

BKC-2, a New BKC Variant Detected in MCR-9.1-Producing *Enterobacter hormaechei* subsp. *Xiangfangensis*

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ABSTRACT

We characterized a multidrug-resistant (MDR) *Enterobacter* spp. isolate highlighting the genetic aspects of the antimicrobial resistance genes. An *Enterobacter* spp. isolate (Ec61) was recovered in 2014 from a transtracheal aspirate sample from a patient admitted to a Brazilian tertiary hospital and submitted to further microbiological and genomic characterization. Ec61 was identified as *Enterobacter hormaechei* subsp. *xiangfangensis* strain ST451, showing an MDR profile and the presence of genes codifying the new β -lactamase variants BKC-2 and ACT-84 and the mobile colistin resistance gene *mcr*-9.1.

KEYWORDS antimicrobial resistance, carbapenemase, phosphoethanolamine transferase, plasmids

In the past decade, the dramatic increase in the prevalence and clinical impact of infections caused by carbapenemase-producing bacteria became a global health concern. Carbapenemase production is especially problematic when encountered in *Enterobacterales* members because of their great ability to spread in healthcare settings, resulting in health and economic burdens (1). Brazilian *Klebsiella* carbapenemase (BKC) is a serine carbapenemase identified for the first time in 2015 from clinical isolates of *Klebsiella pneumoniae* collected in 2008 (2). BKC-1 is able to hydrolyze penicillins, cephalosporins, monobactams, and carbapenems but not cephamycins and has oxacillin as preferential hydrolytic substrate (2). *bla*BKC-1 is usually carried by a 10-kb *IncQ* plasmid and associated with *aph*(3)-VI and *ISKpn23*, an insertion sequence belonging to the IS1380 family; however, this resistance gene has been characterized in only a few strains (2–5).

A mobile colistin resistance gene, *mcr*, which mediates the addition of phosphoethanolamine (pEtN) on bacterial lipopolysaccharide, has been a cause of great concern worldwide (6). So far, 10 variants of *mcr* genes have been deposited in GenBank. The coproduction of carbapenem and colistin resistance drastically hinders therapeutic options in hospital settings because these mechanisms are frequently associated with non- β -lactam antimicrobial resistance (7).

Herein, we report a new allele of BKC, BKC-2, in MCR-9.1-producing *Enterobacter* spp., recovered in a Brazilian hospital in São José do Rio Preto, São Paulo, Brazil. The microbiological and molecular properties of BKC-2 have been assessed to evaluate the spectrum of resistance conferred by BKC-2 and the capability of *bla*BKC-2 to be mobilized and/or transferred.

During a surveillance study conducted from January 2013 to December 2017, a multidrug-resistant (MDR) isolate belonging to the *Enterobacter cloacae* complex (Ec61) was obtained from a transtracheal aspirate culture that was collected in 2014 from a patient admitted to the general intensive care unit. Due to the MDR phenotype, the isolate was screened for the presence of carbapenemases and other antimicrobial resistance genes (ARGs) by PCR (see Text S1 in the supplemental material). Antimicrobial susceptibility testing was performed by broth microdilution (BMD) and interpreted according to EUCAST v.10.0 clinical breakpoints (8). PCR assay of ARG showed the presence of blaBKC, blaSHV, aac(69)-Ib, and aph(39)-IV genes on the Ec61 isolate. Interestingly, to date, only a single isolate of non-Klebsiella pneumoniae carrying blaBKC-1 has been reported (5). Susceptibility profile results confirmed the MDR profile with resistance to most tested antimicrobials (Table 1).

