



High-Level Resistance to Colistin Mediated by Various Mutations in the *crrB* Gene among Carbapenemase-Producing *Klebsiella pneumoniae*

Aurélie Jayol,^{a,b,c,d,e} Patrice Nordmann,^{a,b,c,f} Adrian Brink,^g Maria-Virginia Villegas,^h Véronique Dubois,^{d,e} Laurent Poirel^{a,b,c}

Emerging Antibiotic Resistance Unit, Medical and Molecular Microbiology, Department of Medicine, University of Fribourg, Fribourg, Switzerland^a; INSERM European Unit (LEA Paris, IAME, France), University of Fribourg, Fribourg, Switzerland^b; National Reference Center for Emerging Antibiotic Resistance, University of Fribourg, Fribourg, Switzerland^c; Laboratory of Bacteriology, Bordeaux University Hospital, Bordeaux, France^d; CNRS UMR5234, University of Bordeaux, Bordeaux, France^e; University of Lausanne and University Hospital Center, Lausanne, Switzerland^f; Department of Clinical Microbiology, Ampath National Laboratory Services, Milpark Hospital, Johannesburg, South Africa^g; International Center for Medical Research and Training, CIDEIM, Cali, Colombia^h

ABSTRACT Mutations in *crrAB* genes encoding a two-component regulator involved in modifications of lipopolysaccharide were searched for among a collection of colistin-resistant *Klebsiella pneumoniae* isolates. Four isolates, respectively, producing carbapenemases NDM-1, OXA-181, or KPC-2 showed mutated CrrB proteins compared with those in wild-type strains. Complementation assays with a wild-type CrrB protein restored the susceptibility to colistin in all cases, confirming the involvement of the identified substitutions in the resistance phenotype.

KEYWORDS polymyxin, rapid polymyxin NP test, susceptibility testing, resistance mechanisms, CrrAB, *Klebsiella pneumoniae*

The emergence and spread of carbapenemase-producing *Klebsiella pneumoniae* isolates worldwide has forced clinicians to reintroduce colistin as last-resort therapy (1). Besides the plasmid-mediated *mcr-1* and *mcr-2* genes in *K. pneumoniae* (2, 3), the chromosomally encoded alterations of the *mgrB* gene and the PmrAB and PhoPQ two-component systems are currently the most commonly reported mechanisms of acquisition of polymyxin resistance in this enterobacterial species (4). Recently, mutations in the *crrB* gene, belonging to a third two-component system (named CrrAB for colistin resistance regulation) and involved in lipopolysaccharide (LPS) modifications, were associated with colistin resistance (5, 6). Mutations in the *crrB* gene are responsible for the increase in *crrC* gene transcription, which in turn regulates the expression of the *pmrC* gene and the *pmrHFIJKLM* operon, through the PmrAB two-component system (6). Expression of these genes leads to the addition of cationic groups on the LPS and consequently to colistin resistance.

In previous studies, the colistin resistance mechanisms of a collection of 185 *K. pneumoniae* isolates recovered from human samples worldwide (Europe, Turkey, Colombia, South Africa) were analyzed. Chromosomally encoded modifications of LPS through alterations (mutation, truncation) of the *mgrB* gene (7–9), the *pmrAB* genes (10), or the *phoPQ* genes (11) were identified in 152 isolates. However, 33 isolates did not present any substitutions in these genes, and they were negative for the plasmid-mediated resistance genes *mcr-1* and *mcr-2*.

We analyzed the genes encoding the CrrAB system in these 33 isolates. Amplification of the *crrA* and *crrB* genes was performed using primers *crrAB-extF* (5'-GTGAGGCCATCAAAT

Received 12 July 2017 Returned for modification 31 July 2017 Accepted 30 August 2017

Accepted manuscript posted online 5 September 2017

Citation Jayol A, Nordmann P, Brink A, Villegas M-V, Dubois V, Poirel L. 2017. High-level resistance to colistin mediated by various mutations in the *crrB* gene among carbapenemase-producing *Klebsiella pneumoniae*. *Antimicrob Agents Chemother* 61:e01423-17. <https://doi.org/10.1128/AAC.01423-17>.

Copyright © 2017 American Society for Microbiology. All Rights Reserved.

Address correspondence to Laurent Poirel, laurent.poirel@unifr.ch.

TCTCG-3') and *crrAB*-extR (5'-AAGTCCCAAAGAGGCAAAC-3') located on each extremity of this operon. No amplification was obtained with the primers for 19 of the 33 isolates. We obtained the same result by using internal primers annealing into the *crrB* gene, namely, *crrB*-intF (5'-GTGACTATCTTACGTGGGAG-3') and *crrB*-intR (5'-CACTCAGCATCAAGGAGTAC-3'). This absence of amplification suggested an absence of the *crrAB* operon in these strains, which is in accordance with the variability of the lateral acquisition of the *crrAB* operon in *K. pneumoniae* (5). Amplification of the *crrAB* gene was obtained for the 14 remaining isolates. Subsequent sequencing identified mutations leading to amino acid changes (F84S, N141Y, P151L, and G183V) in the CrrB protein in 4 of the 14 colistin-resistant *K. pneumoniae* isolates (Table 1).

