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Exploiting a conjugative CRISPR/Cas9 system to eliminate plasmid harbouring the *mcr-1* gene from *Escherichia coli*

Haisi Dong[a], Hua Xiang[b], Dan Mu[a], Dacheng Wang[a,*], Tiedong Wang[a,*]

[a] College of Animal Science, Jilin University, Changchun, China
[b] College of Animal Science and Technology, Jilin Agricultural University, Changchun, China

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ABSTRACT

The transfer of multi-drug-resistance plasmids by bacterial conjugation is largely responsible for the development of drug resistance in bacteria, and causes serious problems in the treatment of infectious diseases. Since the first discovery of plasmid-borne colistin resistance gene *mcr-1* was reported in late 2016, this gene has been found in a great number of *Escherichia coli* and other Gram-negative pathogens separated from different types of sources worldwide. The elimination of plasmids carrying *mcr-1* and restoration of polymyxin sensitivity has very important clinical significance because polymyxins are frequently used as last-resort antibiotics to treat extensively drug-resistant Gram-negative bacterial infections. A host-independent conjugative plasmid was constructed in this study, and an engineered CRISPR/Cas9 system was used to remove plasmid harbouring *mcr-1* from bacteria. This study found that this conjugative plasmid can not only be used as a new tool to remove resistance plasmids and sensitize the recipient bacteria to antibiotics, but can also make the recipient cell acquire immunity against *mcr-1*. This strategy provides a novel method to counteract the ever-worsening spread of *mcr-1* among bacterial pathogens.

1. Introduction

The development of antibiotics has saved countless lives; however, over the past several decades, the intensive use of broad-spectrum antibiotics in medicine and animal agriculture has led to rapid bacterial evolution and the emergence of drug-resistant bacteria [1,2]. The polymyxins are a group of cationic polypeptide antibiotics commonly used in clinical veterinary practice to treat Gram-negative bacterial infections [3], and used in humans in limited situations as a last-line option for treating extensively drug-resistant Gram-negative bacterial infections. In late 2016, a mobile colistin resistance gene (*mcr-1*) was discovered on the pHNSHP45 plasmid isolated from porcine *Escherichia coli* strain SHP45 in China [4]. Since then, *mcr-1* has been found on various conjugative plasmids including IncX4-, IncI2-, IncHI2-, IncP-, IncFII- and IncY-type plasmids [5,6], and has spread globally among different Gram-negative bacteria [7]. The elimination of plasmids carrying *mcr-1* and restoration of polymyxin sensitivity has very important clinical significance.

Clustered regularly interspaced short palindromic repeats (CRISPR)/Cas systems offer a promising solution to the spread of *mcr-1* among bacterial pathogens. CRISPR/Cas systems are adaptive immune systems that exist in approximately 40% of sequenced bacterial genomes, and approximately 90% of those from archaea [8]. The type II CRISPR system of *Streptococcus pyogenes* only includes an endonuclease, Cas9 and two RNAs: a crRNA and a trans-activating crRNA (tracrRNA) that facilitates crRNA processing and recruitment to Cas9 [9]. The crRNA/tracrRNA forms a complex with Cas9 which guides Cas9 to bind to a specific DNA sequence and induce double-strand breaks. The dual crRNA:tracrRNA has been engineered as a chimeric single guide RNA (sgRNA) which contains a DNA-specific binding complementary area, a Cas9-binding hairpin structure, and a transcription terminator derived from *S. pyogenes* [10].

A few early studies demonstrated that existing bacterial CRISPR/Cas systems can limit the spread of drug resistance genes by counteracting multiple routes of horizontal gene transfer (HGT) in some pathogenic bacteria [11,12]. Subsequent studies showed that the reprogrammed Cas nuclease can be exploited to prevent the spread of plasmid-borne resistance by targeting antibiotic resistance genes and destroying bacterial plasmids that confer antibiotic resistance [13–15]. These original works provide a convincing demonstration of the potential of the CRISPR/Cas system in solving the problem of multi-drug resistance in clinical and environmental settings. However, to truly exploit the capacities of the CRISPR/Cas9 system to counteract bacterial antibiotic resistance, the delivery vehicles must be developed and optimized because these phage-

* Corresponding authors. Address: College of Animal Science, Jilin University, Changchun 130062, China. Tel.: + 86 431 87836161.
E-mail addresses: wangdc@jlu.edu.cn (D. Wang), wangtd@jlu.edu.cn (T. Wang).

