therefore, the route of acquisition and the mode of bla_{IMP-4} carbapenem resistance transmission in *C. freundii* B38 might differ from those reported for other bla_{IMP-4} producers.

After 454 and Illumina sequencing failed to resolve the two integrons and assemble their respective plasmids, we used the single-molecule real-time (SMRT) sequencing method (Pacific Biosciences, USA) to analyze the whole genome of bla_{IMP-4}-carrying *Citrobacter freundii* B38 isolated from a Guangzhou multicenter surveillance program in order to understand the acquisition, evo-

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TABLE 1 Susceptibilities of *C. freundii* B38

Test date	MIC (mg liter^{-1})[a]											
	IMP	MEM	CAZ	CTX	CRO	FEP	TIM	CFP	TZP	CIP	AMK	GEN
February 1999[b]	24	ND	256	256	256	256	256	256	32	32	256	256
May 2000[c]	0.5	6	256	256	256	256	256	256	32	32	256	256
May 2002[d]	2	0.5	256	256	256	ND	ND	ND	32	32	256	ND

[a] IMP, imipenem; MEM, meropenem; CAZ, ceftazidime; CTX, cefotaxime; CRO, ceftriaxone; FEP, cefepime; TIM, ticarcillin-clavulanic acid; CFP, cefoperazone-sulbactam; TZP, piperacillin-tazobactam; CIP, ciprofloxacin; AMK, amikacin; GEN, gentamicin. ND, not determined.
[b] Tested in Guangzhou with the Etest gradient method.
[c] Tested in Sweden with the Etest gradient method.
[d] Tested in Birmingham with the agar dilution method.

lution, and dissemination of the carbapenem determinant and its associated mobile elements.

MATERIALS AND METHODS

Bacterial strain. The bla_{IMP-4}-carrying *C. freundii* strain B38 is a clinical isolate recovered during a Guangzhou multicenter antibiotic resistance surveillance program (GARSP) (1998 to 2001). The bla_{IMP-4} carrying integron was on a plasmid previously estimated as being 156 kb that was transferable by conjugation into *E. coli* UB1637/R (9). The antibiotic susceptibilities of *C. freundii* B38 are shown in Table 1. The strain had previously been identified as *C. youngae* (9) but was identified in this study as *C. freundii* with a probability of 93% and biochemical profile 4405615565520211 using a Vitek 2 (bioMérieux, Montreal, Canada) and a probability of 99.9% using Vitek matrix-assisted laser desorption ionization mass spectrometry (bioMérieux, Montreal, Canada).

DNA sequencing method. Total DNA was extracted from a culture of the bacterium grown overnight in LB broth at 37°C using the Qiagen Genomic-tip 20/G kit (Qiagen, Toronto, Canada) and quantified by using a fluorometric, PicoGreen-based method as well as on an agarose gel to confirm the quality and high molecular weight of the isolated DNA. The genome was sequenced by the single-molecule real-time (SMRT) technique using a PacBio platform (Pacific Biosciences, Menlo Park, CA) at McGill University Genome Québec Innovation Centre.

Genome assembly and analysis. The genome was assembled *de novo* using the hierarchical genome assembly process (HGAP) and proofread in PacBio (13, 14). Further editing and manual annotation were carried out by using RAST (15, 16), Prodigal (17), GCG (version 11.1; Accelrys Inc., San Diego, CA), CGView (18), and Artemis (release 13.2.0) (19).

Accession number(s). The complete sequence of the chromosome has been submitted to GenBank under accession number CP016762. Plasmids pOZ172, pOZ181, and pOZ182 were submitted under accession numbers CP016763, CP016764, and CP016765, respectively.

