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Abstract

Carbapenemase-producing Enterobacterales expressing OXA-48, KPC, NDM, VIM or IMP enzymes are increasingly reported worldwide. We have characterized LMB-1, a novel metallo-β-latamase (MBL) of Ambler class B3 from *Citrobacter freundii* 164 (*Cf*164) clinical isolate from Buenos Aires, Argentina.

*Cf*164 displayed reduced susceptibility to carbapenems but gave inconsistent results with carbapenemase confirmatory tests, suggesting the presence of a weak carbapenemase. Analysis of WGS of *Cf*164 using Resfinder revealed four β-lactamase genes coding for CTX-M-8, PER-2, TEM-1 and CMY-150, a novel chromosomally-encoded CMY variant. Kinetic parameters of purified CMY-150 did not reveal any carbapenemase activity. However, CMY-150 conferred to *E. coli* higher MIC values for ceftazidime and aztreonam as compared to CMY-2. The in-house developed β-lactamase search software (ResMINER) in WGS data, revealed a novel subclass B3 MBL named LMB-1. LMB-1 conferred to *E. coli*, resistance to penicillins, to expanded-spectrum cephalosporins and reduced susceptibility to carbapenems. The $bla_{\text{LMB-1}}$ gene was located on a 176-kb IncA/C2 plasmid. LMB-1 shared 99% of amino acid sequence identity with the MBL encoded in the chromosome of *Rheinheimera pacifica*, it's likely progenitor. Despite repeated attempts, LMB-1 could not be purified, thus only specific activities could evidence hydrolysis of carbapenems.

Here we report CMY-150, a novel CMY-2 variant that confers increased ceftazidime and aztreonam MICs to *E. coli* and the first description of LMB-1 in Argentina. This work underlines the need for several CPE confirmatory tests, as this novel enzyme might have been missed using only one.

1. Introduction

Gram-negative bacteria (GNB), especially Enterobacterales, *Pseudomonas aeruginosa* and *Acinetobacter baumannii*, have re-emerged as major players in antimicrobial resistance worldwide. In these species, resistance may affect all major classes of anti-gram-negative agents, the multidrug resistance (MDR) being relatively common. Currently, β-lactamase-mediated resistance does not spare even the newest and most powerful β-lactams (carbapenems), whose activity is challenged by carbapenemases [1].

Carbapenem-hydrolyzing β-lactamases encountered in Enterobacterales belong either to (i) the Ambler class A including KPC, IMI and GES enzymes, (ii) Ambler class B metallo-β-lactamases (or MBL) of NDM-, VIM- and IMP-type, and (iii) Ambler class D enzymes including OXA-48 and its variants (mostly OXA-181, OXA-204 and OXA-232) [1]. Even though described for a few class C enzymes, carbapenem-hydrolysis is very rare and weak [2,3].

MBLs are by far the most worrisome β-lactamases since their prevalence is increasing worldwide, their broad substrate profile (including all β-lactams, except monobactams), and the lack of clinically-useful inhibitors. The MBLs are divided into three subclasses (B1, B2, B3) based on primary amino acid sequence homology [4]. There is relatively low sequence identity (< 20%) between the subclasses. Within a subclass the sequence identity is higher and this, along with distinctive structural characteristics within the active sites of B1, B2, and B3 enzymes, is the basis for the establishment of the three subclasses. The B1 and B3 subclasses have a broad-spectrum substrate profile that includes penicillins, cephalosporins, and carbapenems and contain two Zn^{2+} ions in their active site, while the B2 enzymes exhibit a narrow-spectrum profile that includes carbapenems and contain only one Zn^{2+}ion.

MBLs of subclass B1, such as NDM, VIM, and IMP-like are the clinically-most relevant enzymes in Enterobacterales, but sporadic isolations of other class B1 MBLs such as GIM, FIM, KHM, have also be reported [4]. Subclass B2 contains only two enzymes that possess a narrow-spectrum of hydrolysis. The Subclass B3 is a larger group of enzymes, of which the genes are mostly chromosomally-encoded, and display a broad-spectrum of ß-lactam hydrolysis. In this subclass only AIM, found in *P. aeruginosa*, and SMB-1 in *Serratia marcescens* have been reported acquired [5,6].

While the increasing availability of commercial qPCR systems allows for the detection of the most prevalent MBL families, molecular diagnostics clearly overlooks MBLs that belong to less prevalent MBL-families. Furthermore, novel MBLs have recently been identified in clinical isolates: TMB-1 in *Enterobacter hormaechei* and *Citrobacter freundii* from Tunisia [7] and LMB-1 in *Enterobacter cloacae* from Austria [8]. In this work, we have characterized a novel chromosomally-encoded CMY-variant: CMY-150 and the second clinical LMB-1-producing Enterobacterales, a *C. freundii* clinical isolate recovered from a Hospital in Buenos Aires, Argentina.

2. Materials and Methods

2.1. Bacterial strains. *C. freundii* 164 (*Cf*164) was identified with matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) mass spectrometry (MALDI Biotyper CA system, Bruker Daltonics, Billerica, MA, USA). *Escherichia coli* TOP10 (Invitrogen, Saint-Aubin, France) was used for cloning experiments and *E. coli* BL21 was used for over-expression of ß-lactamases.

2.2. Antimicrobial agents, susceptibility testing. Antimicrobial susceptibilities were determined by disk diffusion on Mueller-Hinton agar (Bio-Rad, Marnes-La-Coquette, France) and interpreted according to the EUCAST breakpoints, updated in 2018 (http://www.eucast.org). Minimal inhibitory concentration (MIC) values were determined using the Etest technique (BioMérieux, Marcy-l'Etoile, France).