Molecular techniques were used to better characterize Ec61. Initially, the plasmid was characterized by using S1-pulsed-field gel electrophoresis (S1-PFGE) followed by hybridization to blaBKC (9). Conjugation experiments were performed by using the azide-resistant *Escherichia coli* strain J53 as the recipient cell at room temperature, 37°C, and 42°C in biological triplicate. Transconjugants were selected on UTI ChromoSelect agar (Merck, NJ, USA) containing 200 mg/liter of ampicillin or 0.5 mg/liter of colistin to screen for blaBKC and mcr, respectively. The S1-PFGE pattern suggested the presence of three plasmids in Ec61, with blaBKC located on an ~10-kb plasmid (data not shown) according to the hybridization results. The same results were previously observed for BKC-1-producing *K. pneumoniae* and *Citrobacter freundii* (2, 3). Despite several attempts, conjugation experiments failed to transfer the plasmid carrying blaBKC.

Whole-genome sequencing (WGS) was performed by the Illumina MiSeq platform (MiSeq reagent V3 kit/2 x 300 cycles) and MinION (Oxford Nanopore, UK). Hybrid de novo assembly was performed using Unicycler (v0.4.0) (10), and the automatic annotation was performed using RAST version 2.0 (<https://rast.nmpdr.org/>). Average nucleotide identity (ANI) was performed to identify the Ec61 bacterial species, using an ANI value of .95% as a threshold for species and .98% for subspecies definition (Text S1) (11). ResFinder v.3.2, MLSTfinder v.2.0.4 based on an *E. cloacae* scheme, and PlasmidFinder v.2.1 were used for initial characterization of the ARG, MLST, and plasmid replicons present on the Ec61 isolate. ANI analysis confirmed Ec61 to be *Enterobacter hormaechei* subsp. *xiangfangensis*, with 99.28% nucleotide identity to the reference sequence NZ_CP017183.1 (see Table S1 in the supplemental material). General genome features revealed the presence of a 4.8-Mb chromosome with 55.3% GC content and three plasmids, i.e., pEc61A (149.8 kb), pEc61B (77.6 kb), and pEc61C (8.3 kb) (Fig. 1A). Ec61 belongs to ST451, a sequence type previously associated with blaKPC-3 in the United States (12).

ampR, ompF, and ompC, were investigated by BLASTN using *E. hormaechei* ATCC 49162 as a reference strain (accession no. MKEQ01000001 to MKEQ01000004), a well-known Ser83Phe substitution in GyrA sequence, causing an increase in the MICs for quinolones. No additional amino acid substitutions were detected in the quinolone resistance-determining region (QRDR) region of GyrB, ParC, and ParE. Analysis of the genes encoding the main outer membrane proteins OmpC and OmpF revealed the presence of mutations on the amino acid chain in the OmpC of the Ec61 isolate and the disruption of the ompF nucleotide sequence called ISEcl11.

The ARG analysis initially revealed the presence of blaACT-16*, blaBKC-1*, blaSHV-5, sul1, sul2, mcr-9.1, fosA2, aac(69)-Ib3, aadA1, aph(39)-VIa, aac(69)-Ib-cr, and tetD (see Fig. S1 in the supplemental material). However, due to the 97% similarity between genes blaACT-16* and blaBKC-1*, the

ResFinder database suggested the presence of new gene variants. The cephalosporinase ACT-16 was aligned with all ACT alleles available in the NCBI database, revealing the presence of a new variant of this chromosomal cephalosporinase named ACT-84 (GenBank accession no. MT136764). ACT-84 differs from ACT-83 by a single amino acid substitution (Pro58Ser). Alignment of the BKC-1 nucleotide sequence (GenBank accession no. NG_048710.1) with that of blaBKC detected on Ec61 showed 20 mismatches leading to 11 amino acid modifications (Fig. S2). Thus, we named the new BKC variant as BKC-2. Like BKC-1, the coding sequence of BKC-2 was located in a small plasmid of the IncQ1 group (pEc61C); however, the plasmid was 1.3 kb smaller than p60136 (GenBank accession no. NG_048710.1). Nucleotide comparison of plasmid-encoding blaBKC-1 with blaBKC-2 revealed that ISKpn23 was absent upstream to blaBKC-2 (Fig. 1B). It has been speculated that the association of ISKpn23 with blaBKC-1 would be responsible for the mobilization and expression of blaBKC-1 (2, 4). Although absent, a nucleotide region of almost 250 bp of ISKpn23 was detected upstream to blaBKC-2, suggesting the exit of ISKpn23 from pEc61C. Nucleotide alignment showed that these 250 nucleotides matched the beginning and the end of the ISKpn23 encoding sequence, where the insertion sequence's promoter is located (see Fig. S3 in the supplemental material) (13).