RESULTS

Overview of the genome. The clinical isolate of *C. freundii* B38 had a chromosome of 5,134,500 bp and three plasmids, pOZ172 (127,005 bp), pOZ181 (277,592 bp), and pOZ182 (18,467 bp), assembled as intact circular molecules from SMRT sequencing. The largest plasmid, pOZ181, was identified only using SMRT sequencing and was not recovered in the previous study (9), due to limitations of the rapid plasmid isolation method used (20). The bla_{IMP-4}-carrying plasmid pOZ172 was 127 kb in size. The chromosome had a GC content of 51.7%, with a total of 4,905 open reading frames identified by Prodigal, including 23 pseudogenes. The strain was phenotypically identified as *C. freundii*. Whole-genome sequencing (WGS) revealed B38 to be closest to *C. freundii* strains RLS1, CAV1741, and CAV1321 and more distant from CFNIH1 and P10159. The genome map is shown in Fig. S1 in the supplemental material, and key features of the *C. freundii* B38 genome are listed in Table 2. The genome contains at least 3 prophages not found in other *C. freundii* strains and 15 genomic islands with 10 or more genes and unique to B38. Among these are islands with genes for tellurite resistance and for D-tagatose metabolism (used to differentiate among *Citrobacter* species). Resistance genes identified on the chromosome included genes for β-lactamases, efflux pumps, and resistance to heavy metals (copper, silver, and arsenic) and a multiple antibiotic resistance (MAR) locus.

IncF-like plasmid pOZ172. (i) General features of the pOZ172 sequence. Plasmid pOZ172 contains 127 predicted coding regions, including 4 pseudogenes, with 18% (23/127) of the open reading frames (ORFs) encoding hypothetical proteins, as identified by Prodigal and manually annotated with Artemis. The replication, partitioning, and transfer systems showed similarity to those of other sequenced plasmids in GenBank. A 50-kb block of the plasmid sequence (bp 10663 to 62387), including the replication and transfer region, showed the most similarity (99%) to plasmid pP10164-NDM in *Leclercia adecarboxylata* strain P10164 (21), pNDM-Ec1GN574 and pNDM1_EC14653 of *Enterobacter cloacae* (22, 23), pRJF866 of *Klebsiella pneumoniae* RJF866 (accession no. KF732966) (unpublished), and pKOX_NDM1 of *Klebsiella oxytoca* E718 (24). The plasmid map of pOZ172 shows the genes and their locations (Fig. 1). The IncFIIY maintenance, replication, and transfer modules of pOZ172, homologous to those of these plasmids, are indicated in Fig. 1. The IncFII plasmid replication initiator proteins RepA and RepB are 99% identical to those of pRJF866 and pKOX_NDM1. The RepFIB RepA is identical to those of *K. pneumoniae* KPNIH27 plasmid pKPN-262 (accession no. CP007734), strain 997 pc15-k (HQ202266), and

TABLE 2 Overall features of the *C. freundii* B38 genome

Parameter	Chromosome	pHRB381	pOZ172	pHRB382
Size (bp)	5,134,500	277,592	127,005	18,467
G+C (%)[a]	51.7	45.8	54.4	57.7
No. of predicted coding sequences	4,905	277	127	22
Resistance determinants	*mdtABCD*, MAR, heavy metals (Cu, Ag, As, Zn), efflux pumps	Heavy metals (*ars* operon, *ter* operon, *rcnA* operon), antibiotics [*dfrA1*, *aadA5*, *qacEΔ1*, *sul1*, *armA*, *mph*(*E*), *aphA7*, *msr*(*E*), bleomycin resistance]	bla_{IMP-4}, *qacG*, *aacA4*, *aphA15*	bla_{TEM-1}

[a] Mobile elements and insertions were not excluded.