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based CRISPR/Cas9 delivery strategies currently face various obstacles such as narrow host range, delivery barriers and bacterial resistance to phages [16,17].

Given that bacterial conjugation has the greatest influence on the dissemination of antibiotic resistance genes in the microbiota, it was reasoned that bacterial conjugation may be the best way to deliver the CRISPR/Cas9 antimicrobials into bacteria in their natural environment. This study constructed a host-independent conjugative plasmid, and delivered the engineered CRISPR/Cas9 system among bacteria via conjugation to remove *mcr-1*-carrying plasmids. The results show that this conjugative plasmid can be used as a new tool to destroy *mcr-1*-harbouring plasmid in *E. coli*. Therefore, this approach may offer a novel means to combat the ever-worsening dissemination of antibiotic resistance genes among bacterial pathogens.

2. Materials and methods

2.1. Bacterial strains, plasmids and culture conditions

The bacterial strains and plasmids used or generated in this work are listed in Table 1. *E. coli* DH5α was used as a recipient strain for plasmid construction and propagation. The colistin-susceptible *E. coli* strain MG1655 was used in conjugation experiments. The plasmid pFSEC-01 [18] and the *mcr-1*-harbouring plasmid pHNSHP45 used in the conjugation experiments were gifts from Dr Yang Wang. *E. coli* were grown in Luria-Bertani (LB, 5 g yeast extract, 5 g NaCl and 10 g tryptone/L) broth or on LB agar (LB supplemented with 15 g agar/L) plates at 37°C. When necessary, the appropriate antibiotics were used at the following final concentrations for plasmid selection or maintenance: 100 μg/mL of ampicillin, 12.5 μg/mL of chloramphenicol (Cm), 20 μg/mL of florfenicol or 4 μg/mL of colistin B.

2.2. Plasmid construction

Plasmid pCas9sgRNA- *gfp* was constructed as follows. Plasmid pwtCas9-bacteria (Addgene ID 44250) [19] was chosen as the backbone. A fragment containing the sgRNA cassette was commercially synthesized (GENEWIZ) and cloned into the AflII restriction site of the pwtCas9-bacteria. The sgRNA cassette was designed by fusing tracrRNA and crRNA into a chimeric sgRNA. The transcription of sgRNA is driven by the promoter BBa_J23119 with an unequivocal transcription start site; the sgRNA was designed to target the *gfpmut2* gene. pCas9-ctrl, which does not have the sgRNA cassette, was constructed in the same way as pCas9sgRNA- *gfp* and used as a control plasmid.

Plasmid pGFPmut2 was constructed as follows. The *gfpmut2* gene was polymerase chain reaction (PCR)-amplified from pZA31-sulA-GFP (Addgene ID 78493) [20] with primers *gfp*-f/*gfp*-r and inserted into the two BamHI sites of pZA31-sulA-GFP. The constitutive promoter BBa_J23118 and the ribosome-binding site sequence were added to the 5′ end of the forward primer *gfp*-f, and were inserted in front of the *gfpmut2* coding sequence by PCR amplification.

Plasmid pMCas9-*mcr-1* was constructed as follows. Plasmid pwtCas9-bacteria was selected as the backbone. The fragment containing the sequence of the $oriT_{RP4}$, the sgRNA cassette and the constitutive promoter BBa_J23119 [21] were commercially synthesized (GENEWIZ) and inserted into the AflII and the BglII restriction sites of the pwtCas9-bacteria, yielding pMCas9-*mcr-1*; sgRNA was designed to target *mcr-1*. The fragment containing the sequence of the $oriT_{RP4}$ and the constitutive promoter BBa_J23118 was commercially synthesized (GENEWIZ) and inserted into the AflII and the BglII restriction sites of the pwtCas9-bacteria, yielding pMCas9-ctrl. The non-transmissible plasmid pCas9sgRNA-*mcr-1*

Table 1
Bacterial strains and plasmids used in this study.

