AAC Accepted Manuscript Posted Online 20 February 2018
Antimicrob. Agents Chemother. doi:10.1128/AAC.00006-18

Complete sequence of the IncA/C_1 plasmid pCf587 carrying bla_{PER-2} from *Citrobacter freundii*

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Running title: An IncA/C_1 plasmid carrying bla_{PER-2}

Abstract

The *bla*$_{PER-2}$ harboring plasmid pCf587 (191,541 bp) belongs to lineage IncA/C$_1$ and is closely related to pRA1. It contains a large resistance island including the *bla*$_{PER-2}$ gene between two copies of IS*Kox2*-like elements, the toxin–antitoxin module *pemK-pemI,* several other resistance genes inserted within a Tn*2* transposon, a Tn*21*-like structure, and a class 1 integron. pCf587 belongs into ST 13, a new pMLST.

27 **Text**

28

29 Since the initial report of PER-2 (1), *bla*$_{PER-2}$ was detected in different species including
30 *Klebsiella pneumoniae*, *Enterobacter cloacae*, *Enterobacter aerogenes*, *Vibrio cholerae* and
31 community-acquired enteropathogenic *Escherichia coli*. PER-2 has been the second most
32 frequent ESBL (after the pandemic CTX-Ms) in Argentina (and probably Uruguay), accounting
33 for nearly 10% and 5% of the oxyimino-cephalosporin resistance in *K. pneumoniae* and *E. coli*,
34 respectively (2-4); it has also been sporadically reported in a few other countries (5-10). PER-1
35 and PER-2 are the most frequently reported members of the nine-variants PER family in clinical
36 settings (2).
37 In contrast to *bla*$_{PER-1}$ containing plasmids (11-13), little is known about the genetic
38 organization of the *bla*$_{PER-2}$ gene. We previously reported the immediate flanking sequences in
39 plasmid pCf587 from *Citrobacter freundii* 33587, recovered from a urine sample in 1999 (14).
40 To further delineate the genetic background of *bla*$_{PER-2}$, the aim of this study was to analyze the
41 complete sequence of pCf587 recovered almost 20 years ago.
42 *E. coli* 33587-TC9 is an *E. coli* CAG12177 transconjugant clone harboring the pCf587 plasmid
43 from *C. freundii* 33587 (14). The plasmid sequence was determined with the Illumina MiSeq
44 platform. Full genomic DNA was sequenced by MinION (Oxford Nanopore Technologies). A
45 hybrid *de novo* assembly was performed with SPAdes V3.9.0 using both generated libraries
46 (15). Gaps were closed using a PCR-based strategy and Sanger sequencing. Gene predictions
47 and annotation were performed on classic RAST tool (16, 17) and manually curated by BLAST
48 online. Sequence comparisons were performed by Mauve software (18). Identification of
49 acquired antibiotic resistance genes and the incompatibility group determination were
50 conducted by ResFinder 3.0 (19) and PlasmidFinder (20), respectively. Alignments were
51 constructed using ClustalW with default settings. The phylogenetic tree was produced in
52 MEGA 7.0.26 (21) using maximum likelihood with default settings and 1,000 bootstraps.

