AAC Journals.ASM.org

Polymyxin Resistance Caused by *mgrB* Inactivation Is Not Associated with Significant Biological Cost in *Klebsiella pneumoniae*

Antonio Cannatelli,[a] Alfonso Santos-Lopez,[b] Tommaso Giani,[a] Bruno Gonzalez-Zorn,[b] Gian Maria Rossolini[a,c,d]

Department of Medical Biotechnologies, University of Siena, Siena, Italy[a]; Department of Animal Health and VISAVET, Complutense University of Madrid, Madrid, Spain[b]; Department of Experimental and Clinical Medicine, University of Florence, Florence, Italy[c]; Clinical Microbiology and Virology Unit, Florence Careggi University Hospital, Florence, Italy[d]

The inactivation of the *mgrB* gene, which encodes a negative-feedback regulator of the PhoPQ signaling system, was recently shown to be a common mutational mechanism responsible for acquired polymyxin resistance among carbapenemase-producing *Klebsiella pneumoniae* strains from clinical sources. In this work, we show that *mgrB* mutants can easily be selected *in vitro* from different *K. pneumoniae* lineages, and *mgrB* inactivation is not associated with a significant biological cost.

Global-scale dissemination of carbapenem-resistant *Enterobacteriaceae* (CRE) and carbapenem-resistant *Acinetobacter baumannii* strains has occurred in recent years, resulting in the renewed popularity of polymyxins, which are among the few drugs that retain activity against these resistant Gram-negative organisms (1–3). As a consequence, polymyxin resistance is increasingly being reported among these resistant pathogens (4–8). In *A. baumannii*, however, polymyxin resistance has been shown to be associated with a relevant biological cost, which would reduce the risk of dissemination of resistant strains and explain why polymyxin resistance has remained relatively uncommon among clinical isolates of this species (9, 10).

We recently reported that the inactivation of the *mgrB* gene can be a mutational mechanism responsible for acquired polymyxin resistance in carbapenem-resistant (CR) *Klebsiella pneumoniae* organisms producing the *K. pneumoniae* carbapenemase (KPC) (11). MgrB is a small membrane protein that acts as a negative-feedback regulator of the PhoPQ two-component signaling system (12). The inactivation of the *mgrB* gene is associated with an upregulation of that system, which in turn activates the polymyxin resistance (Pmr) system responsible for the biochemical modification of lipid A, which reduces its affinity for polymyxins (11, 13). Further epidemiological investigations revealed that *mgrB* inactivation is a common mechanism of resistance among polymyxin-resistant clinical isolates of CR *K. pneumoniae* (14–16). Inactivation can be mediated by mutations in *mgrB* or through the integration of an insertion sequence (IS), with the IS integration being more common (14–16). The clonal expansion of *mgrB*-inactivated mutant strains has also been observed in some health care settings (14). A similar epidemiological scenario suggests that *mgrB* inactivation does not impose a relevant fitness cost on the bacterial host, unlike what is observed with *pmr* or *lpx* mutations responsible for polymyxin resistance in *A. baumannii* (9).

In this work, we investigated the stability and the fitness cost of

Received 9 December 2014 **Returned for modification** 7 January 2015
Accepted 8 February 2015

Accepted manuscript posted online 17 February 2015

Citation Cannatelli A, Santos-Lopez A, Giani T, Gonzalez-Zorn B, Rossolini GM. 2015. Polymyxin resistance caused by *mgrB* inactivation is not associated with significant biological cost in *Klebsiella pneumoniae*. Antimicrob Agents Chemother 59:2898–2900. doi:10.1128/AAC.04998-14.