2.3. Confirmatory tests for carbapenemase-producing Enterobacteria. Carbapenemase activity was investigated using the in house Carba NP test, Blue Carba, Modified Hodge test, β Carba Test (BioRad, Marnes-la-Coquette, France), rCIM, Maldi-TOF (MBT STAR-Carba™, Brucker), PCRs and NG Carba5 (NG Biotech, Rennes, France) tests were performed following the manufacturer's recommendations or as previously published [9-12].

2.4. PCR, cloning experiments, and sequencing. Whole-cell DNAs of Cf164 isolates were extracted using the QIAamp DNA minikit (Qiagen, Les Ulis, France) and were used as templates for PCR with the following primers: preCMY-150-A (5'-ATGATGAAAAAATCGAT-3') and preCMY-150-B (5'-TCTGTCAGTTATTGCAGT-3') and preLMB-1-A (5'-ATGACGTTAGCTAAAAGTTTT -3') and preLMB-1-B (5'-TACTGCGTTGTTTCCTTTATTAAA-3') from amplifying $bla_{\text{CMY-150}}$, and $bla_{\text{LMB-1}}$, respectively. The amplicon obtained was then cloned into the pCR®-Blunt II-TOPO® plasmid

(Invitrogen) downstream from the pLac promoter, in the same orientation. The recombinant pR-CMY-150 and pR-LMB-1 plasmids were electroporated into the *E. coli* TOP10 strain; the electroporants were plated on TSA plate containing kanamaycin (50 µg/ml).

The $bla_{\text{CMY-150}}$ and $bla_{\text{LMB-1}}$ gene fragments corresponding to the mature β-lactamases were cloned into the expression vector pET41b (+) (Novagen, VWR International, Fontenay-sous-Bois, France) and $bla_{\text{LMB-1}}$ gene fragment corresponding to the mature β-lactamases was also cloned into pET28a (Novagen) using the PCR generated fragment with primers CMY-150-Mat (5'-ACTTTAAGAAGGAGATATACATATGAAAACAGAACAACAAATTGCCGA-3') and CMY-150-Stop (5'-TGGTGGTGGTGCTCGAATTGCAGTTTTTCAAGAATGCGC-3'), LMB-1-pET41b_Fw (5'-TTTACTTCCAGGGCCAAGGAATGCTGGCCGGTTGC-3'), LMB-1-pET41b-Rv (5'-TGGTGGTGGTGGTGGTGCTCGATTACTGCGTTGTT-3'), LMB_pET28a_Fw (5'-TTTACTTCCAGGGCCAAGGAATGCTGGCCGGTTGC-3'), LMB_pET28a_Rv (5'-TGGTGGTGGTGGTGGTGCTCGATTACTGCGTTGTT 3') and the NEBuilder® HiFi DNA Assembly Cloning Kit (New England BioLabs, Ozyme, Les Ulis, France), following the manufacturer's instructions. The recombinant plasmids pET41-CMY-150 pET41b-LMB-1 and pET28a-LMB-1 were transformed into the chemically-competent *E. coli* strain BL21 (DE3).

Recombinant plasmids were extracted using the Qiagen miniprep kit and both strands of the inserts were sequenced using M13 primers for the pCR®-Blunt II-TOPO® plasmid (Invitrogen), and T7 primers for pET41b(+) (Novagen), with an automatic sequencer (ABI Prism 3100; Applied Biosystems, Les Ulis, France). The nucleotide sequences were analyzed using software available at the National Center for Biotechnology Information website (http://www.ncbi.nlm.nih.gov).

2.5. Whole genome sequencing (WGS) and Bioinformatic analysis. Total DNA was extracted from colonies using the Ultraclean Microbial DNA Isolation Kit (MO BIO Laboratories, Carlsbad, CA, US) following the manufacturer's instructions. The DNA concentration and purity were controlled by a Qubit® 2.0 Fluorometer using the dsDNA HS and/or BR assay kit (Life technologies, Carlsbad, CA, US). The DNA library was prepared using the Nextera XT-v3 kit (Illumina, San Diego, CA, US) according to the manufacturer's instructions and then run on Miseq (Illumina) for generating paired-end 2x150 bp reads. *De novo* assembly was performed by CLC Genomics Workbench v9.5

(Qiagen) after quality trimming (Qs ≥ 20) according the manufacturer's recommendations (Qiagen, Courtaboeuf, France). *C. freundii* Cf164 total DNA was also sequenced using PacBio long read technology (Macrogen, Seoul, South Korea). PacBio subreads were assembled with both Canu [13] and the RS_HGAP_Assembly.3 protocol from the SMRT analysis toolkit v2.3, while consensus accuracy was further polished using Quiver [14] as was previously described [15]. The acquired antimicrobial resistance genes were identified by uploading assembled genomes to the Resfinder server v2.1 that uses BLAST for identification of acquired antimicrobial resistance genes and chromosomal mutations in whole-genome data (http://cge.cbs.dtu.dk/services/ResFinder-2.1) [16]. Additionally, the whole genome sequence of *Cf*164 was also uploaded to an in-house developed software, ResMiner (BI Iorga, pers. comm.), that searches for flags specific of the four molecular classes of β-lactamases.