53 The overall genetic organization of pCf587 is shown in Figure 1. The plasmid (191,541 bp,
54 average G+C content of 49.97 %) contains 253 coding sequences, from which 141 have no
55 assigned annotation. The genes involved in a type IV secretion system, the master regulators
56 *acaDC,* the *traI* gene encoding for the MOB_{H121} relaxase, and the putative maintenance genes
57 *parAB*, *stbA(parM)*, and *kfrA*, were easily recognized (Figure 1). A large resistance island (RI-
58 pCf) was also identified (Figure 2).
59 The *in-silico* analysis established that pCf587 belongs to the IncA/C group. Upon searching for
60 closely related plasmids by nucleotide BLAST in the NCBI database, only three IncA/C plasmids
61 with a query coverage higher than 65% were found: *Enterobacter hormaechei subsp.*
62 *steigerwaltii* strain 34998 plasmid p34998 (73%), *Aeromonas hydrophila* plasmid pRA1 (66%),
63 and *K. pneumoniae subsp. pneumoniae* strain KP4898 plasmid pIncAC-KP4898 (67%) (accession
64 numbers: CP012168.1, FJ705807.1, KY882285.1). The plasmids backbone comparison revealed
65 that pCf587 is more closely related to pRA1 (Figure 3); they have a similar overall genetic
66 arrangement and backbone length with almost 99% nucleotide identity.
67 IncA/C backbones are highly conserved and it has been postulated that they derive from a
68 common ancestor (27). However, two different lineages, A/C_1 and A/C_2, were established
69 based on *rep*A genes sequence similarities (28); $IncA/C_2$ plasmids were further split in two
70 types (29). To date, only the primitive plasmid pRA1 (ST11) (22) and the recently incorporated
71 VIM-encoding pIncAC-KP4898 (ST12) (30) belong to the first lineage.
72 Plasmid pCf587 *repA* gene has 99% nucleotide identity with pRA1 *repA*, with only two
73 nucleotidic changes not resulting in amino acid changes; the *tra* genes are 99-100% identical to
74 the corresponding *tra* genes from pRA1. Therefore, pCf587 belongs to IncA/C1, along with
75 pRA1 and related plasmids.
76 Other features shared with pRA1 are: *(i)* an ORF between *traA* and *dsbC* encoding a 1,828-aa
77 protein (orf1828) with 99% amino acids identity; *(ii)* the TA *hipAB*; *(iii)* absence of *tad* and *ata*

genes from other putative TA system typical of all IncA/C_2 plasmids; *(iv)* lack of *ssb* gene (present in IncA/C_2 plasmids).

In most A/C_2 type 1 plasmids, the antimicrobial resistance island A (ARI-A) is found either embedded in or upstream of the *rhs1* gene. It contains a class 1 integron, multiple transposons, a Tn*21-tnp* module, and a Tn*21-mer* module generally interrupted by IS*4321* which docks the resistance island at that site (29). The RI-pCf (Figure 2) has similar characteristics and location as that for ARI-A, although *rhs1* is absent as expected. RI-pCf comprises: *(i)* a bla_{TEM-1B}-containing Tn*2* whose *tnpA* is interrupted by an IS*As1*-like element; *(ii)* the TA *pemKI* followed by a large region with three IS*1R* copies, an IS*Aba125*-like element, the *aph(3')-VIa*-like and *catA1*-like resistance genes; *(iii)* a Tn*21*-like structure including the *Tn*21-*tnp* and *Tn*21-*mer* modules whose IR_{tnp} is interrupted by a IS*4321*-like element, a class 1 integron carrying the *aadB* and *sul1* resistance genes, and a zone delimited by two similar copies of IS*Kox2*-like elements (sharing 99% nucleotide identity) carrying the bla_{PER-2} gene, a truncated IS*1326* and an IS*6100.* The two IS*Kox2*-like were found 26 bp upstream *abct* and 3,703 bp downstream IS*Pa12* elements previously found as part of the bla_{PER-2} environment (IS*Pa12*/bla_{PER-2}/*gst*-like/*abct*) (14). The region between IS*Kox2*-like and IS*Pa12*, with no homologues in NCBI database, contains 8 ORF including a *traW* gene encoding a putative conjugal transfer pilus assembly protein. We postulate that IS*Kox2*-like elements could have been involved in the recruitment of bla_{PER-2} and its surrounding genes from a still unknown reservoir to an ancient RI-pCf (Figure 2). A recent publication describes bla_{PER-2} in the chromosome of a clinical *Shewanella sp.* isolate Shew256 (31). The *abct* in Shew256 was larger than in pCf587, what may suggest that the IS*Kox2*-like element partially interrupted pCf587-*abct* during recruitment.

Interestingly, there are no copies of IS*26* in pCf587. This IS is found associated with most IncA/C plasmids previously described, including pRA1 (27) and is considered to be implicated in the evolution of ARI-A in type 1 A/C_2 plasmids (29).