Address correspondence to Gian Maria Rossolini, gianmaria.rossolini@unifi.it.

doi:10.1128/AAC.04998-14

TABLE 1 Characteristics of the *K. pneumoniae* strains investigated in this work

Strain	Reference	Clonal lineage	Colistin MIC (mg/liter)	Carbapenemase gene	Phenotype[a]	Mutation frequency[b]	No. (%) of IS in *mgrB*[c]	IS type, position (orientation) (*n*)[d]
KKBO-1	11	ST258 (clade 2)	0.12	bla_{KPC-3}	MDR	7.0×10^{-7}	12 (60)	IS*5*-like, nt 75 (R) (12)
KPB-1	19	ST512	0.06	bla_{KPC-3}	MDR	3.0×10^{-7}	12 (60)	IS*3*-like, nt 75 (R) (11); IS*kpn26*-like, nt 75 (R) (1)
KPGP1	20	ST16	0.12	bla_{OXA-48}	MDR	4.7×10^{-8}	9 (45)	IS*kpn14*-like, nt 124 (F) (6); IS*Ecp1*-like, nt 132 (F) (3)
KPFan	This work	ST674	0.06	None	Susceptible	6.2×10^{-8}	13 (65)	IS*102*-like, nt 69 (F) (13)

[a] MDR, multidrug resistant, i.e., nonsusceptible to carbapenems (imipenem, meropenem, and ertapenem), β-lactamase-inhibitor combinations (amoxicillin-clavulanate and piperacillin-tazobactam), expanded-spectrum cephalosporins (cefotaxime, ceftazidime, and cefepime), trimethoprim-sulfamethoxazole, and fluoroquinolones.
[b] Frequency of COL^r colonies obtained plating 1×10^9 CFU from an early stationary phase.
[c] Number and proportion (%) of 20 analyzed COL^r mutants with an IS in the *mgrB* gene. The remaining mutants in all cases carried a wild-type *mgrB* gene.
[d] Nature of the IS found in the *mgrB* gene in COL^r mutants. The nucleotide (nt) numbers indicate the positions of the ISs; the numbering is based on the coding sequence of the *mgrB* open reading frame (ORF) (GenBank accession no. AVFC01000053, region 155512 to 155655), with number 1 as the first base of the GTG start codon. F indicates that the transposase gene is in the same orientation as the *mgrB* gene, while R indicates that the transposase gene is in the opposite orientation.

TABLE 2 Biological cost of *mgrB* insertional inactivation in *K. pneumoniae* strains belonging to different STs

COL^r strain	Clonal lineage	Chromosomal *mgrB* interruption, position (orientation)[a]	Colistin MIC (mg/liter)	Selection coefficient ± SE[b]
KKBO-1	ST258 (clade 2)	IS*5*-like, nt 75 (R)	8	1.001 ± 0.004
KPB-1	ST512	IS*3*-like, nt 75 (R)	16	1.006 ± 0.0018
KPGP1	ST16	IS*kpn14*-like, nt 124 (F)	4	0.999 ± 0.0017
KPFan	ST674	IS*102*-like, nt 69 (F)	4	1.005 ± 0.003

[a] The nucleotide (nt) numbers indicate the positions of the mutations or ISs; the numbering is based on the coding sequence of the *mgrB* ORF (GenBank accession no. AVFC01000053, region 155512 to 155655), with number 1 as the first base of the GTG start codon. F indicates that the transposase gene is in the same orientation as the *mgrB* gene, while R indicates that the transposase gene is in opposite orientation.

[b] The value of the selection coefficient of the mutant is relative to the progenitor COL^s strain.

mgrB inactivation in polymyxin-resistant mutants of different lineages of *K. pneumoniae*.