Strain or plasmid	Description	Source or reference
Strains		
E. coli strain DH5α	F^-, φ80dlacZΔM15, Δ(lacZYA-argF) U169, deoR, recA1, endA1, hsdR17(rk^-, mk^+), phoA, supE44, λ^-, thi-1, gyrA96, relA1	Laboratory stock
E. coli strain MG1655	F^- lambda$^-$ ilvG$^-$ rfb-50 rph-1	Laboratory stock
E. coli strain S17-1	pro, res^- hsdR17 (rK^- mK^+) $recA^-$ with an integrated RP4-2-Tc:: Mu-Km::Tn7, Tp^r	[27]
Plasmids		
pFSEC-01	*cfr*-harbouring plasmid	[18]
pHNSHP45	*mcr-1*-harbouring plasmid	[4]
pwtCas9-bacteria (addgene, 44250)	Ap^r, derivative of the low-copy vector ColE1 origin harbouring *S. pyogenes* Cas9	[19]
pCas9gRNA-*gfp*	pwtCas9-bacteria derivative with sgRNA targeting *gfp*	This study
pZA31-sulA-GFP	Cm^r, derivative of the low-copy vector p15A origin harbouring GFPmut2	[20]
pMCas9-*mcr-1*	pwtCas9-bacteria derivative with $oriT_{RP4}$, sgRNA targeting *mcr-1*	This study
pMCas9-ctrl	pwtCas9-bacteria derivative with $oriT_{RP4}$	This study
pKD46	Ap^r, oriR101 origin, repA101ts, λ-Red recombination proteins (Exo, Beta and Gam)	[23]
pKD46lacZCm	pKD46 derivative with Cm^r	This study
pMob-cas9	pFSEC-01 derivative with fragment contains the Cas9 expression cassette, the sgRNA targeting *mcr-1*, the ampicillin resistance cassette (bla)	This study
pMob-ctrl	pFSEC-01 derivative with fragment contains the Cas9 expression cassette, the ampicillin resistance cassette (bla)	This study
pCas9sgRNA-*mcr-1*	pwtCas9-bacteria derivative with sgRNA targeting *mcr-1*	This study

E. coli, *Escherichia coli*; *Streptococcus pyogenes*; Ap^r, ampicillin-resistant; Cm^r, chloramphenicol-resistant.

and pCas9sgRNA-ctrl which do not contain the oriT$_{RP4}$ fragment were constructed in the same way as pMCas9-*mcr-1* and pMCas9-ctrl.

2.3. Homologous recombination-based modification of pFSEC-01

To construct the conjugative plasmid pMob-Cas9, the sequence between two IS26 regions of the plasmid pFSEC-01 was substituted with the CRISPR/Cas9-encoding cassette by two-step Red-mediated recombination [22]. First, a linear donor DNA cassette was constructed, carrying the upstream part of the target sequence, the Cas9 expression cassette, the sgRNA cassette, the ampicillin resistance cassette (bla), and the downstream part of the target sequence. DNA fragments corresponding to the upstream region and the downstream region of the target sequence were amplified from pFSEC-01 using the primer pairs TR-up-f/TR-up-r and TR-down-f/TR-down-r, respectively. DNA fragments of the ColE1 origin, DNA fragments of the ampicillin resistance cassette (bla) and the sgRNA cassette were amplified from pMCas9-*mcr-1* using primers Col-f/Col-r and Cbs-f/Cbs-r, respectively. DNA fragments of the Cas9 expression cassette were amplified by PCR from pMCas9-*mcr-1* using primers Cas-f/Cas-r. All five fragments were assembled with GeneArt Seamless Cloning and Assembly Enzyme Mix (Life Technologies, Waltham, MA, USA). The final vector (Fig. S1A, see online supplementary material) was transformed into *E. coli*, and the plasmids were prepared and then treated with restriction enzyme ClaI. A 7576-bp linear DNA fragment was purified using the TIANgel Midi Purification Kit, and finally confirmed by sequencing (Fig. S1B, see online supplementary material).