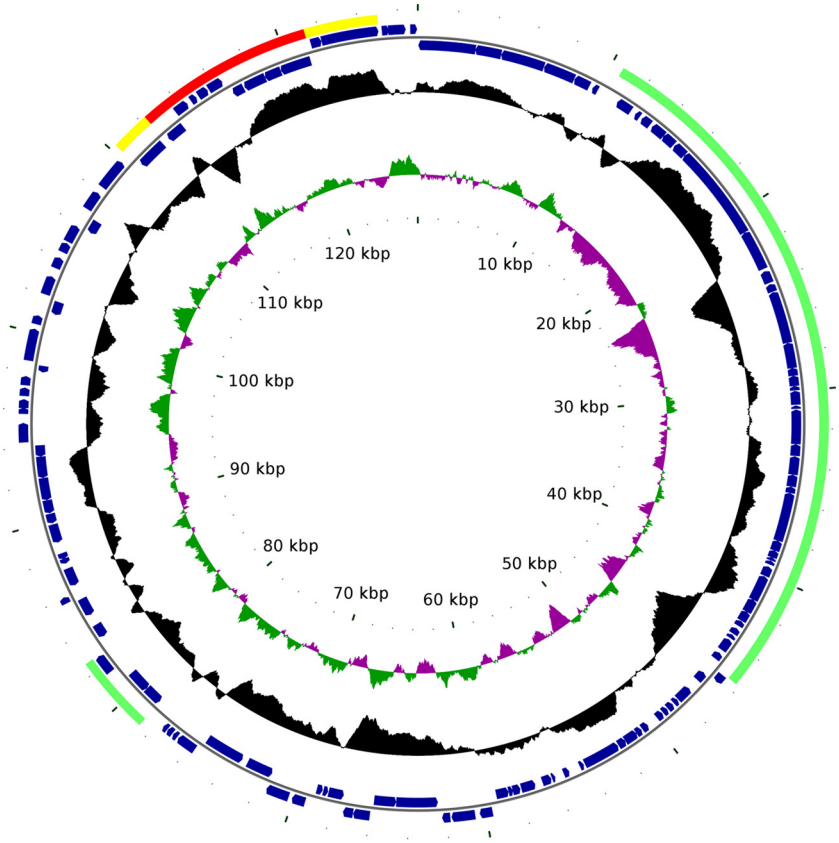

FIG 1 Map of plasmid pOZ172 from *C. freundii* B38. The scale is indicated on the innermost circle. The second circle is G+C skew in green (+) and purple (−), and circle 3 shows G+C content (deviation from the average) in black (+, outward; −, inward). The next two circles illustrate positions of coding sequences in the minus (circle 4) and plus (circle 5) strands in dark blue. The two green arcs represent two replication/transfer regions most similar (99%) to the IncF plasmids in Table 3. The bla_{IMP-4}-carrying integron Tn*6017* (in red) is inserted into Tn*1722* (in yellow).

others (Table 3). Also, three IncFII-type plasmids, pKP02022 (KF719972), pKP09085 (KF719970), and pKP007 (KF719971), that carry $bla_{CTX-M-15}$ and were isolated from three *K. pneumoniae* strains in South Korea also contain a RepFIB RepA that is identical to that found in pOZ172 (25). Plasmid pOZ172 was typed as IncFIIY according to the RST scheme for IncF plasmids (26). The plasmid partitioning proteins ParA and ParB are 99% and 100% identical to their equivalents in plasmid II of *K. pneumoniae* strain Kp52.145 and highly similar to many others (Table 3).

(ii) Antibiotic resistance genes and their mobile elements. The bla_{IMP-4}-carrying class 1 integron in pOZ172 plasmid in *C. freundii* B38 contained four cassettes (*bla_{IMP-4}-qacG2-aacA4-aphA15*) and a complete but hybrid *tni* module (*tniR-tniQ-tniB-tniA*) composed of *tniR* from Tn*402* (identical) and *tniQ-tniB-*

TABLE 3 Comparative analysis of key features of the plasmids in *C. freundii* B38

Plasmid	Function	Genomic coordinates (bp)[a]	Homologs in GenBank
pOZ172	Replication, transfer	*repAB* (11218–12648, c), *tra* operon (14277–45250)	Most similar (99%) to pNDM-Ec1GN574 and pNDM1_EC14653 (*E. cloacae*), pKOX_NDM1 (*K. oxytoca*), pP10164-NDM (*L. adecarboxylata*), pYDC644 and pRJF866 (*K. pneumoniae*)
	Replication, partition	*repFIB* (81538–82548), *parBA* (78650–80787, c)	Most similar (99%) to pCAV1344-250, pKPN-262, pKPN3, pKP007 plasmid II strain Kp52.145, pc15-K, pUUH2392 pKP02022, and pKP09085 (*K. pneumoniae*), pOU7519 (*S. enterica*), etc.
pHRB381	Replication, transfer, partition	*repHI2A* (75780–76868, c), *repH1A* (90277–91152), *tra2* (24119–41412), *tra1* (92763–141366), *parAB* (107267–108722), *parMR* (112426–114859)	Most similar (99–100%) to R478 (*S. marcescens*) (pHRB381 has an 11-kb insertion of hypothetical proteins between *parMR* and *htdA* of R478), pK29 (*K. pneumoniae*), pCAV1151-296 (*Kluyvera intermedia*), pEN-08e, pENT-8a4, pMRVIM0813, pKPC-272, pEC-IMPQ, and pEC-IMP (*E. cloacae*), pSTm-A54650 (*S. enterica*), etc.
	Heavy metal resistance	*ter* operon (166156–182942), *ars* operon (53–2937), *rcnA* operon (263574–265084)	
	Sulfate permease	*sfpAB* operon (275261–277585)	

[a] c, complement (bottom strand).