To verify differences in the susceptibility profile conferred by BKC-1 and BKC-2, plasmids p60136 (carrying blaBKC-1) and pEc61C (carrying blaBKC-2) were transformed into *E. coli* Top10 by chemical transformation and selected on LB agar plates containing 100 mg/liter of ampicillin, respectively. Additionally, blaBKC-2 was amplified using Phusion high-fidelity DNA polymerase (Thermo Fisher Scientific) and cloned into pET-26b1 as previously described (2). Transformant BKC-1 and BKC-2 clones were subjected to antimicrobial susceptibility testing by BMD against β -lactam agents. In general, MICs of the BKC-1 transformant were higher than those presented by the BKC-2 transformant. MICs of ertapenem, meropenem, and imipenem were 4, 2, and 2 times higher, respectively, for the BKC-1 transformant than the BKC-2 transformant. Taking into account the BKC clones, MICs of BKC-1 for β -lactams were higher than those of BKC-2, suggesting better activity of BKC-1 against β -lactams. However, although BKC-2 was weaker than BKC-1, in its natural genetic background, BKC-2 showed activities against β -lactam agents similar to those of BKC-1, as suggested by transformant MICs (TfBKC-1 and TfBKC-2) (Table 1). These MIC variations could be explained by differences in the promoter regions between these two carbapenemase-encoding genes. Interestingly, a newly published article suggested that the origin of carbapenemases BKC-1 and GPC-1 related to the *Shinella* genus (14). Besides, a putative BKC-1 ancestral protein was synthesized in laboratory to antimicrobial susceptibility analysis and named BKC-b (14). BKC-b resulted from a deletion in the duplication segment of amino acids (Ala12 to Ser27) and shares 94% amino acid identity with BKC-2. Our data have been supported by the finding that BKC-b (as well as BKC-2) has weaker hydrolytic activity against the β -lactam agents than BKC-1 (14). Although BKC-2 shows duplication of the peptide Leu24-Arg39, the duplication segment Ala12-Ser27 (present in BKC-1 and responsible for the increase in β -lactamase activity) is absent, explaining the low activity of this new BKC variant.

Ec61 harbored colistin resistance gene mcr-9.1, in addition to novel gene blaBKC-2; however, the isolate showed susceptibility to colistin similar to the previously described mcr-9-positive strains (15). mcr-9.1 was carried on a different plasmid, pEc61A (149.8 kb), belonging to IncFIB without other antimicrobial resistance genes; although genes encoding metal resistance, such as arsenic, tellurium, mercuric, and nickel/cobalt resistance, were detected in the plasmid. Since the initial description of mcr-9, its dissemination has been strongly related to IncHI2 plasmids, as supported by our *in silico* analysis of the GenBank database (16). Only a single plasmid, non-IncHI2, was detected carrying mcr-9.1 (Fig. 2). The mcr-9.1 genetic backbone related to pEc61A contained

IS903B::mcr9.1::wubC::qseC-like::qseB-like::ATPase::integrase::IS26 as previously described in IncHI plasmids (15). Although the mechanism responsible for mcr-9.1 mobilization has not been elucidated, different insertion sequences (IS903-like, IS903B, IS26, and IS1) have been associated with this gene (15, 17).

