

Colistin Resistance Caused by Inactivation of the MgrB Regulator Is Not Associated with Decreased Virulence of Sequence Type 258 KPC Carbapenemase-Producing *Klebsiella pneumoniae*

Fabio Arena,^a Lucia Henrici De Angelis,^a Antonio Cannatelli,^a Vincenzo Di Pilato,^b Marina Amorese,^a Marco Maria D'Andrea,^a Tommaso Giani,^a Gian Maria Rossolini^{a,c,d,e}

Department of Medical Biotechnologies, University of Siena, Siena, Italy^a; Department of Surgery and Translational Medicine, University of Florence, Florence, Italy^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; Don Carlo Gnocchi Foundation, Florence, Italy^e

Using a *Galleria mellonella* animal model, we compared the virulence of two sequence type 258 (ST258) KPC-producing *Klebsiella pneumoniae* strains, which were representative of the two clades of this clonal lineage, with that of isogenic colistin-resistant *mgrB* mutants. With both strains, the *mgrB* mutants did not exhibit modification in virulence. In the *G. mellonella* model, the clade 1 strain (capsular type *cps-1* [*wzi29*, producing KPC-2]) was significantly more virulent than the clade 2 strain (capsular type *cps-2* [*wzi154*, producing KPC-3]).

Colistin is a backbone component of combination antimicrobial regimens for serious infections caused by carbapenemase-producing strains of *Klebsiella pneumoniae* (1, 2), which are among the most challenging antibiotic-resistant pathogens spreading globally (3, 4). However, in settings in which carbapenemase-producing *K. pneumoniae* is endemic, the emergence and rapid dissemination of colistin resistance have been reported, especially among KPC carbapenemase-producing *K. pneumoniae* strains, including those of the pandemic sequence type 258 (ST258) clonal lineage (5–9).

Colistin resistance is mostly due to modification of the antibiotic target (the lipid A moiety of the bacterial lipopolysaccharide [LPS]), which can be mediated by different mutational events up-regulating the endogenous LPS modification systems (10–14), or by the acquisition of exogenous genes encoding LPS modification systems (15). Mutations causing a loss of function in the MgrB protein, a negative-feedback regulator of the PhoP-PhoQ signal transduction system, which controls several biochemical pathways, including those involved in LPS modification, are a frequent mechanism of acquired colistin resistance among KPC carbapenemase-producing *K. pneumoniae* strains circulating in the clinical setting (14, 16, 17).

We previously showed that colistin resistance associated with inactivation of the *mgrB* gene is not associated with a significant biological cost and is stably maintained in the absence of selective pressure (18), a finding consistent with the rapid and efficient dissemination of colistin-resistant (COL^r) KPC carbapenemase-producing *K. pneumoniae* strains carrying this resistance mechanism observed in the clinical setting (7, 9).

In this work, we investigated the impact of colistin resistance, mediated by *mgrB* inactivation, on the virulence of KPC carbapenemase-producing *K. pneumoniae* strains representative of the two clades of the pandemic ST258 clonal lineage (19); we used the *Galleria mellonella* infection model, which is considered to be a validated model for testing the virulence of *K. pneumoniae* (20, 21).

The KPC carbapenemase-producing *K. pneumoniae* strains used in the experiments are described in Table 1. KK207-1 and KKBO-1 are two previously described strains and are representa-

tive of the two clades of ST258 (clades 1 and 2, respectively) (22). The *mgrB* insertional mutant of KKBO-1 (named mutKKBO-1) was previously described (18), while the *mgrB* mutant of KK207-1 (named mutKK207-1) was obtained with the same approach as for mutKKBO-1 and carried an ISKpn18 insertion disrupting the *mgrB* gene. The colistin MICs of the strains were determined by reference broth microdilution (23).