For the reconstruction of pCf164_LMB-1 and pCf164_CTX-M-8 gaps were filled by PCR amplification and Sanger sequencing. Open reading frames (ORFs) were annotated using the RAST server (rast.nmpdr.org) followed by manual comparative curation and determination of sequence similarity using the BLAST web server. Alignments were performed by using the BRIG tool [17]

2.6. Plasmid characterization and conjugation assay. Plasmid DNA of the clinical isolates *Cf*164 was extracted using the Kieser method [18]. The Kieser extract was used to transform *E. coli* TOP10 strain by electroporation. The electroporants were plated on a TSA plate containing 100 µg/ml ampicillin. Transformants were analyzed by PCR using the primers pre-LMB-1A and pre-LMB-1B. From the transformants harboring the $bla_{\text{LMB-1}}$ gene, plasmid DNA extraction was done using the Kieser's method and subsequently analyzed on 0.7% agarose gel stained with ethidium bromide. Plasmids of ca. 154, 66, 48, and 7 kb of *E. coli* NCTC 50192 were used as plasmid size markers [19].

To perform the quantitative filter mating-out assay, the clinical isolates *Cf*164 was used as donor and *E. coli* J53 as recipient. Both isolates were each grown overnight in brain heart infusion (BHI) (BioMérieux). A 0.25 ml of each donor and recipient donor culture were diluted in 4.75 ml BHI broth and incubated at 37°C for 5 h without shaking. Subsequently, 0.25 ml of the donor culture was gently mixed with 2.5 ml of the recipient culture, and 200 µl of this mating mix was spotted on a 0.45-µm filter (Millipore, Paris, France) placed on a prewarmed MH plates and incubated at 37°C for 2 h. The mating assays were terminated by placing the filters in 4 ml of an ice-cold 0.9% NaCl solution,

158 followed by vigorous agitation for 30 s. The number of transconjugants per donor cell was determined
159 by plating dilutions of the mating mixture onto plates containing antibiotics. Strain donor cells were
160 selected with 100µg /ml ampicillin. Transconjugants were selected with 100µg/ml ampicillin and
161 100µg/ml azide. Transfer frequencies were calculated by dividing the number of transconjugants by the
162 number of donor cells.

163 **2.7. β-Lactamase purification.** An overnight culture of *E. coli* strain BL21 (DE3) harboring
164 pET41b-CMY-150, pET41b-LMB-1 and pET28a-LMB-1 were used to inoculate 2 L of LB broth
165 containing 50 µg/ml kanamycin. The bacteria were cultured at 37ºC until reaching an OD of 0.6 at 600
166 nm. Expression of both enzymes was induced overnight at 25ºC with 0.2 mM IPTG, as previously
167 described [20]. For pET41b-LMB-1 additional induction conditions were tested (temperature: 16°, 25°,
168 37°; IPTG: 0.2mM, 0.5mM, 1.0mM; cells: *E. coli* BL21 (DE3), *E. coli* BL21 (DE3) pLysS,
169 *E. coli* Rosetta™ (DE3)). Cultures were centrifuged at 6000 g for 15 min and the pellets were
170 resuspended in 10 mL of Buffer A (20 mM phosphate buffer, 175 mM potassium sulfate (K_2SO_4),
171 40mM Imidazol, pH 7.40). The cells were disrupted by sonication and bacterial debris was removed by
172 two consecutive centrifugation steps: at 10.000 g for 1 h at 4ºC, and at 48.000 g for 1 h at 4ºC. CMY-
173 150 and LMB-1 were purified in one step pseudo-affinity chromatography using a NTA-Ni column (GE
174 Healthcare, Les Ulis, France) [20]. Protein purity was estimated by SDS-PAGE, pure fractions were
175 pooled and dialyzed against 20mM Hepes, K_2SO_4 50mM buffer (pH 7) and concentrated using
176 Vivaspin® columns (GE Healthcare). Protein concentration was determined by Bradford Protein assay
177 (Bio-Rad) [20].

178 **2.8. Steady-state kinetic parameters.** Kinetic parameters of purified CMY-150 were
179 determined at 30ºC in 100mM sodium phosphate buffer (pH 7). The k_{cat} and K_m values were determined
180 by analyzing hydrolysis of β-lactams under initial-rate conditions with an ULTROSPEC 2000 model
181 UV spectrophotometer (GE Healthcare) using the Eadie–Hoffstee linearization of the Michaelis–
182 Menten equation, as previously described [20]. The different β-lactams were purchased from Sigma–
183 Aldrich (Saint-Quentin-Fallavier, France).

The specific activities of the β-lactamase LMB-1 for β-lactams were determined on the supernatant of a whole cell crude extract obtained from an overnight culture of pTOPO-LMB-1 in *E. coli* TOP 10 as previously described [19].

2.9. Nucleotide sequence accession number. The whole genome sequence of *Cf*164 generated in the study have been submitted to the Genbank nucleotide sequence database, under the accession number of NZ_QCWX01000001. The nucleotide sequence of $bla_{\text{CMY-150}}$ genes along with plasmid pCf164_LMB-1 and pCf164_CTX-M-8 have been submitted to the EMBL/Genbank nucleotide sequence database under the accession numbers NG_060513, MH475146 and MN187903, respectively.

3. Results and discussion

3.1. Clinical case. A 68-year-old patient with Non-Hodgkin lymphoma, type l diabetes and history of previous myocardial infarction, was admitted in a tertiary care hospital in Buenos Aires. After 12 days of the hospitalization, he presented febrile neutropenia and a catheter-related bacteraemia due to a methicillin resistant *Staphylococcus haemolyticus*. At the same time, the urine culture revealed a bacterial count of 10^5 CFU/ml of a *C. freundii* isolate. The patient was treated with vancomycin and amikacin that led to the resolution of the urinary tract infection and the bacteraemia.

