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Generation of Newly Discovered Resistance Gene *mcr-1* Knockout in *Escherichia coli* Using the CRISPR/Cas9 System

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The *mcr-1* gene is a new "superbug" gene discoverd in China in 2016 that makes bacteria highly resistant to the last-resort class of antibiotics. The *mcr-1* gene raised serious concern about its possible global dissemination and spread. Here, we report a potential anti-resistant strategy using the CRISPR/Cas9-mediated approach that can efficiently induce *mcr-1* gene knockout in *Escherichia coli*. Our findings suggested that using the CRISPR/Cas9 system to knock out the resistance gene *mcr-1* might be a potential anti-resistant strategy. Bovine myeloid antimicrobial peptide-27 could help deliver plasmid pCas::mcr targeting specific DNA sequences of the *mcr-1* gene into microbial populations.

Keywords: *mcr-1*, knockout, *E. coli*, CRISPR/Cas9 system, BMAP-27

Introduction

The *mcr-1* gene is a new "superbug" gene discovered in China in 2016 that makes bacteria highly resistant to the last-resort class of antibiotics. The *mcr-1* gene has been found in no fewer than 16 countries, including 7 countries in Southeast Asia [1, 2], Japan [3], Vietnam [4], and Cambodia [5], and 9 European countries. In May of 2016, the plasmid-borne *mcr-1* gene was detected in a woman in the USA [6]. The animal-to-human transmission of *mcr-1* colistin resistance has already been found in China [1], Thailand [7], Laos [8], and Denmark [9], which is raising serious concern about its possible global dissemination and spread [10]. Therefore, it is necessary and the right time to develop new strategies against the increasing antimicrobial resistance.

In our study, we engineered the clustered regularly interspaced short palindromic repeats (CRISPR)-associated (Cas) system, which is an effective tool for genome editing in a wide variety of organisms [11–13]. In the CRISPR/Cas9 system, Cas9 complexes with a single guide RNA (sgRNA) and forms the effector complex that binds to and cleaves dsDNA either in vivo or in vitro. After cleavage by Cas9, both dsDNAs undergo a double-strand break (DSB) at 3 bp upstream of a protospacer adjacent motif (PAM). This inspired us to elucidate whether CRISPR/cas9 can mediate the knockout of the new superbug gene *mcr-1* in *Escherichia coli* (*E. coli*).

Materials and Methods

The clinical isolate bacterial strain *E. coli* NJ-15-3 used in this work was identified by 16S rDNA sequencing and biochemistry analysis and grown in LB medium at 37°C. The colistin resistance phenotype was detected by minimum inhibitory concentration (MIC) according to the recommendations of the Clinical and Laboratory Standards Institute document M100-S25 [14]. Then, the NJ-15-3 plasmids were isolated by employing manual extraction with the alkaline lysis method, and the *mcr-1* gene was detected by PCR (primers located upstream and downstream of the *mcr-1* gene target MCRF-ATGATGCAGCATACTTCTGTG, MCRR-TCGGTCTGTAGGGCATTTTGGAG; primers located upstream and downstream of the *mcr-1* gene MCRUF-GTATAATTG

CCGTAATTATC, MCRUR-ATAATACGAATGGAGTGTGC) with Taq DNA Polymerase (M0495L; New England Biolabs, USA)

Plasmid pCas::mcr containing the *mcr-1* gene target was transferred into 10^{10} CFU/ml cells containing the *mcr-1* gene. After electroporation, cultures were screened for surviving recipient cells on LB plates and LB + chloramphenicol (Cm), LB + colistin (Cl) for selection of transconjugants (Cm resistance is encoded by the pCas::mcr plasmid).

E. coli NJ-15-3 cells were co-incubated with phosphate buffered saline (PBS, pH 7.2) or Bovine myeloid antimicrobial peptide-27 (BMAP-27) labeled with WGA 610-X conjugate (Invitrogen, USA). After washing three times with PBS, the *E. coli* NJ-15-3 cells were observed by laser confocal microscopy.

We co-incubated the *E. coli* NJ-15-3 (OD_{600} = 0.3–0.5) with the BMAP-27 (0.1–1 μM) and pCas::mcr (0.1–1 ng/μl) complex in PBS. After 5 h, we screened 10 *E. coli* strains on LB and detected the *mcr-1* gene by PCR and the colistin resistance phenotype by MIC.