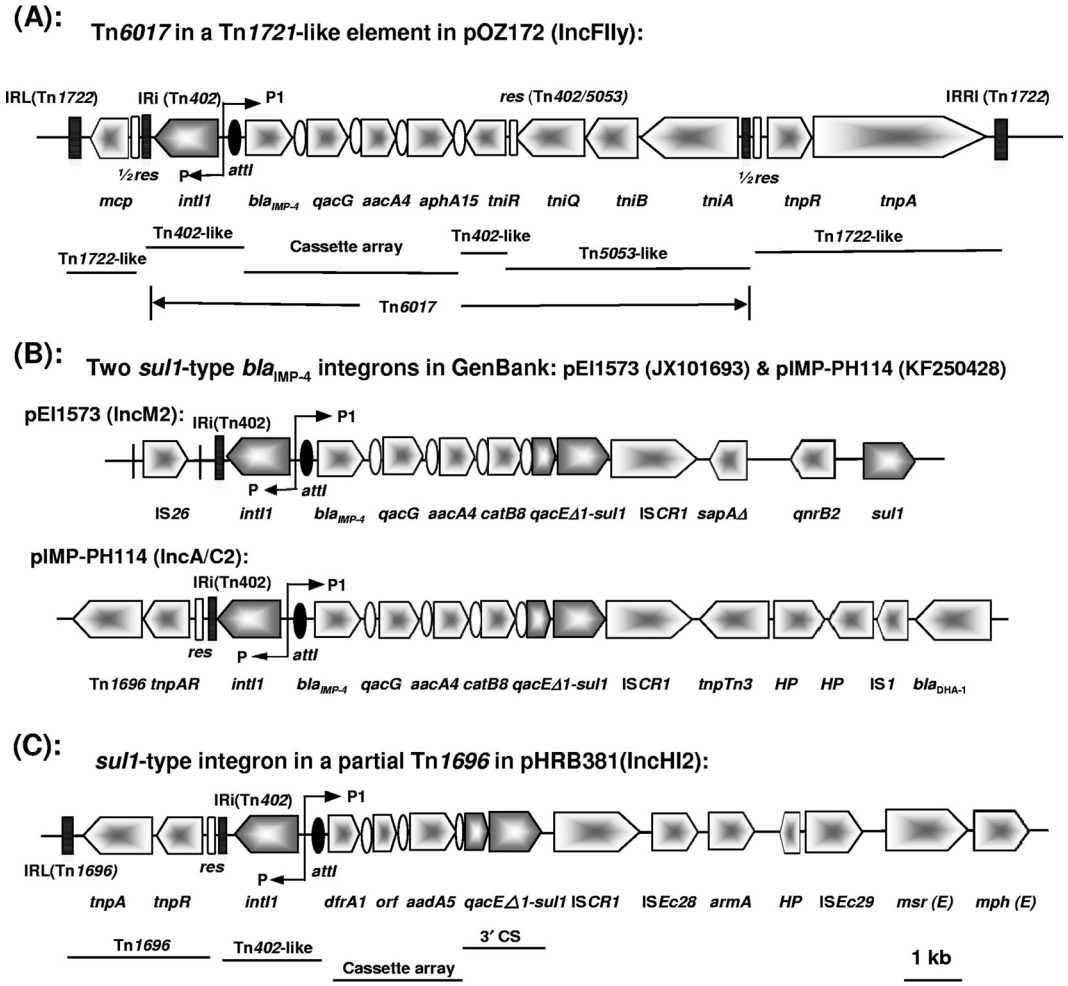

FIG 2 Class 1 integrons identified on pOZ172 and pOZ181 in *C. freundii* B38 and related plasmids. (A) Tn*6017*, a Tn*402*/*5053*-like integron, was identified on pOZ172 and was inserted into the *res* II site of Tn*1722*, splitting the *res* site into two half *res*. Arrow boxes show the genes and their orientations; each solid black oval indicates *attI*, and each white oval represents the *attC* of the preceding gene. Δ, disrupted genes. *mcp* is the gene for the methyl-accepting chemotaxis protein of Tn*1722*. (B) The two most representative *sul1*-type bla_{IMP-4} class 1 integrons found in GenBank. Their cassette arrays are the same but differ from that of Tn*6017*. The black rectangle is 25-nt repeat IRi; the short vertical lines are the 12-nt repeats of IS*26*. (C) A second, *sul1*-type integron was identified on pOZ181 and was linked upstream to Tn*1696* and downstream to an IS*CR1*. The small rectangular white boxes represent the *res* sites adjacent to *tnpR*, and the solid black boxes represent the 25-bp IRi (Tn*402*) and IRt (Tn*5053*) sites; the larger solid black boxes represent the 38-bp IRL and IRRI of Tn*1722*, as well as IRtnp of Tn*1696*. The small arrows represent the direction of promoters (P and P1).