To facilitate identification of the recombinant clones, the plasmid pKD46 [23] was modified, generating pKD46lacZCm. DNA fragments of the LacZ cassette, the chloramphenicol resistance marker (Cat) and the pKD46 backbone were generated using PCR from pUC19, pdCas9-bacteria and pKD46 using primers LacZ-f/LacZ-r, Cat-f/Cat-r and pKD46-f/pKD46-r, respectively. All three fragments were assembled with GeneArt Seamless Cloning and Assembly Enzyme Mix (Life Technologies). The resulting vector was named pKD46lacZCm (Fig. S2, see online supplementary material). For preparation of electrocompetent cells, the plasmid pFSEC-01 was transformed into *E. coli* DH5α cells harbouring pKD46lacZCm. The transformants which contained pKD46lacZCm and the plasmid pFSEC-01 were selected by blue–white screening on LB + X-Gal agar plates with 12.5 μg/mL of chloramphenicol. The competent *E. coli* DH5α cells with pKD46lacZCm were prepared by a method described previously [24] and used for transformation. Fifty nanograms of purified linear donor DNA was electroporated into 100-μL cells in a prechilled electroporation cuvette (0.1 cm) using BTX ECM 399 set at 2.5 kV and 25 μF. Cells that contained the correct recombinants were verified by PCR using primers MT-f/MT-r. A map of the resulting plasmid, pMob-Cas9, is shown in Fig. 3B. pMob-ctrl, which does not have the sgRNA cassette, was constructed in the same way as pMob-Cas9 and used as a control plasmid. All oligonucleotides used for plasmid construction are listed in Table 2.

2.4. Conjugation

The conjugation procedure was carried out as follows. Overnight cultures of the donor and recipient strains grown separately in LB medium with appropriate antibiotics were subcultured 1:100 and grew to an OD_{600} of 0.4 at 37°C. Cells were harvested by centrifugation at 3000 × g for 10 min, washed twice and then resuspended in prewarmed (37°C) fresh sterile LB broth without antibiotics. Equal volumes of the donor culture and the recipient culture were mixed, then incubated at 37°C with gentle shaking on a rotary shaker (100 rpm) to allow for conjugation. After a specific incubation time, samples were collected, vortexed for 10 s to stop conjugation, and then dilutions of the mixtures were spread on three different LB agar plates which contained ampicillin, colistin B, and both ampicillin and colistin B to select for donor, recipient and transconjugant clones, respectively. The plates were incubated overnight at 37°C. Colonies grown on the plates were enumerated and the colony-forming units (CFU) were calculated. Conjugation efficiency was calculated by dividing the number of transconjugant cells by the number of recipient cells.

2.5. Plasmid transformation assays

For the plasmid elimination assay, the competent *E. coli* MG1655 containing pGFPmut2 was prepared using the protocol of Chung et al. [25]. Plasmid pCas9sgRNA-*gfp* or pCas9-ctrl was mixed with a 0.1-mL aliquot of cells, the cells were incubated on ice for 20 min, and subsequently heat shocked at 42°C for 30 s. Following transformation, 0.9 mL of LB broth was added, and the cells were incubated at 37°C with shaking at 220 rpm for 1 h. Serial dilutions of cells were spread on LB agar plates with 100 μg/mL of ampicillin, and the plates were incubated overnight at 37°C to select for transformants. Transformation efficiencies (expressed as CFU per μg of DNA transformed) were calculated. Each experiment was repeated three times.