Results and Discussion

In clinical isolate bacterial strain *E. coli* NJ-15-3 as wild type, the colistin resistance phenotype was detected by MIC, and the *mcr-1* gene was identified by PCR (data not shown). Based on the fact that Liu *et al.* [1] successfully acquired a large plasmid (pHNSHP45) from a pig *E. coli* isolate, we attempted to subject the NJ-15-3 strain to plasmid isolation by employing manual extraction with the alkaline lysis method. It is the same as plasmid pHNSHP45 by sequencing the whole plasmid (data not shown).

Here, we first designed chimeric sgRNA that encodes sequences complementary to a target protospacer of *mcr-1* (shown in Fig. 1A). For sgRNA design, the *mcr-1* gene sequence edited was screened for the presence of a 20-bp guide and PAM sequence. A guide sequence for sgRNA had to meet the rules governing efficient and precise initiation of the T7 promoter. To establish functionality for CRISPR in mediating sequence-specific gene editing, we designed sgRNA to induce a DSB in the *mcr-1* gene, which encodes broad-spectrum and pan-resistance to the colistin antibiotic. Bacterial Cas9-sgRNA expression plasmid pCas was constructed with two T7 promoters to ensure independent expression of Cas9 and sgRNA (Fig. 1A), the same as plasmid BPK764 [15].

Plasmid pCas::mcr containing the *mcr-1* gene target was transferred into 10^{10} CFU/ml cells containing the *mcr-1* gene, and then cultures were screened for surviving recipient cells on LB plates and LB+ chloramphenicol (Cm) (Fig. 1B). Transformation of *E. coli* NJ-15-3 with pCas::mcr containing a copy of *mcr-1* resulted in the same viable recipients in transformation efficiency compared with pCas cells (Fig. 1B). We have repeated the transformation 10 times, and found that the viable recipients of Cm resistance were sensitive to colistin (Cl) for those 10 times (Fig. 1C). These

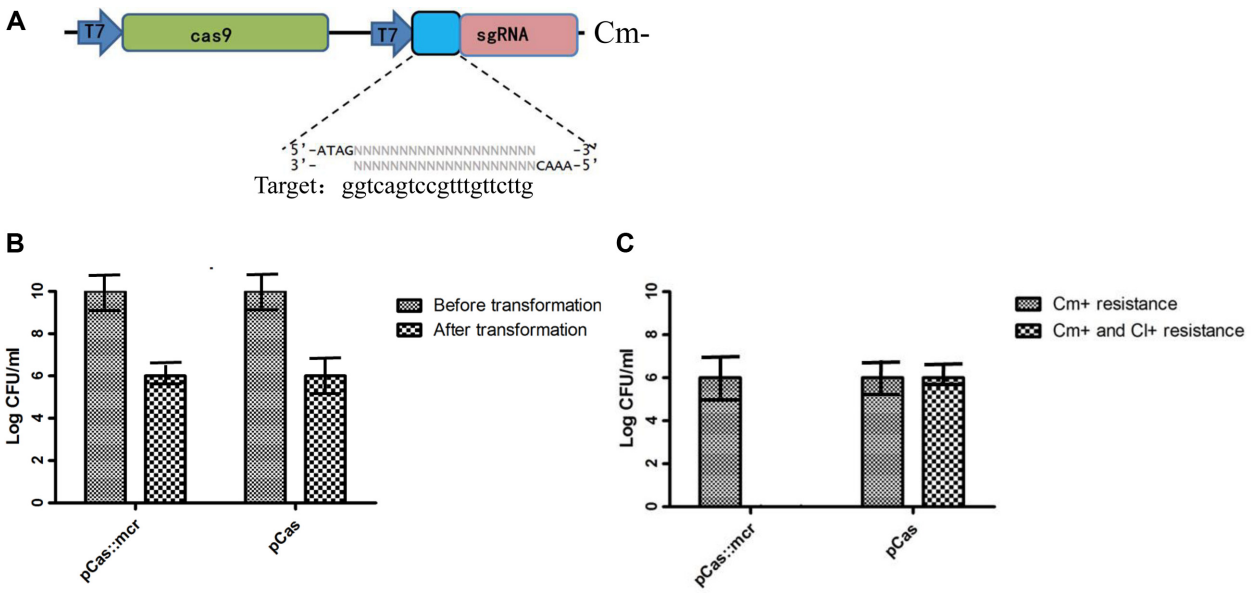

Fig. 1. Design of sgRNA targeting the *mcr-1* gene, and double strand break analysis of the *mcr-1* gene fragment.

