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MECHANISMS OF RESISTANCE

# Application of CRISPR/Cas9-Based Genome Editing in Studying the Mechanism of Pandrug Resistance in *Klebsiella pneumoniae*

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**ABSTRACT** In this study, a CRISPR/Cas9-mediated genome editing method was used to study the functions of the *mgrB*, *tetA*, and *ramR* genes in mediating colistin and tigecycline resistance in carbapenem-resistant *Klebsiella pneumoniae* (CRKP). Inactivation of the *tetA* or *ramR* gene or the *mgrB* gene by CRISPR/Cas9 affected bacterial susceptibility to tigecycline or colistin, respectively. This study proved that the CRISPR/Cas9-based genome editing method could be effectively applied to *K. pneumoniae* and should be further utilized for genetic characterization.

**KEYWORDS** CRISPR/Cas9, *Klebsiella pneumoniae*, drug resistance mechanisms, genome editing, pandrug resistance

*Klebsiella pneumoniae* is one of the ESKAPE (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* species) pathogens which could acquire multiple antimicrobial resistance through either horizontal gene transfer or mutations (1). Tigecycline and polymyxins are last line antibiotics used in clinical settings to treat carbapenem-resistant pathogens (2). Unfortunately, resistance to either one or both tigecycline and colistin has been reported in *K. pneumoniae* (3, 4). Resistance to these two drugs is commonly mediated by chromosomal gene mutations. Mutations in the *ramA*, *ramR*, *acrR*, and *rpsJ* genes that lead to increased expression of the AcrAB-TolC, RND (resistance-nodulation-cell division), or OqxAB efflux pump are known to confer tigecycline resistance (5–8), and resistance to polymyxins was shown to be mediated by the mutations in genes encoding the two-component regulatory systems PmrAB, PhoPQ, and CrrAB, leading to further upregulation of the *pmrHFIJKLM* operon (9–11). Inactivation or downregulation of the *mgrB* gene via IS insertion, nonsense mutations, or mutations is a common mechanism that confers polymyxin resistance (1, 12, 13). A precise and efficient CRISPR/Cas9-mediated *K. pneumoniae* genome editing method was recently developed and could be applied to studying pathogens of clinical importance (14). In this work, we studied and confirmed the functions of genes mediating tigecycline and colistin resistance through genome editing in carbapenem-resistant *K. pneumoniae* (CRKP) strains isolated from a leukemia patient in a previous study (15).

*K. pneumoniae* strain Y4, which harbored an intact *mgrB* gene and was susceptible to colistin (MIC = 0.25 mg/liter), was used to study the mechanism of colistin resistance triggered by *mgrB* mutation. The *K. pneumoniae* Y17 strain, which was resistant to

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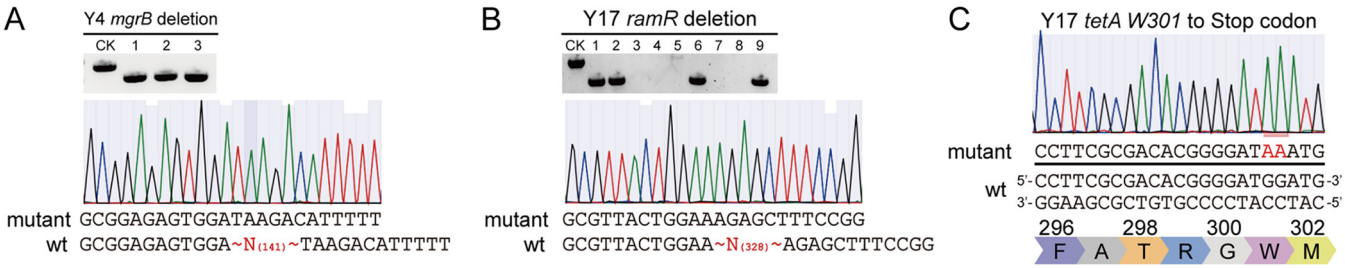


**FIG 1** Genetic manipulation in *K. pneumoniae* using the pCasKP-pSGKP genome editing system and the pBECKP base editing system. (A and B) Deletion of the *mgrB* gene in the *K. pneumoniae* Y4 strain (A) and of the *ramR* gene in the *K. pneumoniae* Y17 strain (B) using the pCasKP-pSGKP system. Lane CK is the PCR band from the wild-type (wt) strain as a control. (C) W301 of the *tetA* gene in the *K. pneumoniae* Y17 strain was successfully mutated to a stop codon using the pBECKP base editing system.

tigecycline (MIC = 8 mg/liter) and harbored a mutated *tetA* gene (S251A) and the wild-type *ramR* gene, was used to study the mechanism of tigecycline resistance.