For experiments with *G. mellonella*, bacteria were grown aerobically in LB broth at 37°C, harvested during exponential phase (optical density at 600 nm [OD₆₀₀], ≈0.7), and washed once with 10 mM phosphate-buffered saline (PBS) (pH 6.5). Bacteria were then suspended in PBS to an OD₆₀₀ of 1.5, corresponding to approximately 1 × 10⁹ CFU/ml. Larvae weighing 450 to 600 mg were used for the experiments. For a comparative evaluation of virulence, groups of 10 larvae were injected with 5 × 10⁵ CFU of each strain and with sterile PBS as a control. The larvae were kept at 37°C in the dark under a humidified atmosphere, with food, and examined daily for pigmentation and mobility. Time of death was recorded at 24, 48, and 72 h. Three independent experiments were performed. The 50% lethal dose (LD₅₀) was determined by inoculating larvae with 10-fold serial dilutions of each strain containing 5 × 10⁴ to 5 × 10⁷ CFU. Ten larvae were injected with each dilution. For each strain, data from three independent experiments were combined. LD₅₀s were calculated using the GraphPad Prism 6.0 software (GraphPad Software, Inc., La Jolla, CA) and were compared using a two-tailed *t* test. *P* values of <0.05 were

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

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TABLE 1 Features of the bacterial strains included in this study

Strain (source or reference)	Sequence type	KPC type	Clade ^a	Capsular type	LD ₅₀ ± standard error ^b	Colistin MIC (µg/ml)	<i>mgrB</i> alteration (orientation) ^c
KKBO-1 (10)	258	<i>bla</i> _{KPC-3}	2	<i>cps-2 (wzi154)</i>	6.02 ± 0.09	0.125	Wild type
mutKKBO-1 (18)	258	<i>bla</i> _{KPC-3}	2	<i>cps-2 (wzi154)</i>	5.92 ± 0.02	8	IS5-like element at nt 75 (R)
KK207-1 (22)	258	<i>bla</i> _{KPC-2}	1	<i>cps-1 (wzi29-K41)</i>	4.68 ± 0.47	0.125	Wild type
mutKK207-1 (this study)	258	<i>bla</i> _{KPC-2}	1	<i>cps-1 (wzi29-K41)</i>	4.88 ± 0.13	32	ISK <i>pn18</i> element at nt 93 (R)
NTUH-K2044 (24)	23		NA ^d	<i>wzi1-K1</i>	4.57 ± 0.23	0.125	Wild type

^a According to reference 19.

^b Expressed as log CFU LD₅₀.

^c R, transposase gene is in the opposite orientation of the *mgrB* gene.

^d NA, not applicable.

considered statistically significant. *K. pneumoniae* NTUH-K2044, an ST23 virulent strain with the K1 capsular serotype (24), was included in all experiments as a virulent control.

All the studied strains caused a time-dependent death of larvae. In the 5 × 10⁵ CFU challenge, KKBO-1 (clade 2) killed, on average, 10% and 17% of larvae at 24 h and 72 h postinfection, respectively. In contrast, KK207-1 (clade 1) caused higher mortality, with average mortality rates of 55% and 65% at 24 h and 72 h, respectively. No differences were seen between the killing rates caused by the colistin-susceptible ST258 strains and the respective *mgrB*-inactivated COL^r mutants (Fig. 1).

In the experiments with scalar inoculum sizes, all tested strains caused larval mortality in a dose-dependent manner. No significant differences were observed in the LD₅₀s of KKBO-1 (colistin susceptible [COL^s]) versus mutKKBO-1 (COL^r) (*P* = 0.13), and of KK207-1 (COL^s) versus mutKK207-1 (COL^r) (*P* = 0.51). On the other hand, the LD₅₀s of KK207-1 and mutKK207-1 (clade 1) were significantly lower than those of KKBO-1 and mutKKBO-1 (clade 2) (*P* < 0.05) (Table 1).

The COL^r phenotype was checked in isolates obtained from larvae infected with mutKKBO-1 and mutKK207-1 at 24 and 72 h postinfection (60 isolates per experiment) and was confirmed in all tested isolates, revealing stability after the *in vivo* passage.