3.2. Characteristics of *C. freundii* 164. Routine antibiogram and MIC values of *Cf*164 revealed penicillins, aztreonam, expanded-spectrum cephalosporin resistance and reduced susceptibility to imipenem (Table 1). As the MIC for imipenem was 0.5 µg/ml and even though the MIC for meropenem was below 0.125 µg/ml, which is the EUCAST screening cut off for carbapenemases, the blue carba carbapenemase confirmatory test was performed. The latter gave a weak positive result suggesting the presence of a carbapenem-hydrolyzing β-lactamase. These results were confirmed using different biochemical confirmatory tests based on imipenem hydrolysis (β Carba, rCIM and MBT STAR-Carba™ tests) which were consistently positive except for the Carba NP that remained repeatedly negative. PCRs detecting the five most prevalent carbapenemases ($bla_{\text{OXA-48}}$, bla_{KPC}, bla_{NDM}, bla_{VIM}, bla_{IMP}) and the immunochromatographic NG Carba5 test were negative, suggesting the presence of a minor carbapenemase. WGS data of *Cf*164 was analyzed using CLC genomic workbench (Qiagen)

and revealed a genome of 5,229,602 bp, with an average 50 X coverage (NZ_QCWX01000001). Resistome was analyzed by searching acquired genes using Resfinder and point mutations involved in chromosomal resistance. Four β-lactamase genes were identified, a novel chromosomal and natural *bla*$_{\text{CMY-2-like}}$ gene, *bla*$_{\text{CTX-M-8}}$ and *bla*$_{\text{PER-2}}$ coding for two ESBLs, and *bla*$_{\text{TEM-1}}$, coding for a broad-spectrum penicillinase. The isolate harbored also two *qnr* resistance genes (*qnrE* and *qnrB38*), a rifampicin resistance gene (*aar-3*), a sulfonamide resistant gene (*sul1*) and a trimethoprim resistance gene (*dfrA27*). The finding of these genes matched well with the resistance phenotype of *Cf*164 to trimethoprim and to nalidixic acid. Mutations in the QRDR, known to confer fluoroquinolone resistance were not found explaining the susceptibility to fluoroquinolones.

3.3. Characterization of β-lactamase CMY-150. CMY-150 differs from CMY-2 by 15 amino-acid substitutions (L6I, V52I, Q55E, Q122R, R125S, R146T, A150E, H153R, A182V, P185H, N214S, D218N, H262L, A273E, V368A) and for CMY-65 by two amino-acid substitutions (P185H, H262L). CMY-150 and CMY-2 were cloned into the pCR®-Blunt II-TOPO® plasmid (pTOPO-CMY-150, and pTOPO-CMY-2) and transformed into *E. coli* TOP10. CMY-150 conferred a higher level of resistance to cefotaxime (>256 µg/ml vs 8 µg/ml for CMY-2), ceftazidime (>256 µg/ml vs 24 µg/ml for CMY-2) and aztreonam (32 µg/ml vs 3 µg/ml for CMY-2) (Table 1). MICs for carbapenems were heterogeneous, with imipenem being the highest (MIC 0.38 µg/ml), meropenem (MIC 0.047 µg/ml) and ertapenem (MIC 0.047 µg/ml) remaining in the susceptibility range. The MICs for carbapenems conferred by pTOPO-CMY-150, even though in the susceptibility range, were slightly higher than those conferred by CMY-2 (Table 1). In front of these results, MICs for imipenem for *Cf*164 was performed on cloxacillin-containing (250 µg/ml) MH plates and revealed the exact same value, suggesting that another enzyme capable of hydrolyzing carbapenems might be present in these bacteria.

To compare the kinetic properties of CMY-2 and the new CMY-150, the steady state parameters of the pure proteins were determined (Table2). Overall, the catalytic efficiencies were very similar between the two enzymes, except for ampicillin (40-fold higher for CMY-2). The kinetic parameters for aztreonam could not be obtained since no hydrolysis was observed. For this reason, we determined the IC_{50} values for CMY-2 and CMY-150, revealing similar results for both enzymes (0.2 µM for CMY-2 and 0.31 µM for CMY-150). These results cannot explain the difference observed in

the MIC values obtained and although further analysis are required to understand this phenotype, we suggest that the CMY-150 could present a higher affinity for aztreonam than CMY-2. Regarding the kinetic parameters for imipenem, in both enzymes the *K*m values were highly similar as well as the activity, further suggesting that another enzyme capable of hydrolyzing carbapenems might be present in *Cf*164.

3.4. Identification of *bla*$_{\text{LMB-1}}$ gene in the WGS of *Cf*164. Using an in-house developed software, ResMiner (BI Iorga, pers. comm.), that searches for flags specific of the four molecular classes of β-lactamases, found in addition to *bla*$_{\text{CMY-150}}$, *bla*$_{\text{CTX-M-8}}$, *bla*$_{\text{PER-2}}$ and *bla*$_{\text{TEM-1}}$ genes, an additional gene coding for a putative MBL of sub class B3. Blast searches revealed that this MBL had 100 % sequence identity with LMB-1, a MBL found only once if an *E. cloacae* isolate from Linz in Austria (Accession number KU646836) [8].

3.5. Susceptibility of LMB-1-producing isolates and kinetic properties of LMB-1. The expression of LMB-1 in *E. coli* Top10 resulted in increased MICs of penicillins, cephalosporins and carbapenems, but not of aztreonam (Table 1). A slight inhibition of β-lactamase inhibitors on the MICs could be detected. Of note, the MIC of cefepime was lower than that of ceftazidime and MICs of aztreonam were comparable to that of *E. coli* TOP 10, confirming the MBL behavior of LMB-1.

