Genome and Plasmid Analysis of blaIMP-4-Carrying Citrobacter freundii B38

Xiong, Jianhui; Déraspe, Maxime; Iqbal, Naeem; Ma, Jennifer; Jamieson, Frances B.; Wasserscheid, Jessica; Dewar, Ken; Hawkey, Peter; Roy, Paul H.

DOI:
10.1128/AAC.00588-16

Document Version
Peer reviewed version

Citation for published version (Harvard):
Xiong, J, Déraspe, M, Iqbal, N, Ma, J, Jamieson, FB, Wasserscheid, J, Dewar, K, Hawkey, PM & Roy, PH 2016, 'Genome and Plasmid Analysis of blaIMP-4-Carrying Citrobacter freundii B38', Antimicrobial Agents and Chemotherapy, vol. 60, no. 11, pp. 6719-6725. https://doi.org/10.1128/AAC.00588-16

Genome and Plasmid Analysis of *bla*$_{IMP-4}$ -Carrying *Citrobacter freundii* B38

Jianhui Xiong[a,b], Maxime Déraspe[a,b], Naeem Iqbal[a,b], Jennifer Ma[c], Frances B. Jamieson[c], Jessica Wasserscheid[d], Ken Dewar[d], Peter M. Hawkey[e,f], and Paul H. Roy[a,b]

Centre de Recherche en Infectiologie, CHU de Québec, Québec, QC. Canada[a]; Department de Biochimie, de Microbiologie, et de Bio-Informatique, Université Laval, Québec, QC, Canada[b]; Public Health Ontario, Toronto, ON. Canada[c]; McGill University and Genome Québec Innovation Centre, Montreal, QC, Canada[d]; PHE Public Health Laboratory Birmingham, Heart of England NHS Foundation Trust, Birmingham B9 5SS, UK[e]; Institute of Microbiology and Infection, University of Birmingham, Birmingham B15 2TT, UK[f].

Corresponding author: Paul H. Roy, Centre de Recherche en Infectiologie, 2705 boul. Laurier, Suite R-0709, Québec, QC G1V 4G2, Canada. Tel.: +1 418 525 4444 ext. 47782; fax: +1 418 654 2715; e-mail: paul.roy@crchul.ulaval.ca

Running title: Genome of *bla*$_{IMP-4}$ -carrying *Citrobacter freundii*

Key words: *Citrobacter freundii*, IMP-4, Tn*402*-like, class 1 integron, SMRT whole genome sequencing, plasmid

ABSTRACT

Sequencing of the *bla*$_{IMP-4}$ -carrying *C. freundii* B38 using 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* (99%) from Tn*5053* 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 IncHI2 (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'CS and IS*CR1*. A Tn*2* transposon with a *bla*$_{TEM-1b}$ β-lactamase was identified on pOZ182. Other interesting resistance determinants on the B38 chromosome included MDR efflux pumps, 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, and 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 suggests a different *bla*$_{IMP-4}$ evolutionary pathway in *C. freundii* B38 compared to other *bla*$_{IMP-4}$ found in Gram-negative bacteria in the Western Pacific Region.

INTRODUCTION

Since the1990s there have been increasing reports of class B metallo-β-lactamase-producing Gram-negative bacteria 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*$_{IMP-1}$ reported in *S. marcescens* from Japan, which were carried by class 3 integrons (1) (AB070224), (2) (AF416297). Most class 1 integrons containing *bla*$_{IMP/VIM/GIM/SIM}$ (but not *bla*$_{NDM}$) are *sul1*-type, containing *a* 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 ICU wards of a university hospital in Hong Kong (7-8) and in *C. youngae* (now identified as *C. freundii* in this study) from a patient with a leg ulcer in Guangzhou, China (9). Now *bla*$_{IMP-4}$-mediated carbapenem resistance has spread to many parts of the world, particularly in Australia where it has caused serious nosocomial outbreaks by different Gram-negative bacteria and 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 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 *E. coli* transconjugant B38T, first described by Hawkey *et al*. in 2001 (9), was further characterized as carrying a class 1 integron with a slightly different cassette array*:* *bla*$_{IMP-4}$*-qacG2-aacA4-aphA15* and a hybridTn*402*-like *tniABQR* module by Xiong *et al*. (12). A second, *sul1*-type integron, was identified in B38 but not 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 from other *bla*$_{IMP-4}$ producers.

After 454 and Illumina sequencing failed to resolve the two integrons and assemble their respective plasmids, we used the (SMRT) sequencing method (Pacific Biosciences, USA) to analyse the whole genome of *bla*$_{IMP-4}$-carrying *Citrobacter freundii* B38 isolated from a Guangzhou multicenter surveillance program in order to understand the acquisition, evolution and dissemination of the carbapenem determinant and its associated mobile elements.