IncFIB plasmids are well recognized as conjugative plasmids (18); however, the conjugation assay for the transfer of IncFIB with mcr-9.1 was not successful. The transfer of IncFIB was only found at 42°C, with a low frequency of 10⁻⁸, by using a low colistin concentration as the selective agent. Because the MIC for colistin of isolate Ec61 was 1 mg/liter, subinhibitory concentrations (0.25 and 0.5 mg/liter) were used in all attempts and did not provide an ideal selection using the *E. coli* J53 AziR strain. The findings suggested the limited dissemination of mcr-9.1 by IncFIB. In contrast, IncHI2 plasmids carrying mcr-9.1 have been widely demonstrated disseminating among Enterobacterales and carrying different antimicrobial resistance genes along with mcr-9.1 (15–17).

We need to pay attention to BKC-2 due to its acquisition on distinct bacterial genera, *Enterobacter* spp., which naturally tend to be microorganisms with high levels of resistance to antimicrobials. Also, the coproduction of a new cephalosporinase, ACT-84, and MCR-9.1 coupled with chromosomal mechanisms revealed a large arsenal of antimicrobial resistance mechanisms in/on Ec61 isolate. Although BKC-2 confers lower β -lactam resistance than BKC-1, its association with chromosomal or other plasmid-acquired mechanisms can lead to carbapenem resistance.

Accession number(s). The nucleotide sequences have been submitted to the GenBank database under accession no. MT427610 for blaBKC-2, MT136764 for blaACT-84, and CP053103 to CP053106 for the Ec61 genome and its respective plasmids

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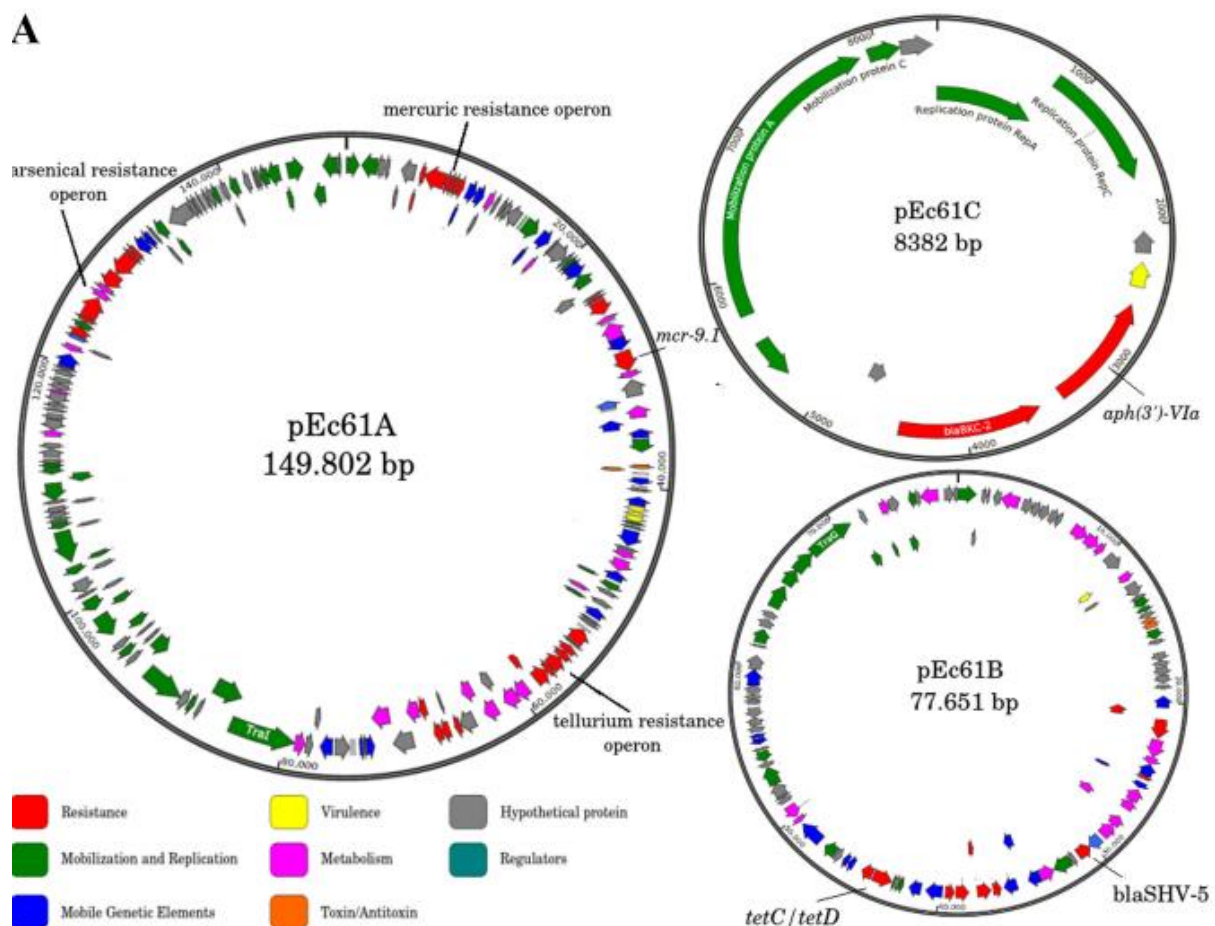
TABLE 1 Susceptibility profile of *Enterobacter hormaechei* subsp. *xiangfangensis* isolates carrying *bla*_{BKC-2} (Ec61), transformants, and clones carrying *bla*_{BKC-1} and *bla*_{BKC-2}