(**A**) Design of sgRNA targeting the *mcr-1* gene. (**B**) Electric transfer of pCas::mcr into 10^{10} CFU/ml cells containing the *mcr-1* gene. After transmission, cultures were plated on LB for surviving recipient cells, and LB + chloramphenicol (Cm) and LB + Colistin (Cl) to select for transconjugants (**C**).

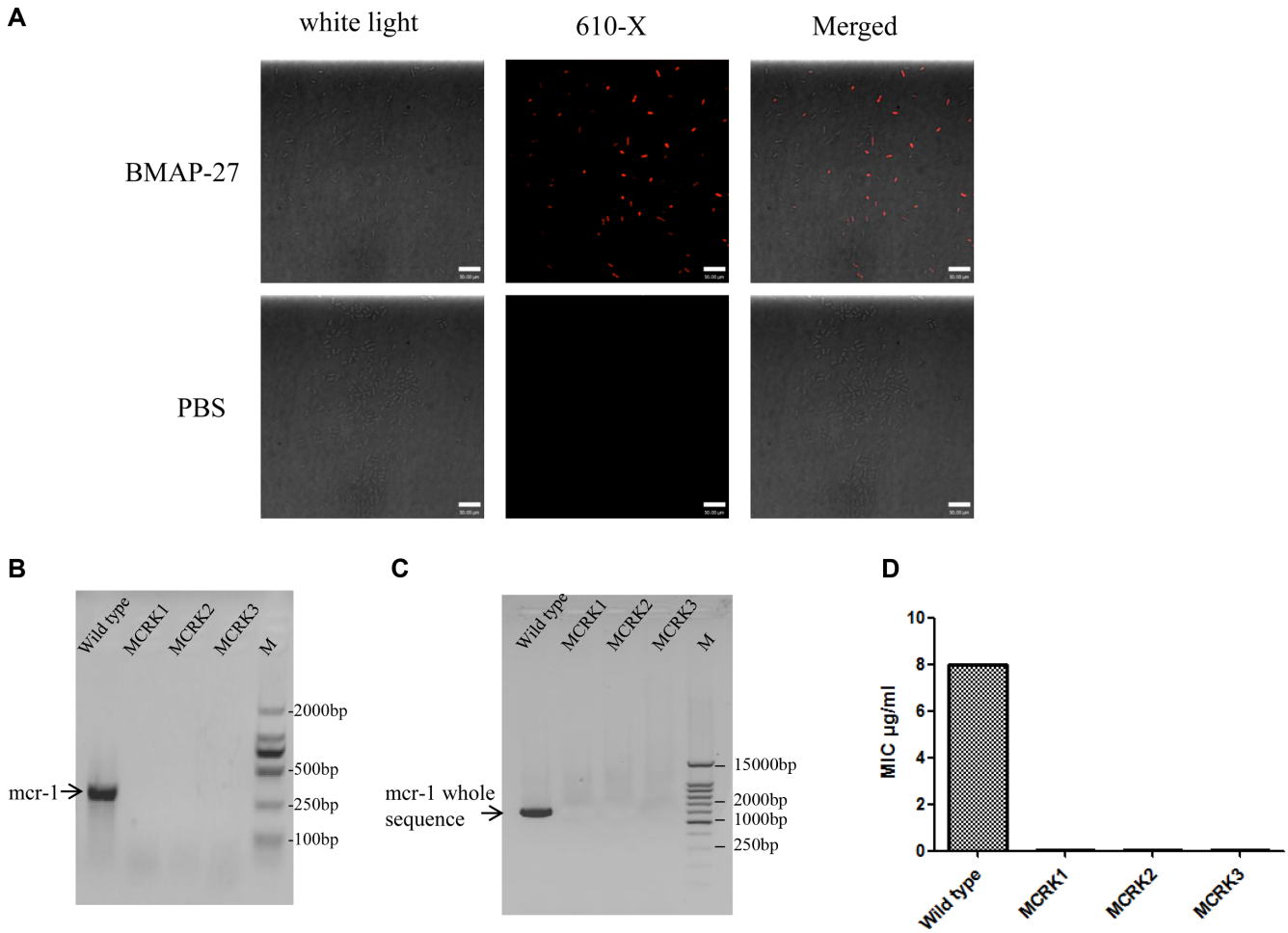

Fig. 2. Cas9-sgRNA delivered by Bovine myeloid antimicrobial peptide-27 (BMAP-27) exhibits efficient and specific antimicrobial effects against strains harboring plasmid target sequences.