MATERIALS AND METHODS

Bacterial strain. The *bla*$_{IMP-4}$-carrying *C. freundii* B38 is a clinical isolate recovered during a Guangzhou (multicenter) antibiotic resistance surveillance program (GARSP, 1998-2001). The *bla*$_{IMP-4}$ carrying integron was on a plasmid previously estimated as 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

(BioMerieux, Montreal, Canada) and 99.9% using a VITEK-MS-MALDI (BioMerieux, 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 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).

Nucleotide sequence accession numbers. 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 only identified 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 4905 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 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, a MAR locus and resistance to heavy metals (copper, silver, and arsenic).

IncF-like plasmid pOZ172

General features of the pOZ172 sequence. Plasmid pOZ172 contains 127 predicted coding regions including 4 pseudogenes, with 18 percent (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 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 *Leclerci adecarboxylata* strain P10164

(21), pNDM-Ec1GN574 and pNDM1_EC14653 of *Enterobacter cloacae* (22-23), pRJF866 of *Klebsiella pneumonia* RJF866 (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 this plasmid, 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 (CP007734), strain 997 pc15-k (HQ202266), etc. (Table 3). Also, three IncFII-type plasmids pKP02022 (KF719972), pKP09085 (KF719970) and pKP007 (KF719971) that carry $bla_{\text{CTX-M-15}}$ and isolated from three *K. pneumonia* in S. Korea also contain 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).

Antibiotic resistance genes and their mobile elements. The $bla_{\text{IMP-4}}$-carrying class 1 integron in pOZ172 plasmid in *C. freundii* B38 contained four cassettes: $bla_{\text{IMP-4}}$-*qacG2-aacA4-aphA15* and a complete but hybrid *tni* module (*tniR-tniQ-tniB-tniA*) composed of *tniR* from Tn*402* (identical) and *tniQ-tniB-tniA* from that of Tn*5053* (6 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* (Fig. S4). 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* (88567-95564). Various insertion sequences were identified in pOZ172 including IS*Cfr12*, IS*Sen*4, IS*1*, IS*Kpn26 (3 copies)*, IS*26*, IS*903B* and IS*Ec36*.

IncHI2-like plasmid pOZ181

General features of the pOZ181 sequence. The plasmid pOZ181 has a length of 277,592 bp and contains 284 predicted coding regions including 7 pseudogenes, with 45 percent (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 several other sequenced plasmids, including the well-characterized IncHI2 plasmid R478 of *S. marcescens* (BX664015, Table 3) (99% identity over 68% of pOZ181) (27). The plasmid map of pOZ181 is shown in Fig. S2. 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 99-100% identical to those of R478. The two transfer/partition regions on pOZ181 are homologous to the *tra*2 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 for ParM and ParR are 99% identical to those of R478 (Table 3).

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

186 The *ter* operon (ca.16.8 kb) consists of *terY3-Y2-X-Y1-W*-as well as *terZ-A-B-C-D-*
187 *E-F*. There were six intervening ORFs identified between *terW* and *terZ*. The operon is
188 highly similar (99%) to the *ter* operon in R478 (27), as well as to those of pK29 (28),
189 pENT-8a4 (CP008899) and pEC-IMP (EU855787) among others (Table 3). The arsenical
190 resistance operon consists of *arsC-B-R-H*. The *ars* operon is identical to those of R478,
191 pENT-8a4 from *Enterobacter cloacae* ECNIH3 (CP008899) and pKPC-272 from ECNIH2
192 (CP008825) as well as to those of pK29 (EF382672) from *K. pneumoniae* NK29 and
193 pSTM-A54650 from *S. typhimurium* (LK056646) (Table 3). A nickel-cobalt efflux system
194 RcnA-RcnR was also identified and was 99% identical to that of pENT-8a4 (CP008899),
195 pKPC-272 (CP008825) from *Enterobacter cloacae* ECNIH3 and ECNIH2, among many
196 others.