Antimicrobial agent	MIC (mg/liter)						
	Ec61 (wild type)	TfBKC-1 (<i>E. coli</i> Top10 + p60136 [<i>bla</i> _{BKC-1}])	TfBKC-2 (<i>E. coli</i> Top10 + pEc61C [<i>bla</i> _{BKC-2}])	Top 10 (<i>E. coli</i> top 10)	CI_BKC-1 (<i>E. coli</i> BL21 + pET-26:: <i>bla</i> _{BKC-1}) ^a	CI_BKC-2 (<i>E. coli</i> BL21 + pET-26:: <i>bla</i> _{BKC-2}) ^a	BL21 + pET26 (<i>E. coli</i> BL21 + pET-26)
Ertapenem	16	0.5	0.125	≤0.03	0.12	0.015	0.015
Meropenem	2	0.25	0.125	≤0.03	0.12	0.06	0.06
Imipenem	4	0.5	0.125	≤0.03	0.5	0.5	0.25
Cefepime	256	16	8	≤0.03	4	0.03	0.03
Ceftazidime	>256	128	64	0.25	8	0.125	0.06
Aztreonam	>256	256	64	0.06	8	0.03	≤0.015
Amoxicillin	>512	>512	256	2	>256	64	2
Ampicillin	>512	>512	>512	1	>256	32	1
Cefotaxime	256	256	64	0.06	16	0.12	≤0.015
Piperacillin-tazobactam	>256/4	NT ^b	NT	NT	NT	NT	NT
Ceftazidime-avibactam	2/4	NT	NT	NT	NT	NT	NT
Amikacin	256	NT	NT	NT	NT	NT	NT
Gentamicin	64	NT	NT	NT	NT	NT	NT
Tobramycin	64	NT	NT	NT	NT	NT	NT
Kanamycin	>256	NT	NT	NT	NT	NT	NT
Levofloxacin	2	NT	NT	NT	NT	NT	NT
Ciprofloxacin	2	NT	NT	NT	NT	NT	NT
Colistin	1	NT	NT	NT	NT	NT	NT

^aBMD was performed in the presence of isopropyl-β-D-thiogalactopyranoside 0.2 mM.

^bNT, not tested.