Three of the CrrB amino acid substitutions were located in the histidine kinase A (HisKA) phosphoacceptor domain (amino acids 136 to 200) (Table 1). Previously, four mutations in this HisKA domain were found to be involved in colistin resistance (6) (Table 1). Among them, two were at the same position as the mutations observed in our strains (amino acids 141 and 151), but the amino acid changes were different. The fourth strain presented a mutation in the HAMP domain of CrrB, whereas a single mutation in this domain has been shown (5). All of the substitutions in the CrrB protein, known to be responsible for colistin resistance, are shown in Table 1.

Determination of the colistin MICs by use of the reference broth microdilution method showed a high level of colistin resistance (MIC of colistin, >128 $\mu\text{g/ml}$ for all four isolates with mutated *crrB* genes) (Table 1). The high MICs of colistin in the *K. pneumoniae* strains are in accordance with results reported by Cheng et al. (6) (Table 1). Furthermore, Wright et al. (5) reported lower MICs of colistin (16 $\mu\text{g/ml}$) for two isolates exhibiting CrrB mutations. However, MICs were determined by use of the Etest strip technique, which is known to underestimate colistin MIC values (12).

We performed complementation assays to confirm the involvement of the mutated *crrB* gene in the colistin resistance phenotype. A recombinant plasmid (pTRIC) was built by cloning a triclosan resistance gene (*mFabI*) (13) into the low-copy-number plasmid pBR322. The wild-type *crrB* gene from the colistin-susceptible *K. pneumoniae* strain Af44a (MIC of colistin, 0.25 $\mu\text{g/ml}$) was amplified by PCR and cloned into this plasmid. The recombinant *crrB*-pTRIC plasmid and the pTRIC plasmid were separately transformed by electroporation into the four resistant strains presenting the mutations in the *crrB* gene. Transformants were selected by overnight incubation at 37°C on Mueller-Hinton agar supplemented with triclosan (1 μM), and the recombinant clones were checked by PCR and sequencing. MICs of colistin for the transformants revealed that production of a wild-type CrrB protein (*crrB*-pTRIC plasmid) restored the susceptibility to colistin in all isolates (MIC, ≤ 0.5 $\mu\text{g/ml}$) (Table 1), confirming that the different substitutions in CrrB were, respectively, responsible for the resistant phenotypes. As expected, transformation with the pTRIC plasmid used as a negative control did not restore any susceptibility to colistin.

All isolates presenting a CrrB amino acid change produced a carbapenemase (NDM-1, OXA-181, or KPC-2) (Table 1). They were recovered from patients who had been treated with colistin in Colombia, France, Greece, and South Africa. For the patient harboring the colistin-resistant isolate Af44b, one colistin-susceptible isolate (Af44a) was recovered before the colistin treatment, and pulsed-field gel electrophoresis analysis confirmed the clonal relationship of the two isolates (data not shown). Sequencing of the *crrB* gene of the Af44a isogenic susceptible strain identified a wild-type CrrB protein, reinforcing the hypothesis that the *in vivo* emergence of colistin resistance under colistin pressure might be related to CrrB mutations in some instances.

In conclusion, four novel mutations in the CrrB protein were identified at the sources of acquisition of high-level colistin resistance among carbapenemase-producing *K. pneumoniae*.

TABLE 1 Features of colistin-resistant *K. pneumoniae* clinical isolates

Isolate ^a	Origin of isolate	MIC of colistin ^b (μg/ml)	Colistin susceptibility ^c	CrrB amino acid change in:												MIC of colistin after complementation ^f (μg/ml)	Carbapenemase	Reference/source
				10 ^e	Q	Y	F	L	W	140 ^e	141 ^e	N	P	G	S			
Af44a ^g	South Africa	0.25	S														OXA-181	This study
UHKPC28	United States	16	R	L													KPC-2	5
Col21	Taiwan	512	R	L													No	6
Col28	Taiwan	>2,048	R	L													No	6
Col44	Taiwan	512	R	H													No	6
20.70	France	>128	R	S													NDM-1	This study
UHKPC26	United States	16	R	M													KPC-2	5
Col36	Taiwan	2,048	R							R							No	6
G104	Greece	>128	R							Y							KPC-2	This study
Col4	Taiwan	>2,048	R							I							No	6
Col20	Taiwan	2,048	R							I							No	6
Af44b	South Africa	>128	R							L							OXA-181	This study
Col7	Taiwan	1,024	R							S							No	6
C7	Colombia	>128	R							V							KPC-2	This study
Col22	Taiwan	2,048	R											N			No	6

^aIsolates from this study are indicated by shading.

^bMICs of colistin were determined using the manual broth microdilution reference method for this study, Etest for the study by Wright et al. (5), and agar dilution for the study by Cheng et al. (6).

^cS, susceptible (MIC, ≤2 μg/ml); R, resistant (MIC, >2 μg/ml), according to EUCAST breakpoints (<http://www.eucast.org/>).