For the plasmid transformation blocking assay, electrocompetent *E. coli* MG1655 containing pCas9sgRNA-*mcr-1* or pCas9sgRNA-ctrl was prepared according to the protocol of Nováková et al. [26]. A 0.1-mL aliquot of electrocompetent cells was mixed with 1 μL (50 ng) of pHNSHP45 and chilled on ice for 5 min, and was then electroporated at 12.5 kV/cm, 25 μF and 300 Ω. Following transformation and recovery, serial dilutions of cells were spread on LB agar plates containing 4 μg/mL of colistin B and incubated overnight at 37°C to select for transformants. Transformation efficiencies (expressed as the number of CFU per μg of DNA transformed) were calculated. Each experiment was repeated three times.

3. Results

3.1. Expression of Cas9 and the specific sgRNA results in plasmid elimination

To test the utility of the CRISPR/Cas9 system in mediating plasmid elimination from *E. coli*, the vector pCas9sgRNA-*gfp* (Fig. S3, see online supplementary material) was generated to express Cas9 with sgRNAs programmed to target the *gfp* gene on the plasmid pGFP*mut2*, which is a p15A-derived vector containing a chloramphenicol resistance marker. When Cas9 and sgRNA are expressed in bacteria, Cas9 forms a complex with sgRNAs and then binds to the *gfp* gene by base-pairing, and results in digestion of the *gfp*-harbouring plasmids (Fig. 1A). The vector pCas9sgRNA-*gfp* and the control vector pCas9-ctrl were transformed into the competent *E. coli* strain MG1655 which contains pGFP*mut2*, and then transformants were selected and cultured in LB with 10 ng/mL of anhydrotetracycline (aTc) at 37°C for 12 h. Fluorescence was measured using a Tecan Microplate reader to determine whether the transformed CRISPR/Cas system can specifically eliminate pGFP*mut2* from the recipient strain. The results showed that the fluorescence intensity of the cells transformed with pCas9sgRNA-*gfp* was reduced approximately 240-fold (39372 AU vs 164 AU, $P < 0.001$) compared with that of the control cells transformed with pCas9-ctrl (Fig. 1B). By microscopy, there was no green fluorescence in the cells transformed with pCas9sgRNA-*gfp*, whereas the cells transformed with pCas9-ctrl showed normal fluorescence, same as the untransformed strains (Fig. 1C). Next, the presence of pCas9sgRNA-*gfp* in the observed colonies on agar plates

Table 2
Oligonucleotides used in this study.

Oligonucleotide	Sequences (5′-3′)
gfp-f	TTGACAGCTAGCTCAGTCCTAGGTATAATGCTAGCCGCGGATCCAAAGAGGAGAAAATGAGTAAAGGAGAAGAAC
gfp-r	GCGGGATCCTTATTTGTATAGTTCATCCATG
TR-up-f	GCCGAATTCGTAGTCTACAATTATAGAGGTCGTATAAATCGCTTAGAGTATTC
TR-up-r	TTATAATATTGTCTGTCATAGGTTTCTAAAGCTG
TR-down-f	GTAAGTTAATGAAATATATACTTGAAGATGTTTATGGCATTC
TR-down-r	ATCGATTTGGCGGCCGCCATGATCGCATTCAATCACGTTGTTC
Cbs-f	AGTTCTGAGGTCATTACTGGAT
Cbs-r	AACTTAAATGTGAAAGTGGGTCTTAA
Cas-f	ATGGAGTTCTGAGGTCATTATAATGCGCTGTTAATCACTTTACTTTTATCTAAT
Cas-r	TTGTAGACTACGAATTCGGCTCACATGTTCTTTCCTGCGTTAT
Cat-f	CAGACCAAAACGATCTCAAGAAG
Cat-r	GATGTAGCCGTCAAGTTGTCATAATAAATCGATGCAGGTGGCACTTTTCGGGGAAATGTGCTTGAATTGATCATATGCGGATTAG
LacZ-f	AGATTATCAAAAAGGATCTATCAGGGTTATTGTCTCATGAGC
LacZ-r	CTTCTTGAGATCGTTTTGGTCTGCAGCGAGTCAGTGAGCGA
pKD46-f	TGCCACCTGCATCGATTTATTATGAC
pKD46-r	CATGACCAAAATCCCTTAACGTGAG
MT-f	AGAAGTACAGACAGGCGGATTCTC
MT-r	CTGTTACGACGGGAGGAGAGATA
sgRNA (*mcr*-1)	ACGCTCGGTCAGTCCGTTTG
sgRNA (*gfp*)	CATCTAATTCAACAAGAATT