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

200 **Plasmid pOZ182**

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

206

207 **DISCUSSION**

208 The *bla*$_{IMP-4}$ in *C. freundii* B38 was identified in the second report of transferable
209 carbapenemase genes in *Enterobacteriaceae* outside of Japan and the first report in the
210 Mainland China (9). Unlike *bla*$_{IMP-9}$ in *P. aeruginosa*, which was described at the same
211 time and place and found to be on a narrow host range IncP2 plasmid of *Pseudomonas*
212 (3, 29), *bla*$_{IMP-4}$ has been observed to spread to a variety of Gram-negative species,
213 plasmid incompatibility groups and genetic contexts in clinical, animal, and environmental
214 isolates in the last 15 years (10, 30-33). Other reported *bla*$_{IMP-4}$ is most commonly
215 encoded by IncL/M and IncA/C2 plasmids (Fig. 2B), typically by the cassette array *bla*$_{IMP-4}$-*qacG2-aacA4-catB3* in a class 1 integron (10, 34). This cassette array was described in
217 *Acinetobacter baumanii* from a Hong Kong outbreak (7-8), and from Singapore (11); *K.*
218 *pneumonie* pIMP-PH114 from Hong Kong (35); *E. cloacae* from Australia (32), *K.*
219 *pneumoniae* pJIBE401 (10); *Enterobacter cloacae* plasmid pEI1573 (34); and
220 *Enterobacteriaceae* in silver gulls in Australia (33) where the *bla*$_{IMP-4}$ cassette is in a *sul1*-
221 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* (*aphA15* instead of *catB3*); second, it is on a *tniABQR*-type class 1 integron, Tn*6017*, on pOZ172; 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* (Fig. S4) (37). The hybrid transposon composed of *tniR* (Tn*402*) and *tniQBA* (Tn*5053*) resulted from an event of site-specific recombination at the position TATACGTTC within the *res* site (Fig. S4 part C). Tn*5053* and Tn*402 tni* genes are known to complement each other (38). A similar Tn*402*/*5053* hybrid exists as an integron encoding *aacA4*-bla_{VIM-2} in plasmid PPV2-2 of *P. putida* (39). The finding of Tn*6017* together with recent reports of *Tn402-liketniABQR*-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*)-type 3′CS and instead descended either 1) directly from a Tn*402*-like element containing only *intI1* and *tniRQBA* (40) or 2) 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 was the traditional *sul1* type and is encoded 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} 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* x *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} gene were also found to co-exist with those encoding a class A serine carbapenemase bla_{KPC-2} in a *K. pneumoniae* 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* was also isolated from three infants in a NICU in the US during the period 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 inter-species levels (47). Tn*21,* Tn*1696* and their relatives are important vehicles for 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 β-lactamase bla_{TEM-1} conferring resistance to ampicillin; aminoglycoside resistance encoded

by *aphA7* and *armA*; heavy metal resistances by multiple mechanisms: Cu, Ag, As, Zn, Te, Co, and Ni. Plasmids of IncHI2 and IncHII 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 99-100% 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 was highly similar to 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 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* encoded by IncF pOZ172 suggests a different bla_{IMP-4} evolution route in *C. freundii* B38 than other bla_{IMP-4} found in Gram-negative bacteria in Western Pacific Region. The co-existence of multiple mobile elements including the IncH and IncF conjugative plasmids with dual

289 replication systems reflects the active horizontal genetic transfer that is taking place. The
290 closed chromosome and plasmid genomes obtained in this study using PacBio
291 technology allows for a better understanding of the relationships among resistance genes,
292 mobile elements and whole plasmids.

293

294 **Funding information:** P.H.R. was supported by a contract from Integrogene Inc.
295 This work was also supported by Canadian Institutes of Health Research operating grant
296 MT-13564 to P.H.R. Initial sequencing at Public Health Ontario was supported by an
297 internal grant. J. X. was a Fellow of CIHR Strategic Training Program STP-53924 and the
298 previous grant holder of the Guangzhou Antibiotic Resistance Study Project (grant no.
299 98-Z-01-22).

300

301 **Acknowledgements:** We also thank Tony Mazzulli, Aimin Li, the DNA Core Lab of
302 Public Health Ontario and the Microbiology Laboratory of the Guangzhou First People's
303 Hospital and AB BIODISK in Sweden for their support of the study.

304

305

REFERENCES

1. **Arakawa Y, Murakami M, Suzuki K, Ito H, Wacharotayankun R, Ohsuka S, Kato N, Ohta M.** 1995. A novel integron-like element carrying the metallo-β-lactamase gene *bla*$_{IMP}$. Antimicrob Agents Chemother **39:**1612-1615.
2. **Collis CM, Kim MJ, Partridge SR, Stokes HW, Hall RM.** 2002. Characterization of the class 3 integron and the site-specific recombination system it determines. J Bacteriol **184:**3017-3026.
3. **Xiong J, Alexander DC, Ma JH, Deraspe M, Low DE, Jamieson FB, Roy PH.** 2013. Complete sequence of pOZ176, a 500-kilobase IncP-2 plasmid encoding IMP-9-mediated carbapenem resistance, from outbreak isolate *Pseudomonas aeruginosa* 96. Antimicrob Agents Chemother **57:**3775-3782.
4. **Vilacoba E, Quiroga C, Pistorio M, Famiglietti A, Rodriguez H, Kovensky J, Deraspe M, Raymond F, Roy PH, Centron D.** 2014. A *bla*$_{VIM-2}$ plasmid disseminating in extensively drug-resistant clinical *Pseudomonas aeruginosa* and *Serratia marcescens* isolates. Antimicrob Agents Chemother **58:**7017-7018.
5. **Marchiaro PM, Brambilla L, Moran-Barrio J, Revale S, Pasteran F, Vila AJ, Viale AM, Limansky AS.** 2014. The complete nucleotide sequence of the carbapenem resistance-conferring conjugative plasmid pLD209 from a *Pseudomonas putida* clinical strain reveals a chimeric design formed by modules derived from both environmental and clinical bacteria. Antimicrob Agents Chemother **58:**1816-1821.
6. **Wang Y, Lo WU, Lai EL, Chow KH, Ho PL.** 2015. Complete sequence of the multidrug-resistant IncL/M plasmid pIMP-HB623 cocarrying *bla*$_{IMP-34}$ and fosC2 in