Four different *K. pneumoniae* strains of a clinical origin were investigated in this study. The strains belonged to different sequence types (STs) and included representatives of the ST258 clonal lineage, which is responsible for the recent global dissemination of KPC-producing *K. pneumoniae* strains, and also included an OXA-48-producing ST16 strain (Table 1). The MICs of colistin and other antimicrobial agents (including carbapenems, cephalosporins, trimethoprim-sulfamethoxazole, and fluoroquinolones) were determined by reference broth microdilution (BMD) (17) and interpreted according to the EUCAST guidelines (http://www.eucast.org). *Escherichia coli* strain ATCC 25922 and *K. pneumoniae* strain KKBO-4 (11) were used as the control strains for antimicrobial susceptibility testing. Colistin-resistant (COL^r) colonies were selected from each colistin-susceptible (COL^s) strain by plating approximately 1×10^9 CFU from an early stationary-phase culture onto Mueller-Hinton agar (MHA) (Oxoid Ltd., United Kingdom) containing 16 mg/liter colistin sulfate (AppliChem GmbH, Germany). The stability of the COL^r phenotype was tested by growing selected mutants in antibiotic-free MHA broth aerobically at 37°C for approximately 50 generations (by diluting the culture 1:200 in fresh medium every day for 6 days); at the end of growth, each culture was plated onto MHA to obtain isolated colonies, and 50 randomly selected colonies were checked by replica plating onto MHA supplemented with colistin (12 μg/ml, a concentration that allowed the growth of the COL^r mutants used in these experiments but not of the COL^s progenitors). With each mutant, the stability experiments were carried out in triplicate. The biological cost assay was performed by coculturing each COL^s strain together with the corresponding COL^r mutant under the same conditions described for the stability assays. At the end of growth, the ratio of COL^r to COL^s cells was measured as described for the stability assays. For each pair of strains, four independent competition experiments were carried out. The selection coefficient (s) was calculated as the slope of the linear regression model: $s = \ln(\mathrm{CI})/\log_2(d)$, where CI is the competition index, calculated as the ratio between the CFU counts of the resistant and susceptible populations each day divided by the same ratio at time zero (t_0), and (d) is the dilution factor (18).

The COL^r colonies were obtained from each COL^s strain at various frequencies between 7×10^{-7} and 4.7×10^{-8} (Table 1). The characterization of the *mgrB* mutations, which was carried out by PCR and sequencing, as described previously (14), with a random sample of 20 COL^r colonies obtained from each strain revealed an insertional inactivation by an IS in 45 to 65% of the mutants, with some diversity in the IS types and sites of insertion. All the remaining analyzed COL^r mutants exhibited a wild-type *mgrB* gene (Table 1).

Of the insertional *mgrB* mutants obtained from each strain, one representative of the most frequent type of insertion event was selected to test the stability and biological cost of the resistance mechanism. The ISs carried by these four mutants were different (IS*5*-like, IS*3*-like, IS*kpn14*-like, and IS*102*-like) and inserted at various positions within the *mgrB* gene (Table 2). The colistin MICs varied from 4 to 16 mg/liter. Despite the MIC values determined by the BMD, all mutants were able to grow onto MHA containing colistin at a concentration of 12 mg/liter. The stability assays revealed that after ca. 50 generations in the absence of antibiotics, the resistance phenotype was stable with each selected resistant mutant. PCR and a sequence analysis of the *mgrB* locus carried out with end-stage cultures confirmed the presence of the original *mgrB* alteration in all cases (data not shown).

The competition assays, which were carried out with each pair of COL^s and COL^r isogenic strains, did not detect any significant biological cost for the COL^r mutants (Table 2).

Together, the present findings indicated that (i) COL^r mutants created by insertional inactivation of the *mgrB* gene can be selected from different strains upon exposure to colistin, (ii) the resistance mechanism based on *mgrB* insertional inactivation was stable even in the absence of antibiotic selection, and (iii) the *in vitro* competition experiments between the COL^s and COL^r strains did not show any significant biological cost associated with the insertional inactivation of the *mgrB* gene.

Although this behavior was observed *in vitro*, these findings are consistent with the epidemiological observations that indicate a major role for *mgrB* inactivation in the evolution of polymyxin resistance in the clinical setting. Further studies will be necessary to investigate the potential effect of *mgrB* inactivation on virulence and the ability to establish persistent colonization in humans.