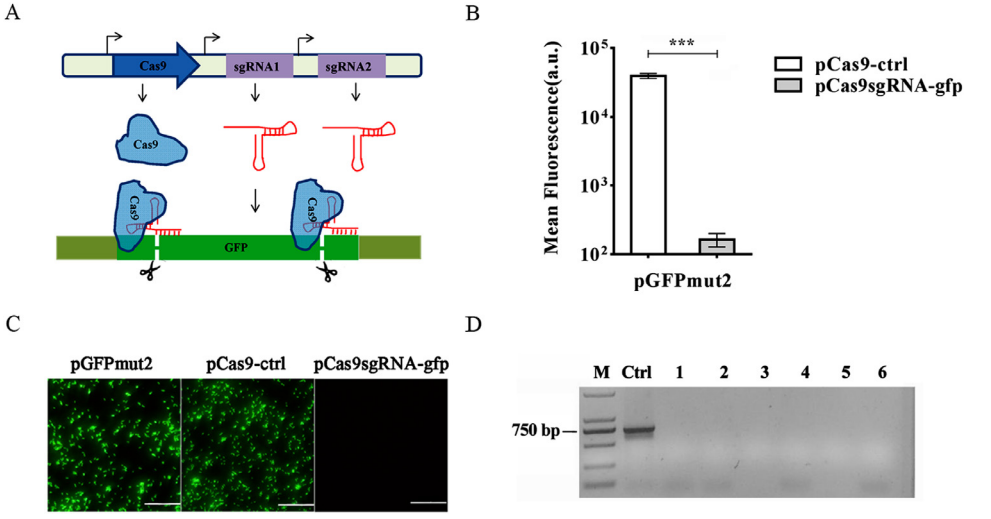

Fig. 1. CRISPR/Cas9-mediated plasmid elimination in *Escherichia coli*. (A) Cartoon representation of CRISPR/Cas9-mediated gene destruction. Gene destruction is achieved when the Cas9 protein forms a complex with the sgRNA chimera; it binds to the specific coding region of the *gfp* gene and destructs the target gene. (B) Reduction of green fluorescence is seen only in the pGFPmut2 cells transformed with pCas9sgRNA-*gfp*, not the cells that transformed with pCas9-ctrl. Error bars represent fluorescence results from at least three biological replicates (mean ± standard error of the mean). (C) Microscopic images of GFP expression in cells. Green fluorescence was not observed in the cells transformed with pCas9sgRNA-*gfp*. pCas9-ctrl is the report strain which contains pGFPmut2 and pCas9-ctrl. PGFPmut2 is the strain which contains pGFPmut2. Scale bar, 10 μm. (D) Plasmid clearance was determined by polymerase chain reaction amplification with *gfp*-specific primers. Ctrl is a GFP report strain transformed with pCas9-ctrl; 1–6 are six separate strains transformed with pCas9sgRNA-*gfp*.

containing 10 ng/mL of aTc was verified by colony PCR using the primers *gfp*-f/*gfp*-r. After transformation, six clones selected at random were chosen for colony PCR. As shown in Fig. 1D, a 750-bp band representing the existence of the *gfpmut2* gene could only be amplified from bacterial clones transformed with pCas9-ctrl, and not those transformed with pCas9sgRNA-*gfp*. Taken together, these data demonstrate that the activity of the CRISPR/Cas system can remove plasmids from *E. coli* cells.