tniA from Tn*5053* (6-nucleotide [nt] difference) (Fig. 2). The recombination of *tniR* from Tn*402* with the *tniQBA* from Tn*5053* occurred within the *res* site of Tn*402*/*5053* (see Fig. S4 in the supplemental material). This integron was flanked upstream by the Tn*1722* methyl-accepting chemotaxis protein (*mcp*) gene and downstream by Tn*1722 tnpRA*. The resistant mobile element was formed by insertion of the Tn*402*/*5053*-like integron, named Tn*6017*, into the *res* II site of Tn*1722*.

A potential transposon contains an ABC transport system and an RND efflux transporter subunit flanked by inverted repeats of IS*4321R* (position 88567 to 95564). Various insertion sequences were identified in pOZ172, including IS*Cfr12*, IS*Sen4*, IS*1*, IS*Kpn26* (3 copies), IS*26*, IS*903B*, and IS*Ec36*.

IncHI2-like plasmid pOZ181. (i) General features of the pOZ181 sequence. Plasmid pOZ181 has a length of 277,592 bp and contains 284 predicted coding regions, including 7 pseudogenes, with 45% (129/284) of the open reading frames (ORFs) encoding hypothetical proteins, as identified by Prodigal and manually annotated. The key features of the plasmid, such as the replication, stability, and transfer systems, showed similarity to those of several other sequenced plasmids, including the well-characterized IncHI2 plasmid R478 of *Serratia marcescens* (accession no. BX664015) (Table 3) (99% identity over 68% of pOZ181) (27). The plasmid map of pOZ181 is shown in Fig. S2 in the supplemental material. The major features of pOZ181 are shown in Table 3. The dual replication and transfer modules encoded on pOZ181 are similar to those of R478, containing two functional iteron-based plasmid replication determinants, *repHI2A* and *repH1A* (27). The plasmid replication proteins encoded by *repHI2A* and by *repH1A* of pOZ181 are 99 to 100% identical to those of R478. The two transfer/partition regions on pOZ181 are homologous to the *tra2* and *tra1* regions of R478 except for an 11-kb insertion between *parMR* and *htdA* in pOZ181. Genes for the plasmid partitioning proteins ParA and ParB, as well as

those for ParM and ParR, are 99% identical to those of R478 (Table 3).

(ii) Resistance genes and their mobile elements. Plasmid pOZ181 carries a *sul1*-type class 1 integron which has a 5′-CS and four cassettes, *orf-dfrA1-gcu37-aadA5*, flanked upstream by a Tn*1696*-like *tnpR-tnpA* and downstream by 3′-CS and an IS*CR1* transposase (Fig. 2). This *sul1*-type integron was not found in the transconjugant *E. coli* B38T and was identified as being located on pOZ181 by SMRT sequencing. Other resistance genes include the 16S rRNA methylase gene *armA* downstream of IS*CR1*, the macrolide efflux pump gene *msr*(*E*), the macrolide 2′-phosphotransferase gene *mph*(*E*), the aminoglycoside 3′-phosphotransferase gene *aphA7*, and a glyoxalase/bleomycin resistance protein/dioxygenase gene.

The *ter* operon (ca.16.8 kb) consists of *terY3-terY2-terX-terY1-terW* as well as *terZ-terA-terB-terC-terD-terE-terF*. There were six intervening ORFs identified between *terW* and *terZ*. The operon is highly (99%) similar to the *ter* operon in R478 (27), as well as to those of pK29 (28), pENT-8a4 (accession no. CP008899) and pEC-IMP (EU855787), among others (Table 3). The arsenical resistance operon consists of *arsC-arsB-arsR-arsH*. The *ars* operon is identical to those of R478, pENT-8a4 from *Enterobacter cloacae* ECNIH3 (CP008899), and pKPC-272 from ECNIH2 (CP008825) as well as to those of pK29 (EF382672) from *K. pneumoniae* NK29 and pSTM-A54650 from *Salmonella enterica* serovar Typhimurium (LK056646) (Table 3). A nickel-cobalt efflux system, RcnA-RcnR, was also identified and was 99% identical to those of pENT-8a4 (CP008899) and pKPC-272 (CP008825) from *Enterobacter cloacae* ECNIH3 and ECNIH2, among many others.