As purification of LMB-1 failed despite repeated attempts in different conditions, crude lysates of *E. coli* TOP10 pTOPO-LMB-1 were used to determine β-lactam specific activities. It revealed strong specific activity against carbapenems, oxyimino cephalosporins and aminopenicillins (Table 3). No specific activity against aztreonam nor cefepime, as previously reported (8), could be detected. When compared to NDM-1 expressed in the same conditions, LMB-1 had slightly higher (2-fold) activity against cefoxitin, whereas the activity against carbapenems was c.a. 8-fold lower (Table3). It is important to highlight that the MIC values for carbapenems in *Cf*164 were lower than those obtained with *E. coli* Top10 carrying the natural plasmid pCf164_LMB-1 and with *E. coli* Top10 carrying pTOPO-LMB-1. The latter results, together with the low specific activities against carbapenems as compared to NDM-1, may explain the negative results obtained with NP Carba test, a test based on imipenem hydrolysis.

Most of these results correlate well with the results of the comparative MIC studies confirming activity of LMB-1 against aminopenicillins, ceftazidime, cefotaxime and carbapenems. However, in the comparative MIC studies, expression of LMB-1 accounted for an 8-fold increase in the MIC of cefepime, which indicates that LMB-1 does indeed present activity against cefepime. The fact that no specific activity could be detected against cefepime within 10 min may indicate that the hydrolysis rates of LMB-1 for this substrate may be very low yet the hydrolysis rate appears to have been sufficiently high to account for a substantial increase in the MIC of cefepime, or the affinity of LMB-1 for cefepime may be high enough to trap the antibiotic, which is reflected in the raise of the MIC value.

3.6. Characterization of plasmids and genetic environments. PacBio assembly analysis revealed 12 contigs, two of them belonging to plasmidic DNA. The presence of two plasmids was confirmed by Kieser extraction (Figure 1). *bla*$_{\text{LMB-1}}$ gene was inserted onto a conjugative IncA/C type plasmid of 175,712bp, pCf164_LMB-1 (accession number MH475146.1) (Figure 1), that also carried *bla*$_{\text{PER-2}}$ gene. pCf164_LMB-1 was different from the IncFIB(K)-type pNRZ-10170-LMB-1 plasmid found in *E. cloacae* NRZ-10170 from Austria (accession number MH056209.1) [8] except for the immediate genetic environment of *bla*$_{\text{LMB-1}}$ gene.

The comparison of the immediate genetic environment of *bla*$_{\text{LMB-1}}$ in pNRZ-10170-LMB-1 and in pCf164_LMB-1 revealed an identical area between a *qacG* gene upstream of *bla*$_{\text{LMB-1}}$ gene and *sul1* gene downstream of *bla*$_{\text{LMB-1}}$ gene (Figure 2). This genetic organization is similar to a complex class 1 integron with the presence of IS*CR*1 element. Toleman *et al.* reported the mobilization of class 1 integrons by IS*CR1* through the transposition of IS*CR1* next to the 3' end of the class 1 integron (*qac/sul* genes) and the transposase misreading of the *ter*IS sequence [21]. The segment of DNA involving the 3' end of the class 1 integron and the 5' end of the IS*CR1* is deleted erasing the normal termination site, *ter*IS and thereby creating and integron-IS*CR1* fusion. From this point on, IS*CR1* is able to mobilize the integron [21]. It is likely that the similarities observed in the genetic environment of *bla*$_{\text{LMB-1}}$, in pNRZ-10170-LMB-1 and in pCf164_LMB-1, might be the result of *bla*$_{\text{LMB-1}}$ gene mobilization mediated by an IS*CR1*, among other events. LMB-1 shares 99% sequence identity with a predicted MBL (SEI10464) from the marine bacterium *R. pacifica*, as previously shown [8].

IncA/C plasmids were among the earliest antimicrobial resistance-encoding plasmids identified in Gram-negative bacteria and have received attention due to their broad host range and their association with the emergence of multidrug resistance in enteric pathogens of humans and animals [22]. IncA/C plasmids are of high-molecular-weight low-copy-number and are considered responsible for the dissemination of the clinically-relevant ß-lactamase genes e.g. *bla*$_{\text{CTX-M}}$, *bla*$_{\text{CMY}}$, *bla*$_{\text{NDM}}$, *bla*$_{\text{IMP}}$, *bla*$_{\text{VIM}}$, and *bla*$_{\text{KPC}}$ genes, among others [23,24]. In a nucleotide BLAST search for closely related plasmids to pCf164_LMB-1, two IncA/C plasmids were found, namely in, *Salmonella enterica* strain CFSAN064034 plasmid pGMI17-002_1 (CP028170) and in *Vibrio cholerae* genome assembly *V. cholerae* 116-17a plasmid pNDM-116-17 both isolated in India (LN831185). In addition, plasmid pCf587 harboring PER-2 (MG053108) recovered in Argentina from *C. freundii* 33587, also belongs to IncA/C type plasmids and is related to pCf164_LMB-1 [25]. PER-2 has been the second most prevalent ESBL, after the pandemic CTX-Ms, in Argentina, accounting for nearly 10% and 5% of the oxyimino-cephalosporin resistance in *K. pneumoniae* and *E. coli*, respectively [25]. Comparative sequence analysis revealed a conserved backbone length and similar overall genetic arrangements (Figure 3). pNRZ-10170-LMB-1 was also included in the analysis, showing similarities only in the immediate environment of *bla*$_{\text{LMB-1}}$ gene (Figure 3).