3.2. Elimination of plasmids using the CRISPR/Cas system delivered by E. coli strain S17-1

Bacterial conjugation is the main mechanism of HGT that is used by various bacteria to obtain foreign DNA. This process requires direct cell-to-cell contact and formation of a channel between the cells. Both *E. coli* strains S17-1 and SM10 contain chromosomally integrated genes from the natural conjugative plasmid RP4, and are always used as donor strains. Bacterial conjugation mediated by plasmid RP4 requires the origin of transfer ($oriT_{RP4}$) on the plasmid to be transferred, while the other proteins necessary for conjugation/mobilization are provided in trans from RP4 integrated into the chromosome of *E. coli* S17-1 or SM10 [27]. To test the possibility that the CRISPR/Cas9 system can mediate plasmid elimination by conjugation, a set of conjugative CRISPR/Cas plasmids was constructed for coexpression of gene-specific sgRNA with *S. pyogenes* Cas9, so the plasmid pMCas9-*mcr-1*, which contains $oriT_{RP4}$, the *mcr-1*-specific sgRNA cassette and the Cas9 expression cassette were generated (Fig. S4, see online supplementary material). Plasmid pMCas9-ctrl, which does not have the sgRNA cassette, was also constructed and used as a control plasmid. All of these plasmids were transformed into competent S17-1 *E. coli* cells. The transformants grown on selective medium were used as donor cells. The *E. coli* strain MG1655, which contains the *mcr-1*-harbouring plasmid pHNSHP45, was used as recipient cells. When bacterial conjugation occurs, mobile plasmid pMCas9-*mcr-1* was transferred into recipient cells to eliminate the plasmid car-

