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MECHANISMS OF RESISTANCE

Characterization of KPC-82, a KPC-2 Variant Conferring Resistance to Ceftazidime-Avibactam in a Carbapenem-Nonsusceptible Clinical Isolate of *Citrobacter koseri*

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ABSTRACT KPC-82 is a KPC-2 variant identified in a carbapenem-nonsusceptible *Citrobacter koseri* that confers high-level resistance to ceftazidime-avibactam. Genomic analysis revealed that bla_{KPC-82} is carried by a chromosomally integrated Tn*4401* transposon (disrupting porin gene *phoE*) and evolved by a 6-nucleotide tandem repeat duplication causing a two-amino-acid insertion (Ser-Asp) within the Ala_{267}-Ser_{275} loop. Similar to related KPC variants, KPC-82 showed decreased carbapenemase activity when expressed in a heterologous background and remained susceptible to carbapenem/β-lactamase inhibitor combinations.

KEYWORDS CRE, *Citrobacter koseri*, KPC, carbapenems, ceftazidime-avibactam

Carbapenem-resistant *Enterobacteriaceae* (CRE) are a significant threat to modern medicine. In particular, isolates producing carbapenem-hydrolyzing β-lactamase enzymes (carbapenemases) are increasingly prevalent and a cause for further concern given their ability to spread, the severity of infections, and the lack of effective therapeutics (1). Though colistin and tigecycline have been used as first-line treatment, newer antimicrobials with better safety profiles and potent activity against CRE are increasingly being employed as preferable therapeutic options (2).

Among them, ceftazidime-avibactam (CZA) is a β-lactam/β-lactamase inhibitor combination recently introduced into clinical practice (2). It has proven active against serine β-lactamases, including *Klebsiella pneumoniae* carbapenemases (KPC), which otherwise confer resistance to most β-lactams and β-lactam/β-lactamase inhibitor combinations (1). Despite limited clinical use worldwide, acquired resistance has been reported in multiple independent occurrences and by several mechanisms in both patients with or without a history of CZA therapy (3–10). Most frequently, resistance is caused by KPC variants exhibiting amino acid substitutions, insertions, or deletions in one of 4 loops (loop Leu_{102} to Ser_{106}, Ω-loop Arg_{164} to Asp_{179}, or loops Cys_{238} to Thr_{243} and Ala_{267} to Ser_{275}) (11). At the time of writing (April 2021), 82 bla_{KPC} alleles have been deposited in GenBank, including 20 conferring CZA resistance. In this report, we use genomic and molecular genetic approaches to characterize KPC-82, a KPC-2 variant that confers CZA resistance.

Citrobacter koseri MRSN 755319 was cultured from the blood of a patient in a U.S. hospital in 2020. The patient had been hospitalized for several months after suffering a gunshot wound to the abdomen. During this time, the patient had frequent infections caused by multidrug-resistant (MDR) bacteria, including a recurrent respiratory infection due to a carbapenem-susceptible *Klebsiella aerogenes* (days 159, 197, and 231) as

Citation Lebreton F, Corey BW, McElheny CL, Iovleva A, Preston L, Margulieux KR, Cybulski RJ, Mc Gann P, Doi Y, Bennett JW. 2021. Characterization of KPC-82, a KPC-2 variant conferring resistance to ceftazidime-avibactam in a carbapenem-nonsusceptible clinical isolate of *Citrobacter koseri*. Antimicrob Agents Chemother 65:e00150-21. https://doi.org/10.1128/AAC.00150-21.