329 an *Enterobacter cloacae* strain associated with medical travel to China. Antimicrob
330 Agents Chemother **59:**5854-5856.
331 7. **Chu YW, Afzal-Shah M, Houang ET, Palepou MI, Lyon DJ, Woodford N,**
332 **Livermore DM.** 2001. IMP-4, a novel metallo-β-lactamase from nosocomial
333 *Acinetobacter* spp. collected in Hong Kong between 1994 and 1998. Antimicrob
334 Agents Chemother **45:**710-714.
335 8. **Houang ET, Chu YW, Lo WS, Chu KY, Cheng AF.** 2003. Epidemiology of
336 rifampin ADP-ribosyltransferase (arr-2) and metallo-β-lactamase (*bla*$_{IMP-4}$) gene
337 cassettes in class 1 integrons in *Acinetobacter* strains isolated from blood cultures
338 in 1997 to 2000. Antimicrob Agents Chemother **47:**1382-1390.
339 9. **Hawkey PM, Xiong J, Ye H, Li H, M'Zali FH.** 2001. Occurrence of a new metallo-
340 β-lactamase IMP-4 carried on a conjugative plasmid in *Citrobacter youngae* from
341 the People's Republic of China. FEMS Microbiol Lett **194:**53-57.
342 10. **Espedido BA, Partridge SR, Iredell JR.** 2008. *bla*$_{IMP-4}$ in different genetic
343 contexts in *Enterobacteriaceae* isolates from Australia. Antimicrob Agents
344 Chemother **52:**2984-2987.
345 11. **Koh TH, Sng LH, Wang GC, Hsu LY, Zhao Y.** 2007. IMP-4 and OXA β-
346 lactamases in *Acinetobacter baumannii* from Singapore. J Antimicrob Chemother
347 **59:**627-632.
348 12. **Xiong J, Tremblay, S., and Roy, P.H.** 2007. Abstr. 107th ASM General
349 Conference **Abstr A-047**
350 13. **Chin CS, Alexander DH, Marks P, Klammer AA, Drake J, Heiner C, Clum A,**
351 **Copeland A, Huddleston J, Eichler EE, Turner SW, Korlach J.** 2013. Nonhybrid,

finished microbial genome assemblies from long-read SMRT sequencing data. Nat Methods **10:**563-569.

14. **Hackl T, Hedrich R, Schultz J, Forster F.** 2014. proovread: large-scale high-accuracy PacBio correction through iterative short read consensus. Bioinformatics **30:**3004-3011.

15. **Aziz RK, Bartels D, Best AA, DeJongh M, Disz T, Edwards RA, Formsma K, Gerdes S, Glass EM, Kubal M, Meyer F, Olsen GJ, Olson R, Osterman AL, Overbeek RA, McNeil LK, Paarmann D, Paczian T, Parrello B, Pusch GD, Reich C, Stevens R, Vassieva O, Vonstein V, Wilke A, Zagnitko O.** 2008. The RAST Server: rapid annotations using subsystems technology. BMC Genomics **9:**75.

16. **Overbeek R, Olson R, Pusch GD, Olsen GJ, Davis JJ, Disz T, Edwards RA, Gerdes S, Parrello B, Shukla M, Vonstein V, Wattam AR, Xia F, Stevens R.** 2014. The SEED and the Rapid Annotation of microbial genomes using Subsystems Technology (RAST). Nucleic Acids Res **42:**D206-214.

17. **Hyatt D, Chen GL, Locascio PF, Land ML, Larimer FW, Hauser LJ.** 2010. Prodigal: prokaryotic gene recognition and translation initiation site identification. BMC Bioinformatics **11:**119.

18. **Stothard P, Wishart DS.** 2005. Circular genome visualization and exploration using CGView. Bioinformatics **21:**537-539.

19. **Carver T, Berriman M, Tivey A, Patel C, Bohme U, Barrell BG, Parkhill J, Rajandream MA.** 2008. Artemis and ACT: viewing, annotating and comparing sequences stored in a relational database. Bioinformatics **24:**2672-2676.

20. **Bennett PM, Heritage J, Hawkey PM.** 1986. An ultra-rapid method for the study of antibiotic resistance plasmids. J Antimicrob Chemother **18:**421-424.

21. **Sun F, Yin Z, Feng J, Qiu Y, Zhang D, Luo W, Yang H, Yang W, Wang J, Chen W, Xia P, Zhou D.** 2015. Production of plasmid-encoding NDM-1 in clinical *Raoultella ornithinolytica* and *Leclercia adecarboxylata* from China. Front Microbiol **6:**458.

22. **Tijet N, Richardson D, MacMullin G, Patel SN, Melano RG.** 2015. Characterization of multiple NDM-1-producing *Enterobacteriaceae* isolates from the same patient. Antimicrob Agents Chemother **59:**3648-3651.