A



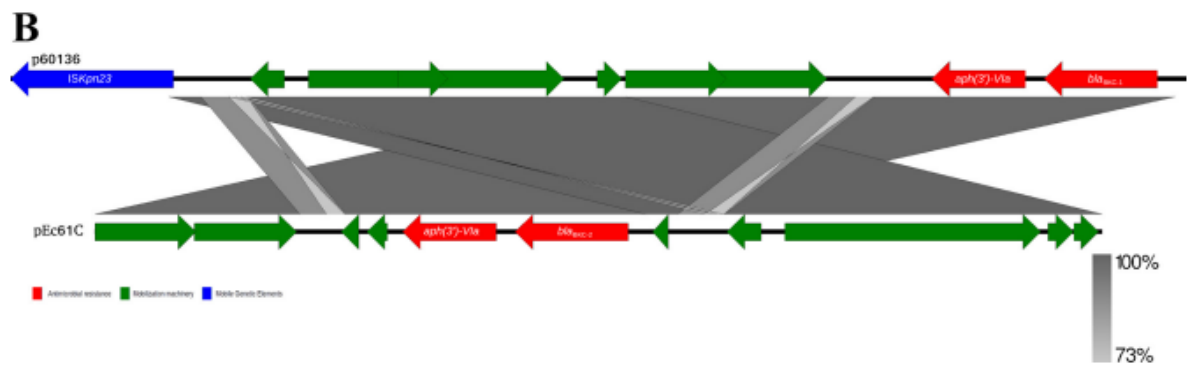


FIG 1 (A) Graphic map of the three plasmids detected in *E. hormaechei* subsp. *xiangfangensis* carrying *bla*_{BKC-2} and *mcr-9.1*. (B) Colinear analysis of the two different plasmids harboring *bla*_{BKC-2}. Arrows, transcription direction of genes and ORFs. Genes were grouped according to their predictive functions, indicated by color.

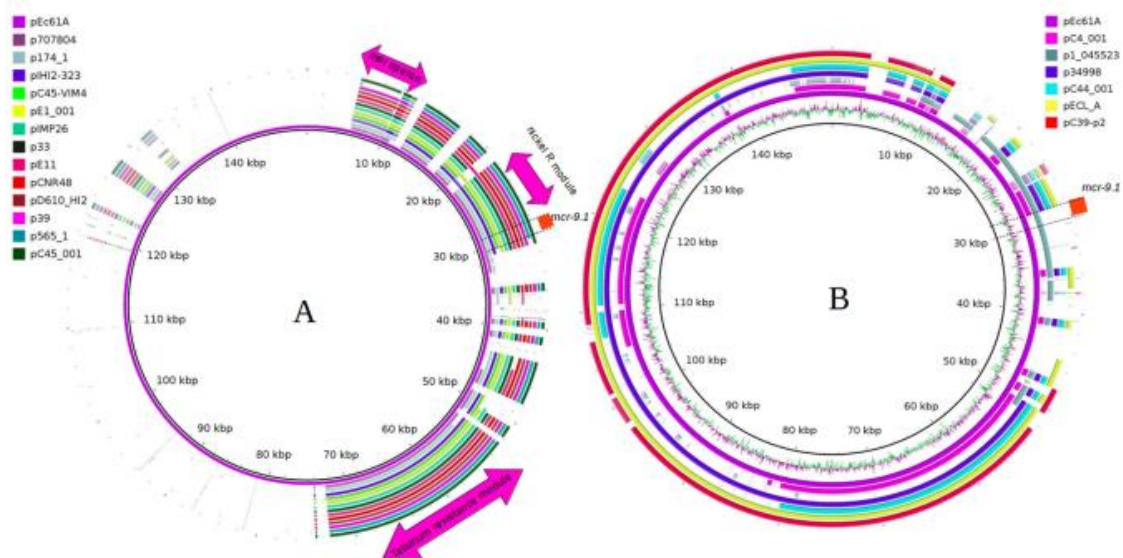


FIG 2 (A) Circular comparison between pEc61A and 13 plasmids carrying *mcr-9.1* belonging to IncH2 and IncH2a. Pink arrows, conserved regions, including *mer* operon, nickel resistance module, and tellurium resistance operon. (B) Circular comparison of the six best hit plasmids related to pEc61A on BLASTN analysis of NCBI database. Five of six analyzed plasmids belonged to the IncFIB group, and only p1_045523 also harbored *mcr-9*. GenBank accession numbers for each plasmid are included in Table S2 in the supplemental material.