The second plasmid carried by *Cf*164, pCf164_CTX-M-8 (accession number MN187903), was fully sequence by PacBio as a single contig. This 68,453 bp plasmid belonged to the IncM incompatibility group and encoded plasmid replication, maintenance, and transfer genes and two resistance gene regions (Figure 4). The first one contained the *bla*$_{\text{TEM-1A}}$ penicillinase and the second one of 3,560 bp, contained the *bla*$_{\text{CTX-M-8}}$ gene bracketed by two copies of IS*26* likely forming a composite transposon [2]. Overall, the nucleotide sequence of pCf164_CTX-M-8 was extremely similar to that of pKp145-11b recovered from a *K. pneumoniae* clinical isolate from Brazil (GenBank accession number KX118608) [26]. The differences between these two plasmids are based on the resistance genes present next to the *bla*$_{\text{TEM-1A}}$ gene. Indeed, pKp145-11b harbored a Tn*1331*-like element, that carried in addition to *bla*$_{\text{TEM-1A}}$ gene, three other resistance genes *bla*$_{\text{OXA-9}}$ gene, *ant(3')-Ia* gene and *aac(6')-Ib* gene [26], as previously described [27]. Finally, as reported pKp145-11b had high nucleotide sequence

identity with plasmid pKp41M (accession number KY781949), another IncM plasmid carried by a *K. pneumoniae* isolate recovered from a parrot in Brazil [26]. To the best of our knowledge, this is the first report of $bla_{\text{CTX-M-8}}$ gene carried by an IncM type plasmid reported in Argentina, suggesting that this epidemic plasmid is not only circulating amongst human and animal in Brazil, but is now also spreading to the south of the continent.

4. Conclusion

In conclusion, this is the first report of LMB-1, a novel MBL of sub class B3, carried by a IncA/C type plasmid. Our data suggests that *R. pacifica* is likely the progenitor of LMB-1, and that two independent mobilization events have occurred on two different plasmids, in two different species, and on two distantly located continents. The dissemination of the conjugative pCf164_LMB-1 represents a public health threat as it combines a prevalent ESBL gene ($bla_{\text{PER-2}}$) with a difficult to detect carbapenemase gene and that both genes are harbored by a broad-spectrum and conjugative IncA/C plasmid that may spread to many human pathogens.

LMB-1 displays hydrolytic activities against most ß-lactams, especially carbapenems and cephalosporins, that are important antimicrobial agents for the treatment of severe nosocomial infections caused by Enterobacterales. Even tough in the susceptibility range, animal models of infections are required to evaluate whether carbapenems may be a suitable therapeutic option. The fact that molecular assays, nor ResFinder, nor immunochromatography tests failed to identify this novel MBLs, and that biochemical tests gave inconsistent results may lead to silent spread of this type of enzyme. Thus, epidemiological studies to evaluate the extend of the dispersion of LMB-1 in Europe and the Americas should be undertaken.

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Conflict of interest

LD is co-inventor of the Carba NP Test, which patent has been licensed to bioMérieux (La Balmes les Grottes, France).

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Table 1 MIC values of β-lactams for *C. freundii 164*, *E. coli* TOP10 pTOPO-CMY-150, *E. coli* TOP10 pTOPO-CMY-2, *E. coli* TOP10 pN-LMB-1, *E. coli* TOP10 pTOPO-LMB-1 and *E. coli* TOP10.

Antibiotic	MIC (µg/mL)					
	C. freundii 164	*E. coli* TOP10 (pTOPO-CMY-150)	*E. coli* TOP10 (pTOPO-CMY-2)	*E. coli* TOP10 (pCf164_LMB-1)	*E. coli* TOP10 (pTOPO-LMB-1)	*E.coli* TOP10
Amoxicillin	>256	>256	>256	>256	>256	2
Amoxicillin + CLA[a]	64	64	32	>256	24	2
Cefotaxime	>32	>32	8	>32	4	0.06
Ceftazidime	128	>256	24	>256	48	0.12
Cefepime	2	0.5	0.125	8	0.38	0.023
Imipenem	0.5	0.38	0.24	1.5	0.75	0.25
Meropenem	0.094	0.047	0.023	0.8	0.25	0.016
Ertapenem	0.064	0.047	0.012	0.5	0.19	0.003
Temocillin	64	24	16	>1024	512	4
Aztreonam	32	32	3	>256	0.064	0.047

[a]CLA, clavulanic acid (2 µg/ml)

Table 2 . Kinetic parameters of CMY-150 and CMY-2.

Substrate	*K*m(μM)		*k*cat(s^{-1})		*k*cat/*K*m (mM^{-1} s^{-1})	
	CMY-150	CMY-2	CMY-150	CMY-2	CMY-150	CMY-2
Ampicillin	55	16	0.30	3.2	5.4	204
Ceftazidime	13	15	0.04	0.05	3.1	3.3
Imipenem	3.7	2.5	0.02	0.02	5.8	9.6
Aztreonam	NH[a]	NH	NH	NH	-	-

[a] No hydrolysis

444 **Table 3**. Specific activities for β-lactamases NDM-1 and LMB-1.

Antibiotic	Specific Activity (mU/mg)	
	NDM-1	LMB-1
Cefalotine	0.41	0.44
Cefoxitine	0.44	0.81
Ceftazidime	0.38	0.24
Imipenem	7.42	0.88
Meropenem	5.95	0.88
Ertapenem	6.55	0.66

445 Experiments were done in triplicate. Error rates were within 10%

446

Figure legends

Figure 1. Plasmid extraction. Migration of the extracted pCf164_LMB-1 from the *Cf*164 (WT) and the transconjugant *E. coli* J53 (Tc). M, marker.

Figure 2. Genetic environment of $bla_{\text{LMB-1}}$. Comparison of the genetic environment of $bla_{\text{LMB-1}}$ gene presents in *Cf*164_LMB-1 with the environment of $bla_{\text{LMB-1}}$-like sequence in *Rheinheimera pacifica* and $bla_{\text{LMB-1}}$ gene in pNRZ-10170-LMB-1. Truncated ORFs are indicated by Δ.