^dDomains of the CrrB protein predicted by SMART software are indicated as follows: TM, transmembrane domain (amino acids 12–34); HAMP, histidine kinase, adenylyl cyclase, methyl binding protein, and phosphatase domain (amino acids 81–135); HisKA, histidine kinase A (phosphoacceptor) domain (amino acids 136–200).

^eAmino acid positions where mutations have been detected.

^fMICs of colistin after complementation with a wild-type CrrB protein (with plasmid crrB-pTRIC).

^gThe colistin-susceptible isolate Af44a is the isogenic colistin-susceptible counterpart of Af44b.

ACKNOWLEDGMENTS

This work was funded by the University of Bordeaux and the University of Fribourg and by grants from the ANIWHWA ERA-NET project, Switzerland, and the Office Fédéral de la Santé Publique, Bern, Switzerland (grant no. 16009294).

REFERENCES

1. Falagas ME, Kasiakou SK. 2005. Colistin: the revival of polymyxins for the management of multidrug-resistant gram-negative bacterial infections. *Clin Infect Dis* 40:1333–1341. <https://doi.org/10.1086/429323>.
2. Liu YY, Wang Y, Walsh TR, Yi LX, Zhang R, Spencer J, Doi Y, Tian G, Dong B, Huang X, Yu LF, Gu D, Ren H, Chen X, Lv L, He D, Zhou H, Liang Z, Liu JH, Shen J. 2016. Emergence of plasmid-mediated colistin resistance mechanism MCR-1 in animals and human beings in China: a microbiological and molecular biological study. *Lancet Infect Dis* 16:161–168.
3. Xavier BB, Lammens C, Ruhel R, Kumar-Singh S, Butaye P, Goossens H, Malhotra-Kumar S. 2016. Identification of a novel plasmid-mediated colistin-resistance gene, *mcr-2*, in *Escherichia coli*, Belgium, June 2016. *Euro Surveill* 21:pil=30280. <http://www.eurosurveillance.org/images/dynamic/EE/V21N27/art22525.pdf>.
4. Poirel L, Jayol A, Nordmann P. 2017. Polymyxins: antibacterial activity, susceptibility testing, and resistance mechanisms encoded by plasmids or chromosomes. *Clin Microbiol Rev* 30:557–596. <https://doi.org/10.1128/CMR.00064-16>.
5. Wright MS, Suzuki Y, Jones MB, Marshall SH, Rudin SD, van Duin D, Kaye K, Jacobs MR, Bonomo RA, Adams MD. 2015. Genomic and transcriptomic analyses of colistin-resistant clinical isolates of *Klebsiella pneumoniae* reveal multiple pathways of resistance. *Antimicrob Agents Chemother* 59:536–543. <https://doi.org/10.1128/AAC.04037-14>.
6. Cheng YH, Lin TL, Lin YT, Wang JT. 2016. Amino acid substitutions of CrrB responsible for resistance to colistin through CrrC in *Klebsiella pneumoniae*. *Antimicrob Agents Chemother* 60:3709–3716. <https://doi.org/10.1128/AAC.00009-16>.
7. Poirel L, Jayol A, Bontron S, Villegas MV, Ozdamar M, Turkoglu S, Nordmann P. 2015. The *mgrB* gene as a key target for acquired resistance to colistin in *Klebsiella pneumoniae*. *J Antimicrob Chemother* 70:75–80.
8. Jayol A, Nordmann P, Desroches M, Decousser JW, Poirel L. 2016. Acquisition of broad-spectrum cephalosporin resistance leading to colistin resistance in *Klebsiella pneumoniae*. *Antimicrob Agents Chemother* 60:3199–3201. <https://doi.org/10.1128/AAC.00237-16>.
9. Nordmann P, Jayol A, Poirel L. 2016. Rapid detection of polymyxin resistance in *Enterobacteriaceae*. *Emerg Infect Dis* 22:1038–1043. <https://doi.org/10.3201/eid2206.151840>.
10. Jayol A, Poirel L, Brink A, Villegas MV, Yilmaz M, Nordmann P. 2014. Resistance to colistin associated with a single amino acid change in protein PmrB among *Klebsiella pneumoniae* isolates of worldwide origin. *Antimicrob Agents Chemother* 58:4762–4766. <https://doi.org/10.1128/AAC.00084-14>.
11. Jayol A, Nordmann P, Brink A, Poirel L. 2015. Heteroresistance to colistin in *Klebsiella pneumoniae* associated with alterations in the PhoPQ regulatory system. *Antimicrob Agents Chemother* 59:2780–2784. <https://doi.org/10.1128/AAC.05055-14>.
12. Humphries RM. 2015. Susceptibility testing of the polymyxins: where are we now? *Pharmacotherapy* 35:22–27.
13. Jang C-W, Magnuson T. 2013. A novel selection marker for efficient DNA cloning and recombineering in *E. coli*. *PLoS One* 8:e57075. <https://doi.org/10.1371/journal.pone.0057075>.