23. **Wu W, Feng Y, Carattoli A, Zong Z.** 2015. Characterization of an *Enterobacter cloacae* Strain Producing both KPC and NDM Carbapenemases by Whole-Genome Sequencing. Antimicrob Agents Chemother **59:**6625-6628.

24. **Huang TW, Wang JT, Lauderdale TL, Liao TL, Lai JF, Tan MC, Lin AC, Chen YT, Tsai SF, Chang SC.** 2013. Complete sequences of two plasmids in a *bla*$_{\text{NDM-1}}$-positive *Klebsiella oxytoca* isolate from Taiwan. Antimicrob Agents Chemother **57:**4072-4076.

25. **Shin J, Soo Ko K.** 2014. Single origin of three plasmids bearing *bla*$_{\text{CTX-M-15}}$ from different *Klebsiella pneumoniae* clones. J Antimicrob Chemother **69:**969-972.

26. **Villa L, Garcia-Fernandez A, Fortini D, Carattoli A.** 2010. Replicon sequence typing of IncF plasmids carrying virulence and resistance determinants. J Antimicrob Chemother **65:**2518-2529.

27. **Gilmour MW, Thomson NR, Sanders M, Parkhill J, Taylor DE.** 2004. The complete nucleotide sequence of the resistance plasmid R478: defining the

backbone components of incompatibility group H conjugative plasmids through comparative genomics. Plasmid **52:**182-202.

28. **Chen CY, Nace GW, Solow B, Fratamico P.** 2007. Complete nucleotide sequences of 84.5- and 3.2-kb plasmids in the multi-antibiotic resistant *Salmonella enterica* serovar Typhimurium U302 strain G8430. Plasmid **57:**29-43.

29. **Xiong J, Hynes MF, Ye H, Chen H, Yang Y, M'Zali F, Hawkey PM.** 2006. *bla*$_{IMP-9}$ and its association with large plasmids carried by *Pseudomonas aeruginosa* isolates from the People's Republic of China. Antimicrob Agents Chemother **50:**355-358.

30. **Leung GH, Gray TJ, Cheong EY, Haertsch P, Gottlieb T.** 2013. Persistence of related *bla*$_{IMP-4}$ metallo-β-lactamase producing *Enterobacteriaceae* from clinical and environmental specimens within a burns unit in Australia - a six-year retrospective study. Antimicrob Resist Infect Control **2:**35.

31. **Peleg AY, Franklin C, Bell JM, Spelman DW.** 2005. Dissemination of the metallo-β-lactamase gene *bla*$_{IMP-4}$ among gram-negative pathogens in a clinical setting in Australia. Clin Infect Dis **41:**1549-1556.

32. **Sidjabat HE, Heney C, George NM, Nimmo GR, Paterson DL.** 2014. Interspecies transfer of *bla*$_{IMP-4}$ in a patient with prolonged colonization by IMP-4-producing *Enterobacteriaceae*. J Clin Microbiol **52:**3816-3818.

33. **Dolejska M, Masarikova M, Dobiasova H, Jamborova I, Karpiskova R, Havlicek M, Carlile N, Priddel D, Cizek A, Literak I.** 2016. High prevalence of *Salmonella* and IMP-4-producing *Enterobacteriaceae* in the silver gull on Five Islands, Australia. J Antimicrob Chemother **71:**63-70.

34. **Partridge SR, Ginn AN, Paulsen IT, Iredell JR.** 2012. pEI1573 Carrying *bla*$_{IMP-4}$, from Sydney, Australia, is closely related to other IncL/M plasmids. Antimicrob Agents Chemother **56:**6029-6032.

35. **Ho PL, Lo WU, Chan J, Cheung YY, Chow KH, Yam WC, Lin CH, Que TL.** 2014. pIMP-PH114 carrying *bla*$_{IMP-4}$ in a *Klebsiella pneumoniae* strain is closely related to other multidrug-resistant IncA/C2 plasmids. Curr Microbiol **68:**227-232.

36. **Liu Y, Zhang B, Cao Q, Huang W, Shen L, Qin X.** 2009. Two clinical strains of *Klebsiella pneumoniae* carrying plasmid-borne *bla*$_{IMP-4}$, *bla*$_{SHV-12}$, and *armA* isolated at a Pediatric Center in Shanghai, China. Antimicrob Agents Chemother **53:**1642-1644.

37. **Minakhina S, Kholodii G, Mindlin S, Yurieva O, Nikiforov V.** 1999. Tn*5053* family transposons are *res* site hunters sensing plasmidal *res* sites occupied by cognate resolvases. Mol Microbiol **33:**1059-1068.

38. **Kholodii GY, Mindlin SZ, Bass IA, Yurieva OV, Minakhina SV, Nikiforov VG.** 1995. Four genes, two ends, and a res region are involved in transposition of Tn*5053*: a paradigm for a novel family of transposons carrying either a *mer* operon or an integron. Mol Microbiol **17:**1189-1200.