ACKNOWLEDGMENT

This work was supported by a grant from the FP7 project EvoTAR (HEALTH-F3-2011-2011-282004) to B.G.-Z. and G.M.R.

REFERENCES

1. **Rossolini GM, Arena F, Pecile P, Pollini S.** 2014. Update on the antibiotic resistance crisis. Curr Opin Pharmacol **18C:**56–60. http://dx.doi.org/10.1016/j.coph.2014.09.006.
2. **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. http://dx.doi.org/10.1086/429323.
3. **Petrosillo N, Giannella M, Lewis R, Viale P.** 2013. Treatment of carbapenem-resistant *Klebsiella pneumoniae*: the state of the art. Expert Rev Anti Infect Ther **11:**159–177. http://dx.doi.org/10.1586/eri.12.162.
4. **Cai Y, Chai D, Wang R, Liang B, Bai N.** 2012. Colistin resistance of

Acinetobacter baumannii: clinical reports, mechanisms and antimicrobial strategies. J Antimicrob Chemother **67:**1607–1615. http://dx.doi.org/10.1093/jac/dks084.

5. **Kim Y, Bae IK, Lee H, Jeong SH, Yong D, Lee K.** 2014. *In vivo* emergence of colistin resistance in *Acinetobacter baumannii* clinical isolates of sequence type 357 during colistin treatment. Diagn Microbiol Infect Dis **79:**362–366. http://dx.doi.org/10.1016/j.diagmicrobio.2014.03.027.
6. **Ah YM, Kim AJ, Lee JY.** 2014. Colistin resistance in *Klebsiella pneumoniae*. Int J Antimicrob Agents **44:**8–15. http://dx.doi.org/10.1016/j.ijantimicag.2014.02.016.
7. **Pena I, Picazo JJ, Rodríguez-Avial C, Rodríguez-Avial I.** 2014. Carbapenemase-producing *Enterobacteriaceae* in a tertiary hospital in Madrid, Spain: high percentage of colistin resistance among VIM-1-producing *Klebsiella pneumoniae* ST11 isolates. Int J Antimicrob Agents **43:**460–464. http://dx.doi.org/10.1016/j.ijantimicag.2014.01.021.
8. **Monaco M, Giani T, Raffone M, Arena F, Garcia-Fernandez A, Pollini S, Network EuSCAPE-Italy, Grundmann H, Pantosti A, Rossolini GM.** 2014. Colistin resistance superimposed to endemic carbapenem-resistant *Klebsiella pneumoniae*: a rapidly evolving problem in Italy, November 2013 to April 2014. Euro Surveill **19:**pii=20939. http://www.eurosurveillance.org/ViewArticle.aspx?ArticleId=20939.
9. **Beceiro A, Moreno A, Fernández N, Vallejo JA, Aranda J, Adler B, Harper M, Boyce JD, Bou G.** 2014. Biological cost of different mechanisms of colistin resistance and their impact on virulence in *Acinetobacter baumannii*. Antimicrob Agents Chemother **58:**518–526. http://dx.doi.org/10.1128/AAC.01597-13.
10. **López-Rojas R, McConnell MJ, Jiménez-Mejías ME, Domínguez-Herrera J, Fernández-Cuenca F, Pachón J.** 2013. Colistin resistance in a clinical *Acinetobacter baumannii* strain appearing after colistin treatment: effect on virulence and bacterial fitness. Antimicrob Agents Chemother **57:**4587–4589. http://dx.doi.org/10.1128/AAC.00543-13.
11. **Cannatelli A, D'Andrea MM, Giani T, Di Pilato V, Arena F, Ambretti S, Gaibani P, Rossolini GM.** 2013. *In vivo* emergence of colistin resistance in *Klebsiella pneumoniae* producing KPC-type carbapenemases mediated by insertional inactivation of the PhoQ/PhoP *mgrB* regulator. Antimicrob Agents Chemother **57:**5521–5526. http://dx.doi.org/10.1128/AAC.01480-13.