(**A**) BMAP-27 delivered pCas::mcr into cells. *E. coli* NJ-15-3 cells were co-incubated with PBS or BMAP-27 labeled with WGA 610-X conjugate (Invitrogen, USA). The labeled BMAP-27 was observed in *E. coli* NJ-15-3 cells but was not found in control cells by laser confocal microscopy. (**B–D**) *mcr-1* gene analysis of *E. coli* NJ-15-3 *mcr-1* knockout monoclonal. *E. coli* NJ-15-3 (OD_{600} = 0.3–0.5) was co-incubated with the BMAP-27 (0.1–1 μM) and pCas::mcr (0.1–1 ng/μl) complex in PBS. After 5 h, we screened 10 *E. coli* strains on LB and found that 3 *E. coli* strains were colistin-sensitive, and no *mcr-1* gene was detected by PCR using primers located upstream and downstream of the *mcr-1* gene target and *mcr-1* gene in screened *E. coli* NJ-15-3. Consequently, we determined the sensitivity of the screened *E. coli* strains to a range of concentration of colistin. The results indicated the screened *E. coli* strains recovered to be sensitive to colistin, and the minimum inhibitory concentration (MIC) of the wild-type *E. coli* NJ-15-3 was 8 μg/ml of colistin.

results indicated that bacterial plasmid pCas::mcr enabled knockout of the *mcr-1* gene in the *E. coli* isolate with the help of electroporation.

Owing to the application limitation of electroporation, we attempted to explore a plasmid-delivery system. Cathelicidins are small, cationic, antimicrobial peptides found in mammalian species. BMAP-27 contains 27 amino acid residues and an α-helical C-terminus with structural attributes of antimicrobial activity. It was demonstrated that BMAP-27 induces mitochondrial permeability by forming transition pores [16]. Here, we investigated whether BMAP-27 could enhance the efficiency of plasmid transfer. As shown in Fig. 2, with the help of BMAP-27, pCas::mcr exhibited better efficient and specific antimicrobial effects against strains harboring plasmid or chromosomal target sequences. To determine how BMAP-27 work it out for the gene transfer, *E. coli* NJ-15-3 cells were co-incubated with PBS or BMAP-27 labeled with WGA 610-X conjugate. The labeled BMAP-27 was observed in *E. coli* NJ-15-3 cells but not in control cells by laser confocal microscopy (Fig. 2A).

We co-incubated the *E. coli* NJ-15-3 (OD_{600} = 0.3–0.5) with the BMAP-27 (0.1–1 μM) and pCas::mcr (0.1–1 ng/μl) complex in PBS. After 5 h, we screened 10 *E. coli* strains on LB and found that 3 *E. coli* strains were colistin-sensitive and no *mcr-1* gene was detected by PCR using primers located upstream and downstream of the *mcr-1* gene target and the *mcr-1* gene in screened *E. coli* NJ-15-3 (Figs. 2B–2C). Consequently, we determined the sensitivity of the screened *E. coli* strains to a range of concentration of colistin according to the recommendations of the Clinical and Laboratory Standards Institute document M100-S25 [14]. The *E. coli* strain NJ-15-3 (wild type) was used for quality control. The results indicated that the screened *E. coli* strains recovered to be sensitive to colistin, and the MIC of the wild-type *E. coli* NJ-15-3 was 8 μg/ml of colistin (Fig. 2D). These results demonstrated that the *mcr-1* gene in *E. coli* NJ-15-3 was successfully knocked out using the CRISPR/Cas9 system, and with BMAP-27, the CRISPR/Cas9 system exhibited better efficiency of intracellular gene editing. BMAP-27, as a small, cationic, antimicrobial peptide, can combine with the plasmid and deliver it to the bacteria.

The CRISPR/Cas9 system can be developed into new microbial gene therapy technology; however, these highly anionic nucleic acids and proteins could not penetrate the cell membrane into the cell by themselves. Because of the lack of an efficient delivery system for CRISPR/Cas9 systems, this technique is limited to the in vitro treatment [17]. Citorik *et al.* [18] delivered CRISPR/Cas9 systems into cells by phage or conjugation plasmid. However, the phage host spectrum is narrow, there is the risk of gene recombination, and the efficiency of the conjugation plasmid has the risk of the existence and recombination of drug resistance genes. CRISPR/Cas9-mediated knockout of the *mcr-1* gene in *E. coli* NJ-15-3 in our study provides a potential solution to resistance genes. Because CRISPR/Cas9 systems are widely conserved in bacteria, the development and optimization of delivery vehicles will be required to improve the efficiency of Cas9-sgRNA targeting in other strains.

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