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Received 27 January 2021
Returned for modification 15 March 2021
Accepted 2 May 2021
Accepted manuscript posted online 10 May 2021
Published 17 June 2021

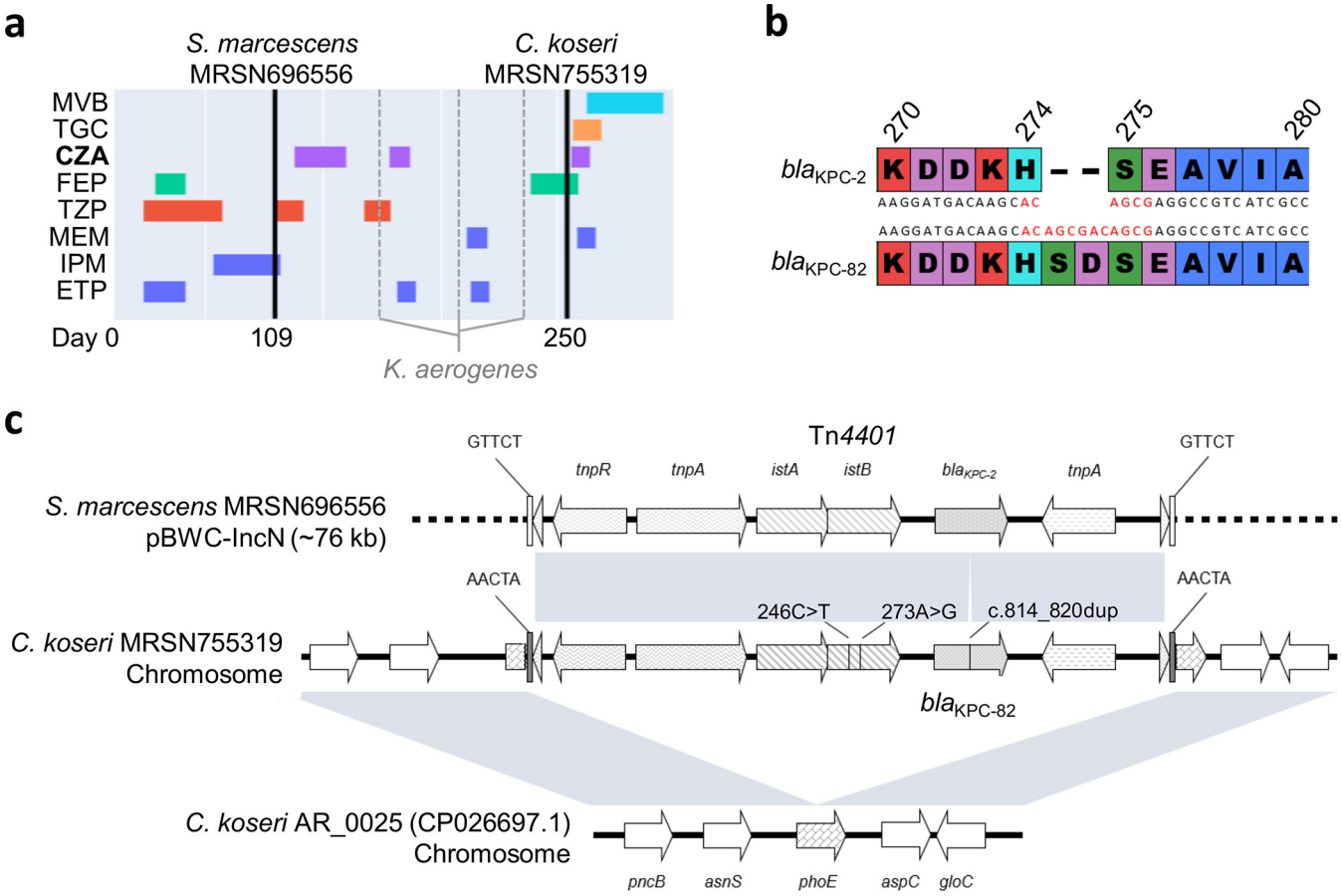

FIG 1 Identification and characterization of KPC-82. (a) Patient treatment course and sample collection. (b) Nucleotide and amino acid alignment (Ambler numbering and Clustal color scheme) of KPC-2 and KPC-82. (c) Alignment of the plasmid-borne KPC-2 carrying Tn*4401* (MRSN 696556) with the chromosomally integrated KPC-82 carrying Tn*4401* (MRSN 755319) and its corresponding insertion site in reference genome *C. koseri* AR_0025.