39. **Juan C, Zamorano L, Mena A, Alberti S, Perez JL, Oliver A.** 2010. Metallo-β-lactamase-producing *Pseudomonas putida* as a reservoir of multidrug resistance elements that can be transferred to successful *Pseudomonas aeruginosa* clones. J Antimicrob Chemother **65:**474-478.

40. **Post V, Recchia GD, Hall RM.** 2007. Detection of gene cassettes in Tn*402*-like class 1 integrons. Antimicrob Agents Chemother **51:**3467-3468.

Antimicrobial Agents and Chemotherapy

41. **Gillings MR.** 2014. Integrons: past, present, and future. Microbiol Mol Biol Rev **78:**257-277.

42. **Collis CM, Recchia GD, Kim MJ, Stokes HW, Hall RM.** 2001. Efficiency of recombination reactions catalyzed by class 1 integron integrase IntI1. J Bacteriol **183:**2535-2542.

43. **Wei Z, Yu T, Qi Y, Ji S, Shen P, Yu Y, Chen Y.** 2011. Coexistence of plasmid-mediated KPC-2 and IMP-4 carbapenemases in isolates of *Klebsiella pneumoniae* from China. J Antimicrob Chemother **66:**2670-2671.

44. **Peleg AY, Franklin C, Walters LJ, Bell JM, Spelman DW.** 2006. OXA-58 and IMP-4 carbapenem-hydrolyzing β-lactamases in an *Acinetobacter junii* blood culture isolate from Australia. Antimicrob Agents Chemother **50:**399-400.

45. **Chen Z, Wang Y, Tian L, Zhu X, Li L, Zhang B, Yan S, Sun Z.** 2015. First Report in China of *Enterobacteriaceae* Clinical Isolates Coharboring bla_{NDM-1} and bla_{IMP-4} Drug Resistance Genes. Microb Drug Resist **21:**167-170.

46. **Limbago BM, Rasheed JK, Anderson KF, Zhu W, Kitchel B, Watz N, Munro S, Gans H, Banaei N, Kallen AJ.** 2011. IMP-producing carbapenem-resistant *Klebsiella pneumoniae* in the United States. J Clin Microbiol **49:**4239-4245.

47. **Roy PH.** 1999. Horizontal transfer of genes in bacteria. Microbiology Today **26:**168-170.

48. **Liebert CA, Hall RM, Summers AO.** 1999. Transposon Tn*21*, flagship of the floating genome. Microbiol Mol Biol Rev **63:**507-522.

49. **Partridge SR, Brown HJ, Stokes HW, Hall RM.** 2001. Transposons Tn*1696* and Tn*21* and their integrons In4 and In2 have independent origins. Antimicrob Agents Chemother **45:**1263-1270.

50. **Summers AO, Jacoby GA.** 1977. Plasmid-determined resistance to tellurium compounds. J Bacteriol **129:**276-281.

51. **Taylor DE, Summers AO.** 1979. Association of tellurium resistance and bacteriophage inhibition conferred by R plasmids. J Bacteriol **137:**1430-1433.

52. **Bradley DE.** 1985. Detection of tellurite-resistance determinants in IncP plasmids. J Gen Microbiol **131:**3135-3137.

53. **Carattoli A, Bertini A, Villa L, Falbo V, Hopkins KL, Threlfall EJ.** 2005. Identification of plasmids by PCR-based replicon typing. J Microbiol Methods **63:**219-228.

480 **FIGURE LEGENDS**

481 **FIG 1.** Map of plasmid pOZ172 from *C. freundii* B38. The scale is indicated on the
482 innermost circle. The second circle is G+C skew in green (+) and purple (-), and circle 3
483 shows G+C content (deviation from the average) in black (+, outward and -, inward). The
484 next two circles illustrate positions of CDSs in minus (circle 4) and plus (circle 5) strands
485 in dark blue. The two green arcs represent two replication/transfer regions most similar
486 (99%) to the IncF plasmids in Table 3, The bla_{IMP-4}-carrying integron Tn*6017* (in red) is
487 inserted into Tn*1722* (in yellow).

488 **FIG 2.** Class 1 integrons identified on pOZ172 and pOZ181 in *C. freundii* B38 and related
489 plasmids. (A) Tn*6017*, a Tn*402*/*5053*-like integron, was identified on pOZ172 and was
490 inserted into the *res* II site of Tn*1722*, splitting the *res* site into two ½ *res*. Arrow boxes
491 show the genes and their orientations; each solid black oval indicates *attI* and each white
492 oval represents the *attC* of the preceding gene; delta (Δ) represents disrupted genes.
493 *mcp* is the gene for the methyl-accepxis protein of Tn*1722*. (B): Two most representative
494 sul1-type bla_{IMP-4} class 1 integrons found in GenBank. Their cassette array is the same
495 but differs from that of Tn*6017*. The black rectangle is 25-nt repeat IRi; the short vertical
496 lines are the 12-nt repeats of IS*26*. (C) A second, *sul1*-type integron was identified on
497 pOZ181 and was linked upstream to Tn*1696* and downstream to an IS*CR1*. The small
498 rectangular white boxes represent the *res* sites adjacent to *tnpR* and the solid black
499 boxes represent the 25-bp IRi (Tn*402*) and IRt (Tn*5053*) sites; the larger solid black
500 boxes represent the 38-bp IRL and IRRI of Tn*1722*, as well as IRtnp of Tn*1696*; the small
501 arrows represent the direction of promoters (P and P1).