104 For pCf587, the database (https://pubmlst.org/plasmid/) could not recognize a specific ST,
105 according to the Plasmid Multilocus Sequence Typing Scheme (pMLST) for IncA/C plasmids
106 developed by Hancock *et al* (25). We built a phylogenetic tree using concatenates of the four
107 pMLST genes (Figure 4), and observed that, as expected, pCf587 was related to ST11 which
108 includes pRA1 as well as other IncA/C_1 plasmids like the recently described ST12 (pIncAC-
109 KP4898) (30), and p34998, all separate from the rest of the STs including the IncA/C_2 lineage
110 (type 1 and type 2). The pMLST alleles of pCf587 were assigned as ST13, and the core gene
111 PMLST (cgPMLST) ST13.1.
112 IncA/C plasmids are high-molecular size, low-copy number plasmids initially described in fish
113 pathogens as *A. hydrophila* and *Vibrio spp*. around 1970 (22-24), and are now disseminated
114 among *Enterobacteriaceae* (25, 26).
115 Currently, IncA/C plasmids are considered as an important healthcare problem (32), and
116 responsible for dissemination of *bla*$_{CTX-M}$, *bla*$_{CMY}$, *bla*$_{NDM}$, *bla*$_{IMP}$, and *bla*$_{VIM}$, and *bla*$_{KPC}$ genes,
117 among others (25, 26).
118 It is noteworthy that IncA/C_1 plasmids like pCf587 could have been circulating among
119 pathogens in Argentina since at least the late 90s, and even so, their dissemination seems to
120 be not as proficient as other resistance plasmids like IncA/C_2 involved in mobilization of CTX-M
121 or metallo-β-lactamases, which are much more widespread enzymes. The presence of efficient
122 TA-systems in their backbone may provide some stability even in the absence of selective
123 pressure. This plasmid lineage may also have a role in mobilization of other (still unrecognized)
124 resistance markers, as shown by the recent finding of some MBL associated with similar
125 backbones in recent isolates (30) (Elena *et al.* unpublished results).
126 Even if further studies on the mechanisms involved in *bla*$_{PER-2}$ mobilization are still needed, this
127 study provides some insights on the genetic elements that could have facilitated the
128 recruitment of *bla*$_{PER-2}$ in IncA/C_1 plasmids.
129

Nucleotide sequence accession number

The annotated complete sequence of pCf587 plasmid has been deposited in GenBank under accession number MG053108.

Acknowledgments

This work was funded by grants from the University of Buenos Aires (UBACyT 2014-2017 to PP; and 2013-2015 to GG), Agencia Nacional de Promoción Científica y Tecnológica (BID PICT 2011-0742 to GG, and PICT 2014-0457 to PP) and the Assistance Publique – Hôpitaux de Paris, by a grant from the Université Paris Sud (EA 7361), and by the LabEx LERMIT supported by a grant from the French National Research Agency (ANR-10-LABX-33). This work was also funded in part by a grant from Joint Programme Initiative on Antimicrobial Resistance (ANR-14-JAMR-0002). M. Ruggiero is a Post-doctoral fellow, CONICET, Argentina. P. Power and G. Gutkind are members of Carrera del Investigador Científico, CONICET, Argentina.

Transparency declarations

None to declare

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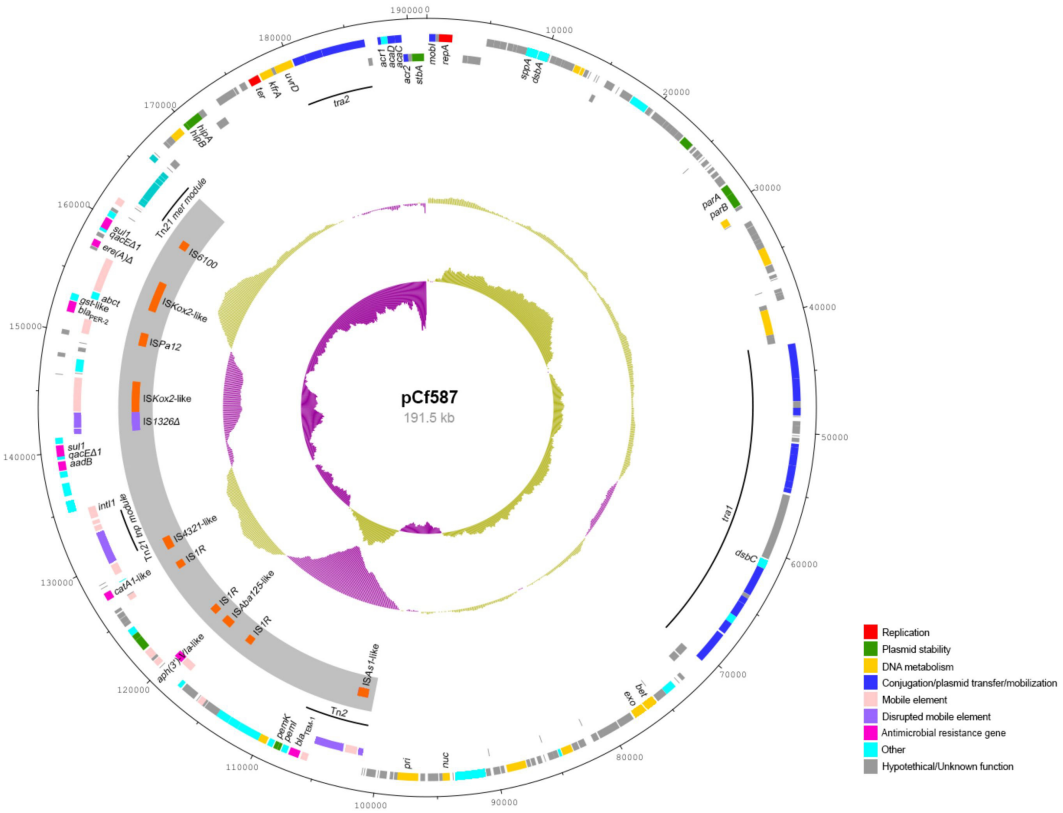
Figure 1. Schematic representation of the genetic organization of pCf587. The two outer rings show the coding sequences (CDSs) on the forward and reverse strand of the plasmid. Each CDS is color-coded by its predicted function as shown in the figure. The grey arc depicts the resistance island (RI-pCf) with the IS identified on the plasmid. The two inner rings represent the GC plot and GC skew graph, respectively. For both plots, magenta and olive green indicate the measures below and above the average, respectively.