The *mgrB* gene deletion mutant in the *K. pneumoniae* Y4 strain and the *ramR* gene deletion mutant in the *K. pneumoniae* Y17 strain were successfully obtained using the pCasKP-pSGKP genome editing system (Fig. 1A and B) according to methods described previously (14). Briefly, the pCasKP-apr plasmid was first transformed into the wild-type *K. pneumoniae* Y4 strain using electroporation (2,500 V, 200 Ω, 25 μF, 2-mm cuvette). After induction of L-arabinose, cells harboring the pCasKP-apr plasmid were then prepared as the competent cells. The annealed *mgrB* spacer oligonucleotides were inserted into the BsaI sites of the pSGKP-spe plasmid by Golden Gate assembly. The *mgrB* spacer-introduced pSGKP-spe plasmid and the corresponding 90-nucleotide (nt) single-strand DNA (ssDNA) repair template were then coelectroporated into the aforementioned pCasKP-apr-harboring competent cells. The colonies were selected on an LB agar plate containing 50 mg/liter apramycin and 100 mg/liter spectinomycin at 30°C. The successful *mgrB* deletion strain (*K. pneumoniae* Y4-ΔmgrB) was confirmed by PCR and sequencing. After obtaining the *mgrB* deletion mutant, both the pCasKP-apr and the *mgrB* spacer-introduced pSGKP-spe plasmids were cured by culturing the cells at 37°C with 5% (wt/vol) sucrose. The *K. pneumoniae* Y17-ΔramR strain was a *ramR* gene deletion mutant of the *K. pneumoniae* Y17 strain created using the same method.

We used the base editor pBECKP-spe to inactivate the plasmid-borne *tetA* gene. The pBECKP-spe system executed editing by introduction of a single-stranded DNA break instead of a double-stranded DNA break, which would not result in plasmid removal during editing (14). Briefly, the annealed *tetA* spacer oligonucleotides were inserted into the BsaI sites of the pBECKP-spe plasmid using Golden Gate assembly. Then, the *tetA* spacer-introduced pBECKP-spe plasmid was transformed into the wild-type *K. pneumoniae* Y17 competent cells using electroporation (2,500 V, 200 Ω, 25 μF, 2-mm cuvette). The colonies were selected on an LB agar plate containing 100 mg/liter spectinomycin at 37°C. The successful *tetA* edited colonies (*K. pneumoniae* Y17-ΔtetA) were confirmed by PCR and sequencing. The *tetA* spacer-introduced pBECKP-spe plasmid was cured by culturing the cells in the presence of 5% (wt/vol) sucrose. As shown in Fig. 1C, the *tetA* gene was successfully mutated by the introduction of a premature stop codon (TAA). Whole-genome sequencing was further utilized to rule out the off-target effects in all three mutants generated by the CRISPR/Cas9 method. The sequencing data accession numbers of the *K. pneumoniae* Y17-ΔtetA, Y17-ΔramR, and Y4-ΔmgrB strains are SAMN11053200, SAMN11053202, and SAMN11053203 in BioSample (NCBI), respectively.

We examined the tigecycline and colistin susceptibility of the wild-type *K. pneumoniae* Y17 strain, *K. pneumoniae* Y4 strain, and the *K. pneumoniae* Y17-ΔtetA, Y17-ΔramR, and Y4-ΔmgrB mutant strains (Table 1) using the broth dilution method; the resistance breakpoints for colistin and tigecycline were both >2 mg/liter according to the 2017 EUCAST breakpoint guideline (available at http://www.eucast.org/clinical_breakpoints/). *Pseudomonas aeruginosa* ATCC 27853 and *Escherichia coli* NCTC 13846

**TABLE 1** MIC values of colistin and tigecycline against bacterial strains used in this study

| *K. pneumoniae* strain | Description | MIC (mg/liter) | |
|---|---|---|---|
| | | Colistin | Tigecycline |
| Y4 | Wild-type clinically isolated *K. pneumoniae* strain Y4 | 0.25 | 1 |
| Y4-ΔmgrB | *K. pneumoniae* Y4 △*mgrB* | 16 | 1 |
| Y17 | Wild-type clinically isolated *K. pneumoniae* strain Y17 | 64 | 8 |
| Y17-ΔtetA | *K. pneumoniae* Y17 *tetA* W301 mutation to stop codon | 64 | 2 |
| Y17-ΔramR | *K. pneumoniae* Y17 △*ramR* | 64 | 64 |

served as susceptible (0.5 to 2 mg/liter) and nonsusceptible (4 to 8 mg/liter) quality control strains for colistin, respectively. In addition, we performed a whole spectrum of antibiotic susceptibility tests against the strains (see Table S1 in the supplemental material). Inactivation of the *tetA* gene significantly increased bacterial susceptibility to tigecycline, while deletion of the *ramR* gene resulted in a dramatic increase in the MIC, from 8 to 64 mg/liter (Table 1). The deletion of the *mgrB* gene resulted in a significant decrease in bacterial susceptibility to colistin. These results verified that the genetic status of the *tetA* and *ramR* genes were important factors that determine tigecycline susceptibility in the wild-type *K. pneumoniae* Y17 strain and that the *mgrB* gene was an important factor that confers colistin resistance in the *K. pneumoniae* Y4 strain.