12. **Lippa AM, Goulian M.** 2009. Feedback inhibition in the PhoQ/PhoP signaling system by a membrane peptide. PLoS Genet **5:**e1000788. http://dx.doi.org/10.1371/journal.pgen.1000788.
13. **Cheng HY, Chen YF, Peng H-L.** 2010. Molecular characterization of the PhoPQ-PmrD-PmrAB mediated pathway regulating polymyxin B resistance in *Klebsiella pneumoniae* CG43. J Biomed Sci **17:**60–76. http://dx.doi.org/10.1186/1423-0127-17-60.
14. **Cannatelli A, Giani T, D'Andrea MM, Di Pilato V, Arena F, Conte V, Tryfinopoulou K, Vatopoulos A, Rossolini GM, COLGRIT Study Group.** 2014. MgrB inactivation is a common mechanism of colistin resistance in KPC-producing *Klebsiella pneumoniae* of clinical origin. Antimicrob Agents Chemother **58:**5696–5703. http://dx.doi.org/10.1128/AAC.03110-14.
15. **Poirel L, Jayol A, Bontron S, Villegas MV, Ozdamar M, Türkoglu S, Nordmann P.** 2014. The *mgrB* gene as a key target for acquired resistance to colistin in *Klebsiella pneumoniae*. J Antimicrob Chemother **70:**75–80. http://dx.doi.org/10.1093/jac/dku323.
16. **Olaitan AO, Diene SM, Kempf M, Berrazeg M, Bakour S, Gupta SK, Thongmalayvong B, Akkhavong K, Somphavong S, Paboriboune P, Chaisiri K, Komalamisra C, Adelowo OO, Fagade OE, Banjo OA, Oke AJ, Adler A, Assous MV, Morand S, Raoult D, Rolain JM.** 2014. Worldwide emergence of colistin resistance in *Klebsiella pneumoniae* from healthy humans and patients in Lao PDR, Thailand, Israel, Nigeria and France owing to inactivation of the PhoP/PhoQ regulator *mgrB*: an epidemiological and molecular study. Int J Antimicrob Agents **44:**500–507. http://dx.doi.org/10.1016/j.ijantimicag.2014.07.020.
17. **Clinical and Laboratory Standards Institute.** 2012. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically; approved standard, 9th ed. CLSI document M07-A9. Clinical and Laboratory Standards Institute, Wayne, PA.
18. **Gutierrez B, Escudero JA, San Millan A, Hidalgo L, Carrilero L, Ovejero CM, Santos-Lopez A, Thomas-Lopez D, Gonzalez-Zorn B.** 2012. Fitness cost and interference of Arm/Rmt aminoglycoside resistance with the RsmF housekeeping methyltransferases. Antimicrob Agents Chemother **56:**2335–2341. http://dx.doi.org/10.1128/AAC.06066-11.
19. **Cannatelli A, Di Pilato V, Giani T, Arena F, Ambretti S, Gaibani P, D'Andrea MM, Rossolini GM.** 2014. *In vivo* evolution to colistin resistance by PmrB sensor kinase mutation in KPC carbapenemase-producing *Klebsiella pneumoniae* associated with low-dosage colistin treatment. Antimicrob Agents Chemother **58:**4399–4403. http://dx.doi.org/10.1128/AAC.02555-14.
20. **Giani T, Conte V, Mandalà S, D'Andrea MM, Luzzaro F, Conaldi Grossi P, Rossolini GM.** 2014. Cross-infection of solid organ transplant recipients by a multidrug-resistant *Klebsiella pneumoniae* isolate producing the OXA-48 carbapenemase, likely derived from a multiorgan donor. J Clin Microbiol **52:**2702–2705. http://dx.doi.org/10.1128/JCM.00511-14.