Figure 2. Schematic representation of the pCf587 resistance island (RI-pCf).. Color codes match those used in Figure 1. The elements highlighted in the figure are further discussed in the text. The box indicates the close environment of the bla_{PER-2} gene. Direct repeats bracketing the postulate IS*Kox2*-like-madiated transposition event of bla_{PER-2} and its surrounding genes are shown in capitals. Inverted repeats from ISKox2-like are marked with yellow (IRL) and orange (IRR) rectangles.

Figure 3. Local collinear blocks (LCBs) comparison between pCf587, pRA1, p34998 and pIncAC-KP4898 by MAUVE software. Each LCB represents regions with homologous sequences without rearrangements. Same LCB are identified with the same color. The height of each LCB is proportional to the identity level between them. Grey and red lines indicate the plasmids backbone and the resistance islands respectively. The dotted grey line indicates an insertion in the p34998 backbone. The black arrow indicates the position of a deletion on pIncAC-KP4898 backbone. The *repA* gene of each plasmid is identified.

Figure 4. Phylogenetic relationships between pCf587, p34998 and the different IncA/C STs to date, based on maximum likelihood and Bayesian methods. The tree was created based on the alignment of concatenated gene sequences of *repA*, *parA*, *parB* and 053.



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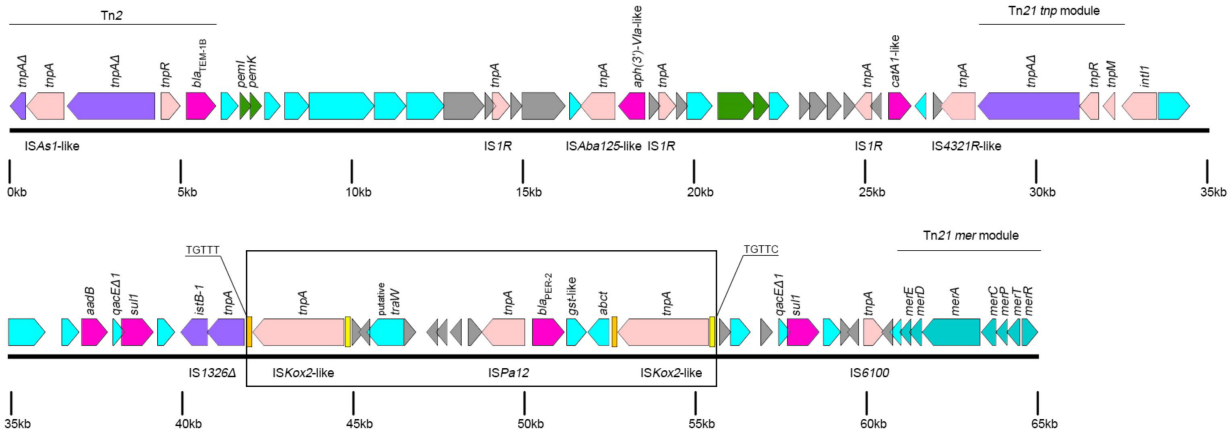