Figure 3. Comparative genome analyses of several IncA/C group plasmids. Structural comparison of pCf164_LMB-1 with those of the most similar IncA/C group plasmids and pNRZ-10170-LMB-1.

Figure 4. Plasmid sequence schematic representation. Major structural features of the plasmids pKp145-11b (KX118608) and pCf164_CTX-M-8 (MN1879903). Common structures are highlighted in gray. Resistance genes and boxed in black. The $bla_{\text{CTX-M-8}}$ composite transposon is also indicated.

Figure1

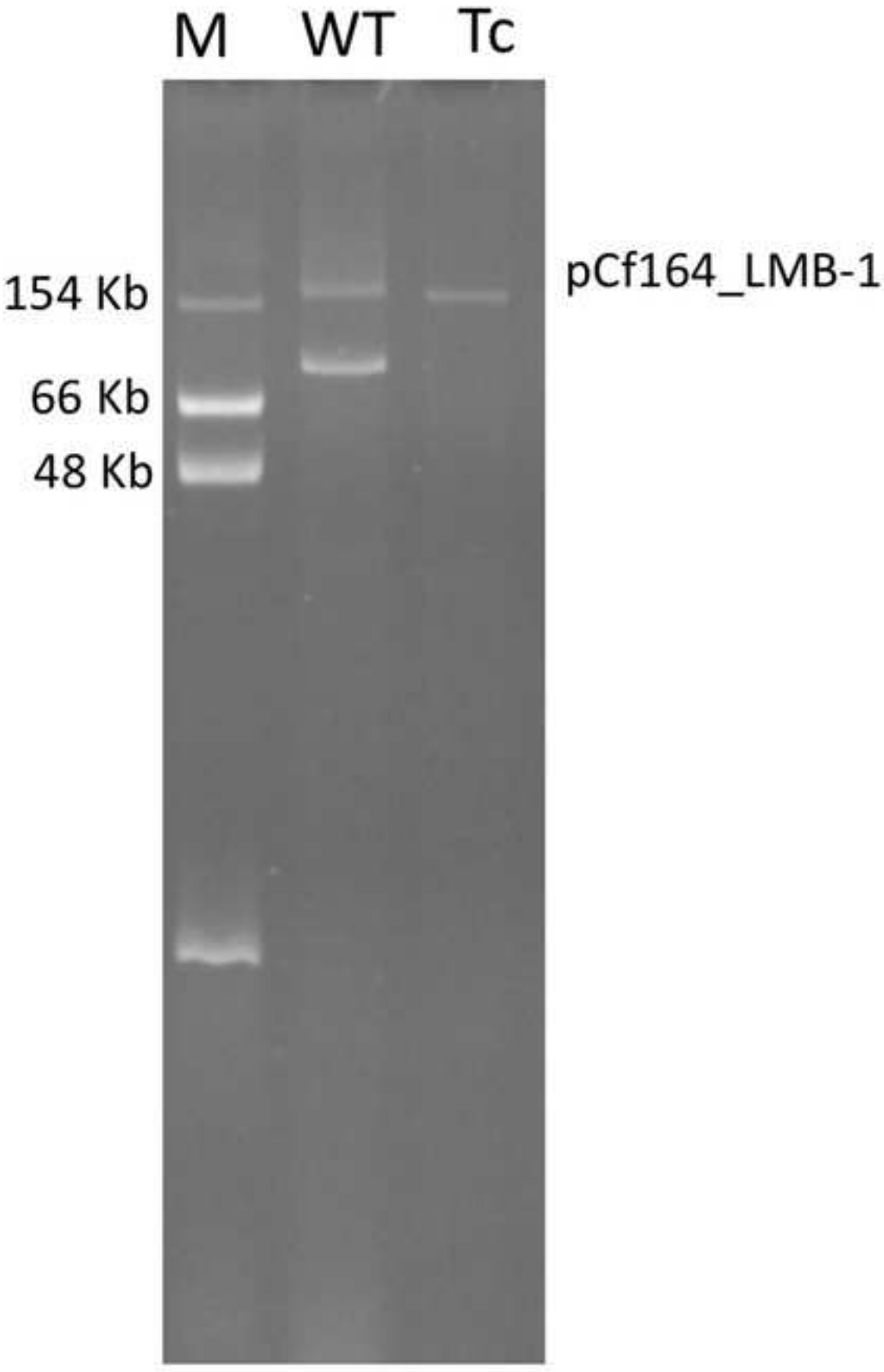

Figure2

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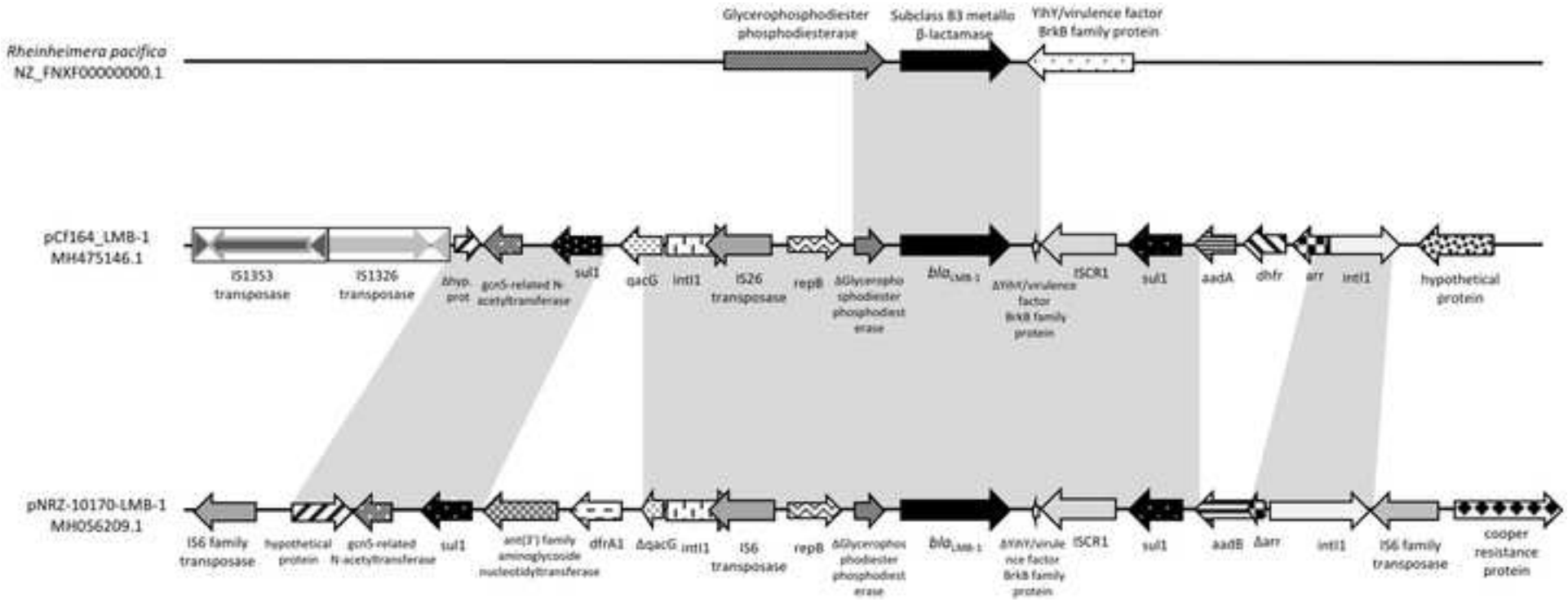

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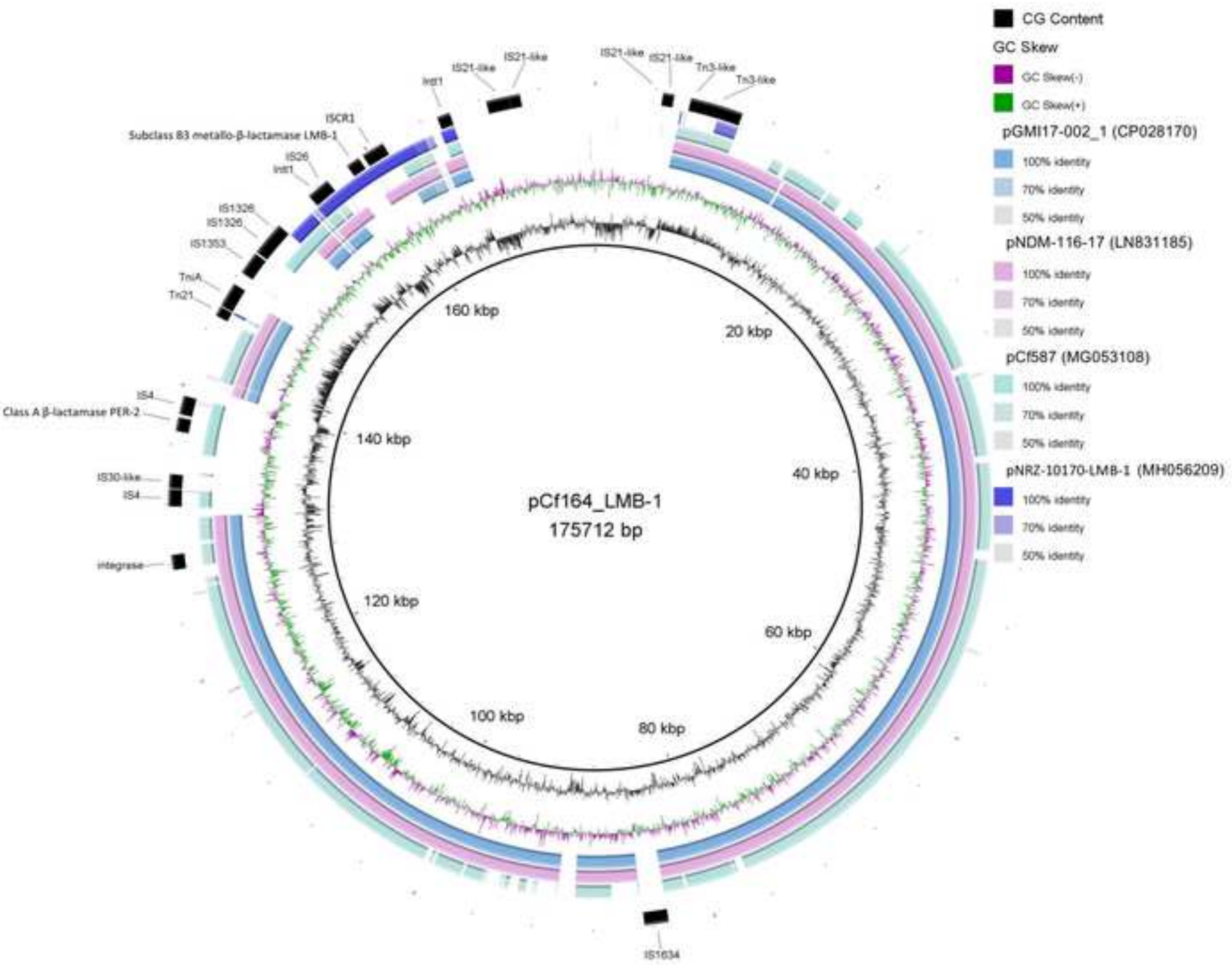

Figure4

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