Altogether, the results of these experiments indicated that the emergence of colistin resistance mediated by inactivation of the *mgrB* gene is apparently not associated with significant

modifications to the virulence potential of KPC carbapenemase-producing *K. pneumoniae* representative of the two clades of the ST258 clonal lineage. This finding is notably different from what was observed with COL^r mutants of *Acinetobacter baumannii*, which exhibit a remarkable impairment of virulence potential (25–27), and it is consistent with the observation that COL^r KPC carbapenemase-producing *K. pneumoniae* strains of clonal complex 258 (CC258) are capable of causing severe invasive infections and large outbreaks (8, 9), similar to their colistin-susceptible counterparts. In fact, recent clinical studies have reported that mortality rates among patients with infections caused by COL^r KPC carbapenemase-producing *K. pneumoniae* strains were higher than those among patients infected by colistin-susceptible KPC carbapenemase-producing *K. pneumoniae* strains (8, 28). The present results suggest that this reported mortality rate excess is likely attributable to the further narrowing of available therapeutic options rather than to an increased virulence potential of COL^r KPC carbapenemase-producing *K. pneumoniae* strains.

Our data also confirmed previous findings that ST258 strains of clade 2 (with a *cps-2* capsular gene cluster) exhibit an overall low virulence potential in the *G. mellonella* model (29) compared with that of highly virulent *K. pneumoniae* strains, such as NTUH-K2044. On the other hand, for the ST258 strain of clade 1 (with a *cps-1* capsular gene cluster), we documented a higher virulence potential than that for clade 2 strains, which was similar to that of NTUH-K2044. This finding apparently differs from that of a previous study that reported a low virulence potential for a KPC-2-producing ST258 strain of clade 1 (deduced by the K41 serotype) in a mouse septicemia model (30). Further studies will be necessary to confirm these discrepancies, which might be dependent on strain-specific differences and/or differences in the animal infection models and their immune systems. In fact, *G. mellonella* moths lack an adaptive immune response, and the model can be used only to approximate mammals' innate immune response to bacterial infections. However, the difference in virulence among the clade 1 and clade 2 strains observed in this work is consistent with the different prevalences observed for the two clades, with an overall predominance of clade 2 strains in settings of high-level endemicity of KPC carbapenemase-producing *K. pneumoniae* strains of CC258 (22, 29).

In conclusion, our findings suggest that COL^r KPC carbapenemase-producing *K. pneumoniae* strains of ST258 carrying an in-

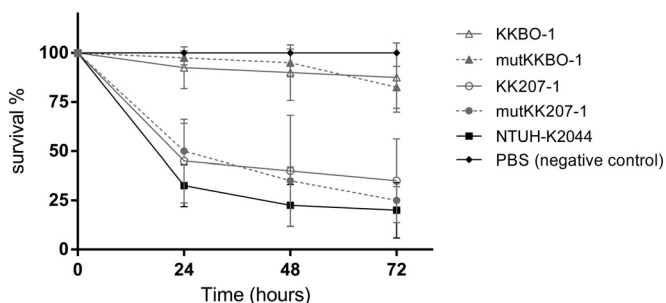


FIG 1 Results of *G. mellonella* infection experiments. Larvae were injected with PBS or with 5 × 10⁵ CFU of *K. pneumoniae* strain NTUH-K2044, KKBO-1, mutKKBO-1, KK207-1, or mutKK207-1 (10 larvae per strain), and survival was monitored over 72 h postinfection. The data are mean values from the results of three independent experiments, and the error bars represent standard deviations from the mean.

activated *mgrB* gene might be as virulent as their COL^S ancestors. Efforts focused at containing their dissemination should therefore be maximized. Our findings also suggest that significant differences might exist in the interaction of ST258 strains of KPC carbapenemase-producing *K. pneumoniae* of clades 1 and 2 with the innate immune response, regardless of their colistin susceptibility status.

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