There are three copies of IS*1* and of IS*Kpn26* and three identical copies of IS*26*. Two of the IS26 copies flank the *aphA7* gene and form a potential transposon. There are two copies of IS*903B*. The others include IS*4321*, IS*CR1*, IS*Ec28*, and ISEc29.

Plasmid pOZ182. Plasmid pOZ182, the smallest plasmid in *C. freundii* B38, is 18,467 bp in length. It has 22 open reading frames, of which 73% encode hypothetical proteins. The plasmid backbone shows no similarity to plasmid sequences in GenBank. The key features of this plasmid are that it contains a Tn*2* transposon and a TEM-1 β-lactamase gene. The genetic map of pOZ182 is shown in Fig. S3 in the supplemental material.

DISCUSSION

The bla_{IMP-4} gene in *C. freundii* B38 was identified in the second report of transferable carbapenemase genes in *Enterobacteriaceae* outside Japan and the first report in the People's Republic of China (9). Unlike bla_{IMP-9} in *Pseudomonas aeruginosa*, which was described at the same time and place and found to be on a narrow-host-range IncP2 plasmid of *Pseudomonas* (3, 29), bla_{IMP-4} has been observed to spread to a variety of Gram-negative species, plasmid incompatibility groups, and genetic contexts in clinical, animal, and environmental isolates in the last 15 years (10, 30–33). bla_{IMP-4} is most commonly carried on IncL/M and IncA/C2 plasmids (Fig. 2B), typically in the cassette array bla_{IMP-4}-*qacG2-aacA4-catB3* in a class 1 integron (10, 34). This cassette array was described in *Acinetobacter baumannii* from a Hong Kong outbreak (7, 8) and from Singapore (11), *K. pneumoniae* pIMP-PH114 from Hong Kong (35), *Enterobacter cloacae* from Australia (32), *K. pneumoniae* pJIBE401 (10), *E. cloacae* plasmid pEl1573 (34), and *Enterobacteriaceae* in silver gulls in Australia (33), where the bla_{IMP-4} cassette is in a *sul1*-type class 1 integron (i.e., with a 3′-CS). Additionally, there is a *sul1*-type class 1 integron from a *K. pneumoniae* isolated from Shanghai that has a single bla_{IMP-4} cassette with a group II intron in its *attC* site (36).

The context of the IMP-4 carbapenem resistance cassette in *C. freundii* B38 is unique and has evolved in a different manner. First, the cassette array is bla_{IMP-4}-*qacG2-aacA4-aphA15* (with *aphA15* instead of *catB3*); second, it is on a *tniABQR*-type class 1 integron, Tn*6017*, on pOZ172; and finally, the element is on an IncFIIY plasmid (Fig. 2).

Tn*6017* may be the product of a Tn*402*-like element, bearing a class 1 integron and a hybrid T*402*/Tn*5053*-like *tni* module, inserted into the *res* II site of Tn*1722* (see Fig. S4 in the supplemental material) (37). The hybrid transposon composed of *tniR* (Tn*402*) and *tniQBA* (Tn*5053*) resulted from an event of site-specific recombination at position TATACGTTC within the *res* site (see Fig. S4C in the supplemental material). The Tn*5053* and Tn*402 tni* genes are known to complement each other (38). A similar Tn*402*/*5053* hybrid exists as an integron carrying *aacA4*-bla_{VIM-2} in plasmid PPV2-2 of *Pseudomonas putida* (39). The finding of Tn*6017* together with recent reports of *Tn402*-like *tniABQR*-type $bla_{VIM/IMP}$-carrying class 1 integrons, e.g., those of pDCPR1 (4) and pOZ176 (3), may represent the emergence of a distinct evolutionary lineage of class 1 integrons lacking a 3′-CS (*qacEΔ1-sul1*) and instead descended either (i) directly from a Tn*402*-like element containing only *intI1* and *tniRQBA* (40) or (ii) from such an element already carrying *qacE* (41). Until the recent appearance of carbapenemase-encoding *tniABQR*-type integrons, the only example of a resistance integron of this type was Tn*402* itself. These integrons would have escaped the detection of class 1 integrons with primers 5′-CS and 3′-CS.