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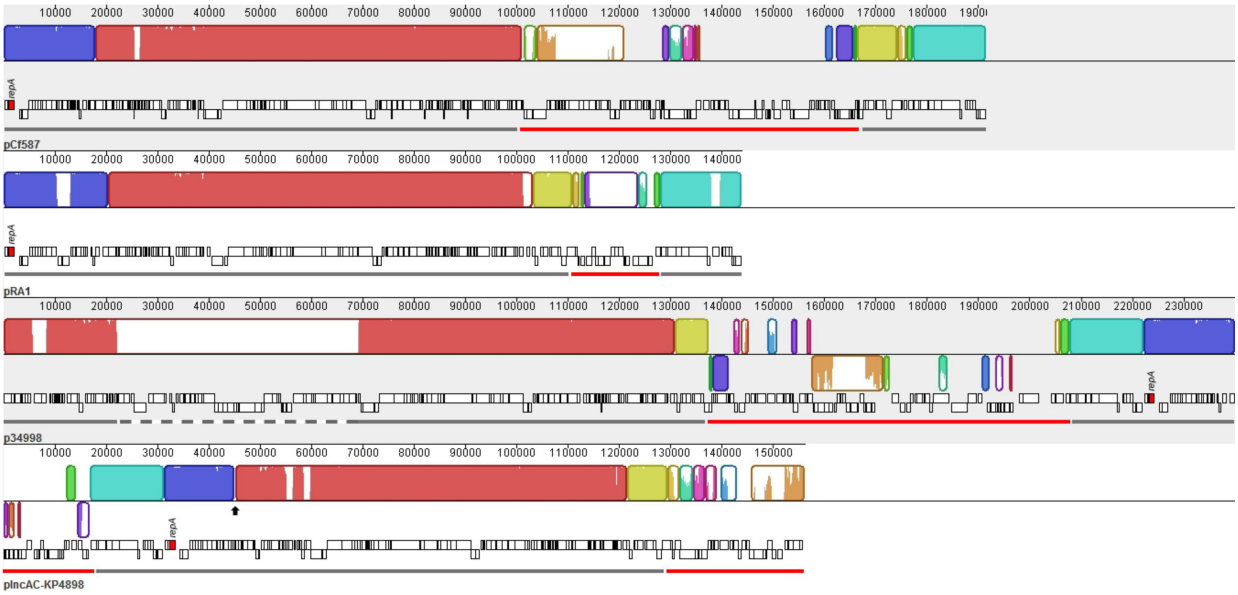

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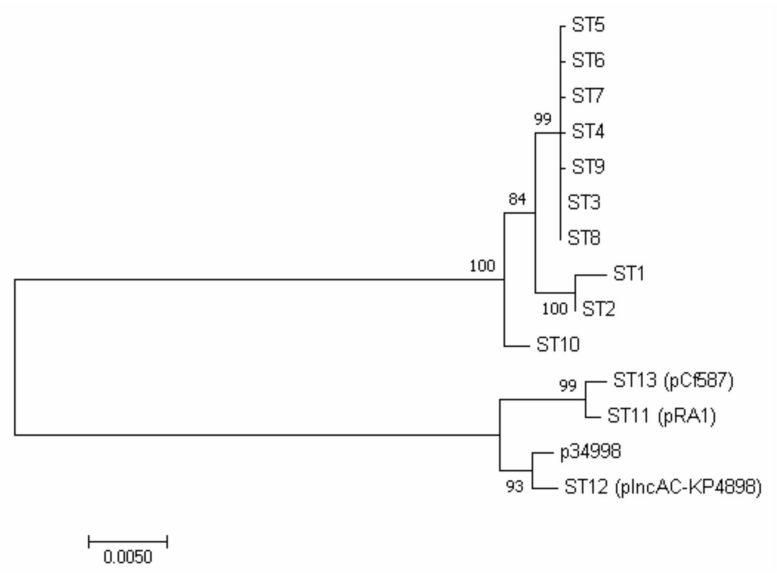

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