Quantitative real-time PCR **(**qRT-PCR) was performed to determine the expression levels of the efflux pumps AcrAB and OqxAB and their transcriptional regulators RamA, MarA, RarA, and SoxS. The primers used for real-time PCR and the procedure were described previously (16). The biological replicates and three technical replicates of experiments were carried out in triplicate. The expression level of each target gene was normalized to that of a housekeeping gene (16S rRNA, *rrsE*), and *K. pneumoniae* ATCC 13883 was used as the reference strain with a MIC of tigecycline of 0.5 mg/liter. The relative gene expression data were analyzed on the basis of the $2^{-\triangle\triangle Ct}$ method as previously described (17). Overexpression of *ramA* (53.3-fold), *acrB* (6.42-fold), *acrA* (5.08-fold), and *oqxB* (1.73-fold) were detected in the *K. pneumoniae* Y17-ΔramR mutant strain (Table S2 and Fig. S1). No positive effect on the overexpression of the efflux pumps AcrAB and OqxAB in the *K. pneumoniae* Y17-ΔtetA mutant strain was identified.

Genome editing has been proved to be a powerful tool for the study of multidrug resistance mechanisms in pathogens, including multidrug resistant *K. pneumoniae*, which posed serious threat to public health. Mutations in chromosomal genes frequently confer resistance to the last line antibiotics colistin and tigecycline in *K. pneumoniae*, and should therefore be closely monitored (18). The recently developed CRISPR/Cas9-based gene editing method enables precise and rapid genetic manipulation in *K. pneumoniae* strains (14). In this study, we successfully verified the functions of genes related to tigecycline and colistin resistance in a carbapenem-resistant *K. pneumoniae* strain isolated from a leukemia patient using the CRISPR/Cas9-based gene editing method (15).

Tigecycline resistance was previously found to be predominantly associated with mutations that cause overexpression of AcrAB-TolC, OqxAB, and RND efflux pumps, including the reported regulator genes *acrR*, *marA*, *soxS*, *rarA*, *rob*, and *ramA* and the effector genes *acrA*, *acrB*, *tolC*, *oqxA*, and *oqxB* (19). Mutations in the *ramR* gene were previously demonstrated to cause overexpression of the AcrAB-TolC efflux pump, thus triggering tigecycline resistance (5). Deletion of the *ramR* gene resulted in significant overexpression of the *ramA* gene, which could further enhance the expression level of the AcrAB efflux pump and lead to tigecycline resistance (8). Genomic analysis indicated that the tigecycline resistance in *K. pneumoniae* Y17 strain was due to the carriage of the *tetA* variant gene (15). However, a previous study showed that carriage of the *tetA* variant did not result in variation in the expression level of any efflux pump-related genes (20). Likewise, deletion of the *tetA* variant also did not result in a change in expression level of the efflux pump genes. The mechanism of tigecycline resistance triggered by *tetA* variant needs further study.

MgrB was produced upon activation of the PhoPQ signaling system. Inactivation of the *mgrB* gene leads to the upregulation of the PhoPQ two-component system, consequently upregulating the expression of *pmrHFIJKLM* (12). Deletion of *mgrB* via CRISPR/Cas9-based genome editing resulted in a marked increase in MIC of colistin, further confirming the findings in previous studies.

In summary, this study applied the recently developed CRISPR/Cas9-based method to studying the functions of *ramR*, *tetA*, and *mgrB* genes in conferring tigecycline or colistin resistance in carbapenem-resistant *K. pneumoniae*. The related mutants were constructed efficiently and precisely. Mutations in *tetA* and *ramR* genes were found to be associated with tigecycline resistance through different mechanisms, while deletion of the *mgrB* gene is related to colistin resistance. The CRISPR/Cas9-based genome editing method was proved to be effective in characterization of multidrug resistance genes in *K. pneumoniae*.

**Accession number(s).** All the plasmids in this study are available in Addgene with numbers 117231 (pCasKP-apr), 117234 (pSGKP-spe), and 117236 (pBECKP-spe) and in GenBank under accession numbers MH587687 (pCasKP-apr), MH587684 (pSGKP-spe), and MH587686 (pBECKP-spe). Whole-genome sequencing was further utilized to rule out the off-target effects in all three mutants generated by the CRISPR/Cas9 method. The sequencing data accession numbers of the *K. pneumoniae* Y17-ΔtetA, Y17-ΔramR, and Y4-ΔmgrB strains are SAMN11053200, SAMN11053202, and SAMN11053203 in BioSample (NCBI), respectively.

## SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AAC.00113-19.

**SUPPLEMENTAL FILE 1**, PDF file, 0.3 MB.

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We declare that we have no conflict of interest.

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