well as a bloodstream infection caused by a carbapenem-resistant (CR), bla_{KPC-2}-carrying *Serratia marcescens* (MRSN 696556, day 109), that ultimately resolved after ~4 weeks of treatment with CZA (Fig. 1A). Two and a half months after CZA was discontinued, the patient developed another infection, and blood cultures yielded *C. koseri* (MRSN 755319, day 250). The isolate was carbapenem resistant (Table 1), and the bla_{KPC} gene was detected using the Cepheid Xpert Carba-R assay. On day 252, the patient was prescribed tigecycline and CZA, which was substituted on day 260 with meropenem-vaborbactam (MVB) following extended antibiotic susceptibility testing (AST) that indicated the isolate was nonsusceptible to CZA (MIC, 128 μg/ml) but susceptible to MVB (MIC, 0.125 μg/ml).

As part of routine surveillance of MDR organisms, isolates *S. marcescens* 696556 and *C. koseri* 755319 were forwarded to the Multidrug-Resistant Organism Repository and Surveillance Network (MRSN). Whole-genome sequencing was performed on an Illumina MiSeq sequencer (Illumina, Inc., San Diego, CA), and genomes were processed as previously described (12). For *S. marcescens* 696556, long-read sequencing was performed using a MinION sequencer (Oxford Nanopore Technologies). Base-calling was performed using Guppy (configuration r9.4.1_450bps_hac) and filtered using Filtlong (https://github.com/rrwick/Filtlong), and hybrid assembly was performed using Unicycler (13).

Genome analysis revealed that CR and CZA-susceptible (Table 1) *S. marcescens* MRSN 696556 carried the bla_{KPC-2} allele. In contrast, CR and CZA-nonsusceptible *C. koseri* MRSN 755319 carried a mutated bla_{KPC-2} allele (hereby named bla_{KPC-82}; GenBank accession no. MW485086) and no other acquired β-lactamase. The mutated allele was identical to bla_{KPC-2} with the exception of a 6-nucleotide (ACAGCG) tandem

TABLE 1 MICs of β-lactams for isolates *S. marcescens* MRSN 696556, *C. koseri* MRSN 755319, and recombinant strains *E. coli* TOP10 with or without KPC-82 or KPC-2

	MIC (μg/ml) of:				
β-lactam(s)[a]	*S. marcescens*[b] MRSN 696556 (KPC-2)	*C. koseri*[b] MRSN 755319 (KPC-82)	*E. coli*[c] TOP10 (KPC-82)	*E. coli*[c] TOP10 (KPC-2)	*E. coli*[c] TOP10 (pBCSK)
Ampicillin	>16	>16	>16	>16	≤8
Ampicillin-sublactam	>16	>16	>16	>16	≤4
Piperacillin-tazobactam	>64	>128	32	>128	≤8
Ceftriaxone	>64	>32	32	>32	≤0.5
Cefepime	>16	>32	16	32	≤4
Ceftazidime	16	>16	>16	>16	4
Ceftazidime-avibactam	8	128	64	0.5	0.25
Ceftolozane-tazobactam	ND[d]	>16	>16	>16	≤1
Aztreonam	ND	>16	>16	>16	≤1
Ertapenem	>8	4	0.5	4	≤0.25
Imipenem	>4	4	1	>8	≤0.5
Meropenem	>4	2	≤0.5	>8	≤0.5
Meropenem-vaborbactam	0.125	0.125	ND	ND	ND

[a]Tazobactam and avibactam were added at a fixed concentration of 4 μg/ml.
[b]Performed in duplicate using a Vitek 2 in the MRSN College of American Pathologists (CAP)-accredited laboratory.
[c]Performed in two biological duplicates (distinct transformants confirmed by Sanger sequencing) using a Gram-negative GN4F AST plate (Thermo Fisher).
[d]ND, not determined.

repeat (TR) insertion causing a two-amino-acid insertion (Ser-Asp) between positions 274 and 275 (Ambler numbering) in the KPC protein (Fig. 1B). TR insertions within the KPC Ala_{267} to Ser_{275} loop have been reported previously (11), including KPC-50, a KPC-3 variant with a three-amino-acid insertion (Glu-Ala-Val) at this exact position (3).