502

503 **TABLE 1.** Susceptibilities (MICs (mgL^{-1}) of *C. freundii* B38

Strain	Test date	IMP	MEM	CAZ	CTX	CRO	FEP	TIM	CFP	TZP	CIP	AMK	GEN
C. freundii	1999.2[a]	24	ND	256	256	256	256	256	256	32	32	256	256
B38	2000.5[b]	0.5	6	256	256	256	256	256	256	32	32	256	256
	2002.5[c]	2	0.5	256	256	256	ND	ND	ND	32	32	256	ND

504 a: tested in Guangzhou with Etest gradient method; b: tested in Sweden with Etest
505 gradient method; c: tested in Birmingham with agar dilution method. IMP, imipenem;
506 MEM, meropenem; CAZ, ceftazidime; CTX, cefotaxime; CRO, ceftriaxone; FEP,
507 cefepime; TIM, ticarcillin/clavulanic acid; CFP, cefoperazone/sulbactam; TZP, piperacillin/
508 tazobactam; CIP, ciprofloxacin; AMK, amikacin; GEN, gentamicin. ND, not determined.

509
510
511 **TABLE 2.** The overall features of the *C. freundii* B38 genome

	Chromosome	pHRB381	pOZ172	pHRB382
Size (bp)	5,134,500	277,592	127,005	18,467
G+C%*	51.7	45.8	54.4	57.7
Predicted CDS	4905	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^R	bla_{IMP-4}; *qacG*; *aacA4* , *aphA15*	bla_{TEM-1}

512 *Mobile elements and insertions were not excluded.