The second class 1 integron in B38 is of the traditional *sul1* type and is carried by pOZ181, an IncHI2 plasmid (Fig. 2). It contains four cassettes in the order *orf-dfrA1-gcu37-aadA5*. There are three distinct plasmids in B38, with pOZ172 carrying bla_{IMP-4} and being transferable into *E. coli* UB1637/R (9). The order of integron and cassette arrival in B38, with the latter usually from right to left (due to the preference for *attI* × *attC* for cassette integration) (42), may reflect the history of antibiotic selective pressure in this strain, with bla_{IMP-4} the most recent acquisition.

In the past decade, genes encoding the class B metallo-carbapenemase bla_{IMP-4} were also found to coexist with those encoding a class A serine carbapenemase (bla_{KPC-2}) in a *K. pneumoniae* strain from China (43), a class D carbapenemase (bla_{OXA-58}) in *Acinetobacter* spp. in Australia and Singapore, (11, 44), and another class B metallo-carbapenemase (bla_{NDM-1}) (45). The IMP-4-producing *K. pneumoniae* strain was also isolated from three infants in a neonatal ICU in the United States during the period from November 2009 to June 2010, and the patients had no foreign travel histories; however, the genetic contexts flanking the bla_{IMP-4} genes in these strains were not characterized (46). The association of integrons with mobile elements such as transposons and/or plasmids facilitates horizontal transfer of resistances at the intra- and interspecies levels (47). Tn*21*, Tn*1696*, and their relatives are important vehicles for the acquisition and horizontal transfer of resistance in Gram-negative bacteria (48, 49).

Analysis of the *C. freundii* B38 genome revealed many other antibiotic and heavy metal resistance determinants besides the cassettes in the two integrons. They were found not only on plasmids but also on the chromosome (Table 2). They included the β-lactamase gene bla_{TEM-1} conferring resistance to ampicillin,

aminoglycoside resistance encoded by *aphA7* and *armA*, and heavy metal resistances by multiple mechanisms (Cu, Ag, As, Zn, Te, Co, and Ni). Plasmids in the IncHI2 and IncHII groups in *Enterobacteriaceae* and the IncP2 group in *Pseudomonas* are often associated with tellurite resistance (50–52). The *ter* and *ars* operons identified on pOZ181 were 99 to 100% identical to those of R478 and other plasmids that carry carbapenem resistance genes such as bla_{IMP}, bla_{KPC} and bla_{VIM} (Table 3).

Both large plasmids, pOZ181 and pOZ172, contain dual replication/transfer systems. The replication, partitioning and stability, and conjugative transfer systems of the IncHI2 plasmid pOZ181 were highly similar to those of R478; the former contains a unique 11-kb insertion near ParMR. The corresponding IncFIIY region of pOZ172, identified by replicon sequence typing (RST) (26, 53), is very similar to those of NDM-1-producing IncFIIY plasmids; however, the second replication protein, RepFIB, in pOZ172, while homologous to many RepFIB proteins from IncFII plasmids (Table 3), was only 60% similar to those of some NDM-1 producers.

This is the first report of a *tniRQBA* module linked to bla_{IMP}-$_4$-carrying class 1 integrons on an IncF plasmid. Together with other recent findings of Tn*402*-like *tniR* associated with bla_{VIM-2}-carrying class 1 integrons, they may represent the emergence of a distinct evolutionary lineage of class 1 integrons lacking the usual *qacEΔ1-sul1* 3′-CS and instead descended directly from a Tn*402*-like element containing only *intI1* and *tniRQBA*. The unique cassette array linked to a complete *tni* module in Tn*6017* carried by IncF pOZ172 suggests a different bla_{IMP-4} evolution route in *C. freundii* B38 than for other bla_{IMP-4} genes found in Gram-negative bacteria in the Western Pacific region. The coexistence of multiple mobile elements, including the IncH and IncF conjugative plasmids with dual replication systems, reflects the active horizontal genetic transfer that is taking place. The closed chromosome and plasmid genomes obtained in this study using PacBio technology allow for a better understanding of the relationships among resistance genes, mobile elements, and whole plasmids.

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