To investigate whether the two-amino-acid insertion identified within KPC-82 was responsible for the phenotypic resistance to CZA, the bla_{KPC-82} gene was cloned into vector pBCSK (Stratagene, La Jolla, CA) and expressed in *E. coli* TOP10. AST showed that KPC-82 conferred resistance to all β-lactams, including ceftazidime, as well as high-level resistance to CZA (Table 1). Importantly, and similar to KPC-50 (3), *E. coli* expressing bla_{KPC-82} remained susceptible to the carbapenems (ertapenem, imipenem, and meropenem).

Further investigations into the genetic context of bla_{KPC-82} in MRSN 755319 revealed that it was carried by an ~10-kb Tn*4401*-like transposon that inserted into the chromosome and disrupted the gene coding for the outer membrane protein PhoE (Fig. 1C and D). Porin loss, such as OprD in *P. aeruginosa* (14) and OmpK36 in *K. pneumoniae* (15), has been widely implicated in β-lactam and carbapenem resistance in other bacterial species. Notably, PhoE downregulation has been hypothesized as a possible reason for carbapenem resistance in *K. pneumoniae* (16), suggesting that its inactivation in MRSN 755319 could cause the otherwise unexplained low-level carbapenem resistance (Table 1).

Interestingly, in *S. marcescens* MRSN 696556 from the same patient, the bla_{KPC-2} allele was also carried by a nearly identical Tn*4401* (only 2 synonymous mutations in *istB* in addition to the TR insertion in bla_{KPC}). However, unlike MRSN 755319 but similar to previous reports (17), this transposon was not chromosomally located and was instead carried by an ~76-kb IncN plasmid named pBWC01 (Fig. 1C and D). The backbone of pBWC01 was absent in MRSN 755319, but both *S. marcescens* and *C. koseri* isolates carried an identical ~4-kb Col440i-type plasmid (Fig. 1C). Similar Col440i cryptic plasmids have been identified in a variety of *Enterobacteriaceae* and have been documented to coconjugate with a larger IncN KPC-carrying plasmid (including across genus, *in vitro*) (18). Altogether, and despite missing intermediate isolates, a hypothesis for the emergence of bla_{KPC-82} would be that (i) both plasmids cotransferred from *Serratia* to *Citrobacter* within the host, and (ii) Tn*4401* inserted into the chromosome of *Citrobacter* while the remaining of pBWC01 was lost. In this proposed chain of events, whether bla_{KPC-82} evolved from bla_{KPC-2} in *Serratia*, as a result of CZA exposure, or once acquired by *Citrobacter* MRSN 755319 still remains unresolved.

In summary, a novel KPC-type enzyme conferring resistance to CZA was identified from a multidrug-resistant *C. koseri*. Similar to other KPC mutants conferring resistance to CZA, KPC-82 showed decreased carbapenemase activity and remained susceptible to carbapenem/β-lactamase inhibitor combinations, including meropenem-vaborbactam, which successfully cleared the infection in this patient.

Data availability. Genomes of *S. marcescens* MRSN 696556 and *C. koseri* MRSN 755319 have been deposited at NCBI (BioProject accession no. PRJNA692233).

ACKNOWLEDGMENTS

This study was funded by the U.S. Army Medical Command and the Defense Medical Research and Development Program. The manuscript has been reviewed by the Walter Reed Army Institute of Research. There is no objection to its presentation. The opinions or assertions contained herein are the private views of the authors and are not to be construed as official or reflecting the views of the Department of the Army or the Department of Defense.

A.I. was supported through Physician Scientist Incubator Program at the University of Pittsburgh sponsored by the Burrows Wellcome Fund. Y.D. was supported by research grants from the National Institutes of Health (R01AI104895, R21AI151362, and R21AI035522).

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