513

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

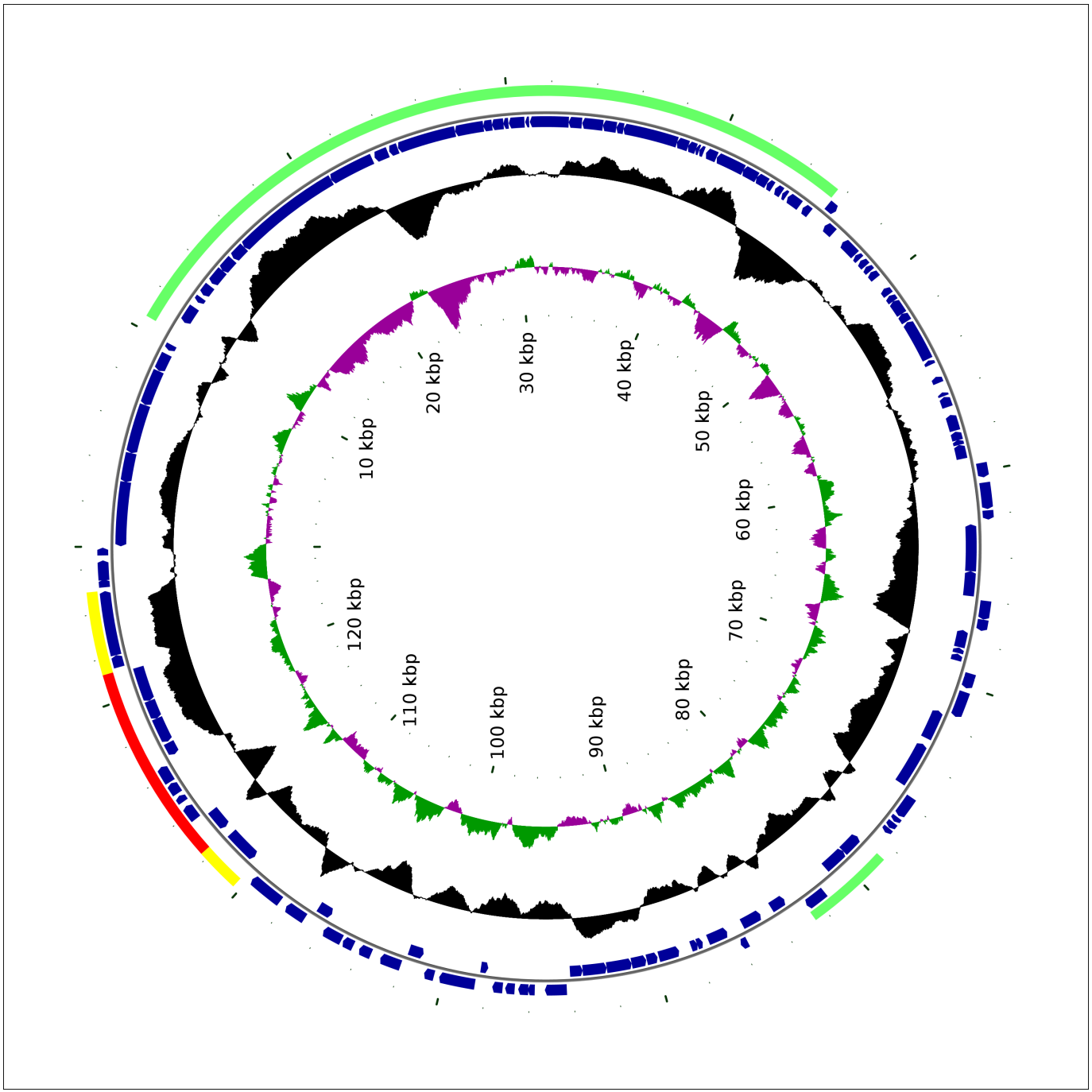
515

Function	Genomic(coordinates)	Homologs in GenBank
pOZ172		
Replication Transfer	*repAB* (bp: 11218-12648, c); *tra* operon (bp:14277-45250)	most similar (99%) to: pNDM-Ec1GN574, pNDM1_EC14653 (*E. cloacae*); pKOX_NDM1 (*K. oxytoca*); pP10164-NDM (*Leclericia adecarboxylata*); pYDC644, pRJF866 (*K. pneumonia*)
Replication Partition	*repFIB* (bp: 81538-82548); *parBA* (bp:78650–80787, c)	most similar (99%) to: pCAV1344-250, pKPN-262, pKPN3 ,pKP007 plasmid II str. Kp52.145, pc15-K, pUUH2392 pKP02022, pKP09085 (*K. pneumoniae*); pOU7519 (*S. enterica*); etc.
pHRB381		
Replication Transfer Partition	*repHI2A* (bp:75780-76868,c); r*epH1A* (bp:90277-91152); *tra2* (bp:24119-41412); *tra1*(bp:92763-141366); *parAB*(107267-108722); *parMR*(112426-114859)	most similar (99-100%) to: R478* (*S. marcesens*); 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. *pHRB381 has an 11 kb insertion of hypothetical proteins between *parMR* and *htdA* of R478.
Heavy metal resistance	*ter* operon(bp:166156-182942); *ars* operon (bp:53-2937); *rcnA* operon (bp:263574-265084)	
Sulphate permease	*sfpAB* operon (bp:275261-277585)	

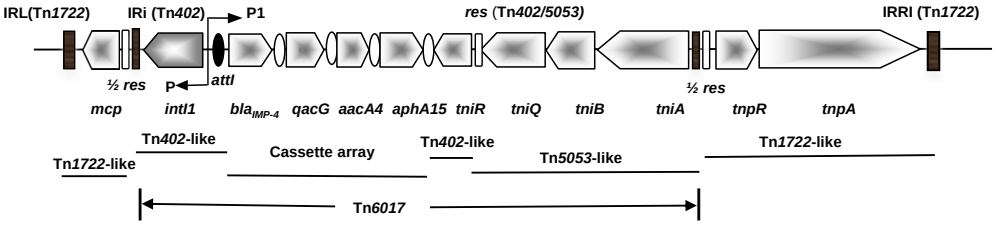
516

517

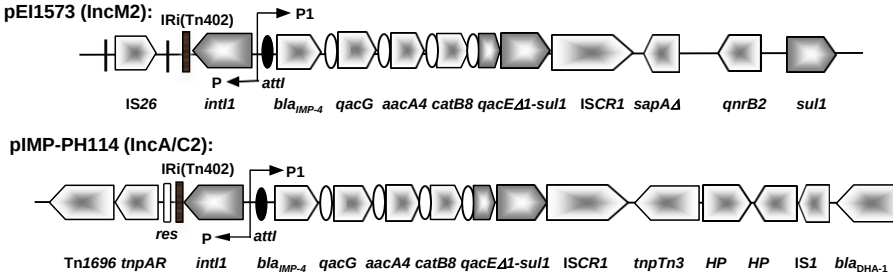
Antimicrobial Agents and Chemotherapy



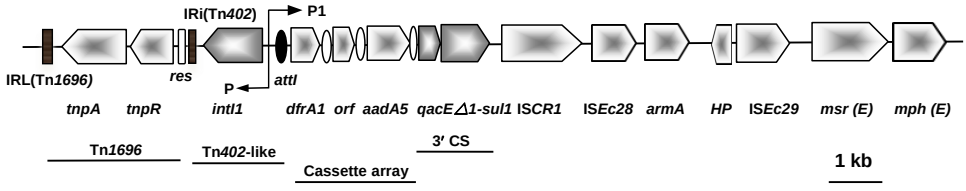
(A): **Tn*6017* in a Tn*1721*-like element in pOZ172 (IncFIIy):**



(B): **Two *sul1*-type *bla*$_{IMP-4}$ integrons in GenBank: pEI1573 (JX101693) & pIMP-PH114 (KF250428)**



(C): ***sul1*-type integron in a partial Tn*1696* in pHRB381(IncHI2):**



Antimicrobial Agents and Chemotherapy