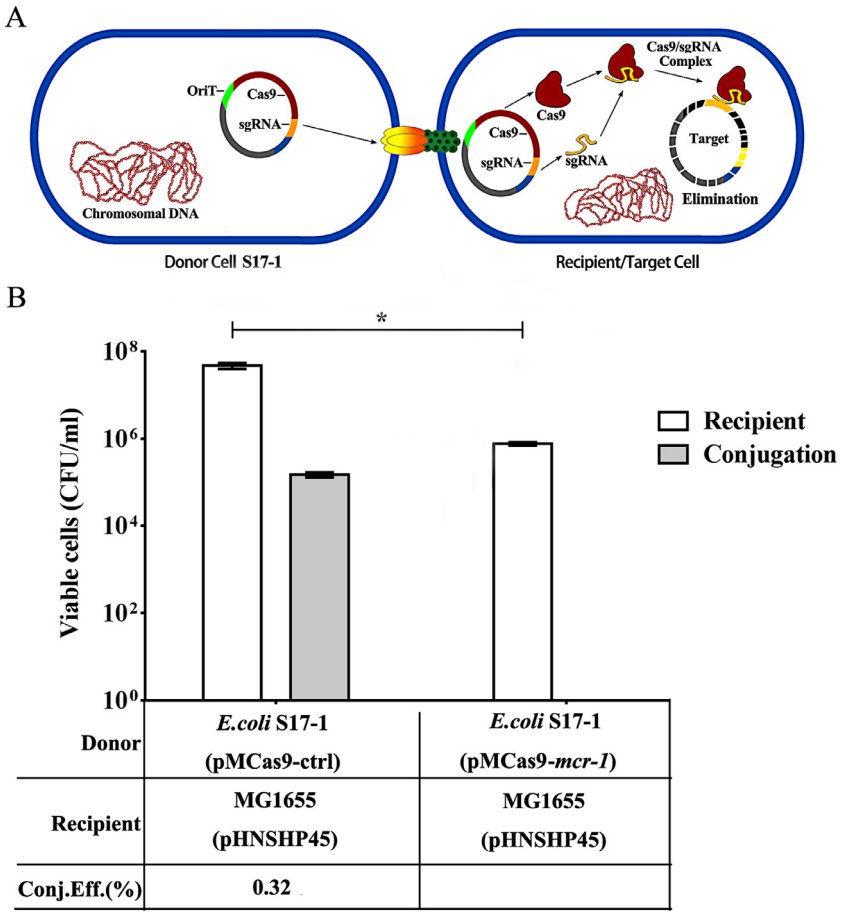

Fig. 2. CRISPR/Cas9 constructs can be delivered into target bacteria by S17-1 for curing plasmid harbouring target sequences. (A) Cartoon representation of the conjugative CRISPR/Cas plasmid-mediated plasmid curing. The plasmid harbouring the oriTRP4 and CRISPR/Cas constructs can be delivered into recipient cells by *Escherichia coli* S17-1; the plasmid harbouring the target sequence in the recipient cell was destroyed by the Cas9/sgRNA complex. (B) Conjugated pMCas9-*mcr-1* cause specific clearance of plasmid pHNSHP45. *E. coli* S17-1 donor cells containing pMCas9-*mcr-1* or pMcas9-ctrl were mated at a donor:recipient ratio of 1:1 for 8 h with *E. coli* MG1655 recipient cells that contain pHNSHP45. Cultures were plated on Luria-Bertani (LB) + colistin B to select for surviving recipient cells, and LB + colistin B + ampicillin to select for transconjugants (ampicillin resistance is encoded by pMCas9-*mcr-1*). The colony-forming units (CFU) per millilitre values of recipient strains and transconjugants were calculated. Conjugation efficiency (Conj. Eff.) was calculated by counting the number of transconjugant cells divided by the number of recipient cells. Error bars indicate mean ± standard error of the mean of three independent experiments.

rying *mcr-1*. After 8 h of coculture of donor bacteria and the recipient bacteria, transconjugant cells and recipient cells were selected, respectively, on plates containing specific antibiotics. The results indicated that conjugative transfer of pMCas9-*mcr-1* to the corresponding recipient cells resulted in a 62-fold (4.75 + E07 vs 7.7 + E05 CFU/mL; $P < 0.05$) reduction in the number of survival recipient cells compared with the control plasmid pMCas9-ctrl (Fig. 2B). The conjugation efficiency was estimated at 0.32% after 8 h of coculture of donor bacteria containing the control plasmid pMCas9-ctrl and the recipient bacteria (Table S1, see online supplementary material). These results suggest that the CRISPR/Cas9 system mediated a double-strand break of the antibiotic resistance gene, which induced elimination of the whole plasmid in the recipient *E. coli* cells.

3.3. Elimination of plasmids from E. coli by the CRISPR/Cas system delivered by the host-independent conjugation

Although pMCas9-*mcr-1* can eliminate the plasmids from the recipient cells, the transferability of the vector that contains the CRISPR/Cas9-encoding cassette depends on the performance of the particular strain as a donor. To circumvent this restriction, a host-independent conjugative plasmid pMob-Cas9 was developed (Fig. 3B), which contains the constructs encoding all proteins necessary for conjugation and the CRISPR/Cas9-encoding cassette. When this host-independent CRISPR/Cas9 plasmid is transformed into any *E. coli* strain, that strain can act as a donor. Therefore, the conjugative plasmid can be transferred continuously in the microbiota and eliminate the corresponding antibiotic resistance plasmids from recipient cells (Fig. 3A). Plasmid pMob-ctrl, which does not have the sgRNA cassette, was also constructed and used as a control plasmid. pMob-Cas9 and pMob-ctrl were transformed into competent MG1655 *E. coli* cells. The transformants were grown on selective medium and used as donor cells. The *E. coli* strain SHP45, which contains the *mcr*-1-harbouring plasmid pHNSHP45 [4], was used as recipient cells. For the conjugation assay, the donor bacteria and the recipient bacteria were mixed at a ratio of 1:1, and were incubated at 37°C for 2, 4, 6, 8 and 10 h to allow for conjugation. The cocultures were plated on solid LB medium containing colistin B (4 μg/mL), or containing both ampicillin (100 μg/mL) and colistin B (4 μg/mL) to select the recipients and transconjugants (recipient strain with the conjugative plasmid), respectively. As shown in Fig. 3C, the conjugative transfer of pMob-Cas9 to the recipient cell resulted in a 101-fold reduction (5.81 + E07 vs 5.75 + E05 CFU/mL; $P < 0.05$) in the number of survival recipient cells compared with control plasmid pMob-ctrl. Conjugation efficiency was estimated as above described at 1.43% after 8 h of coculture (Table S2, see online supplementary material).

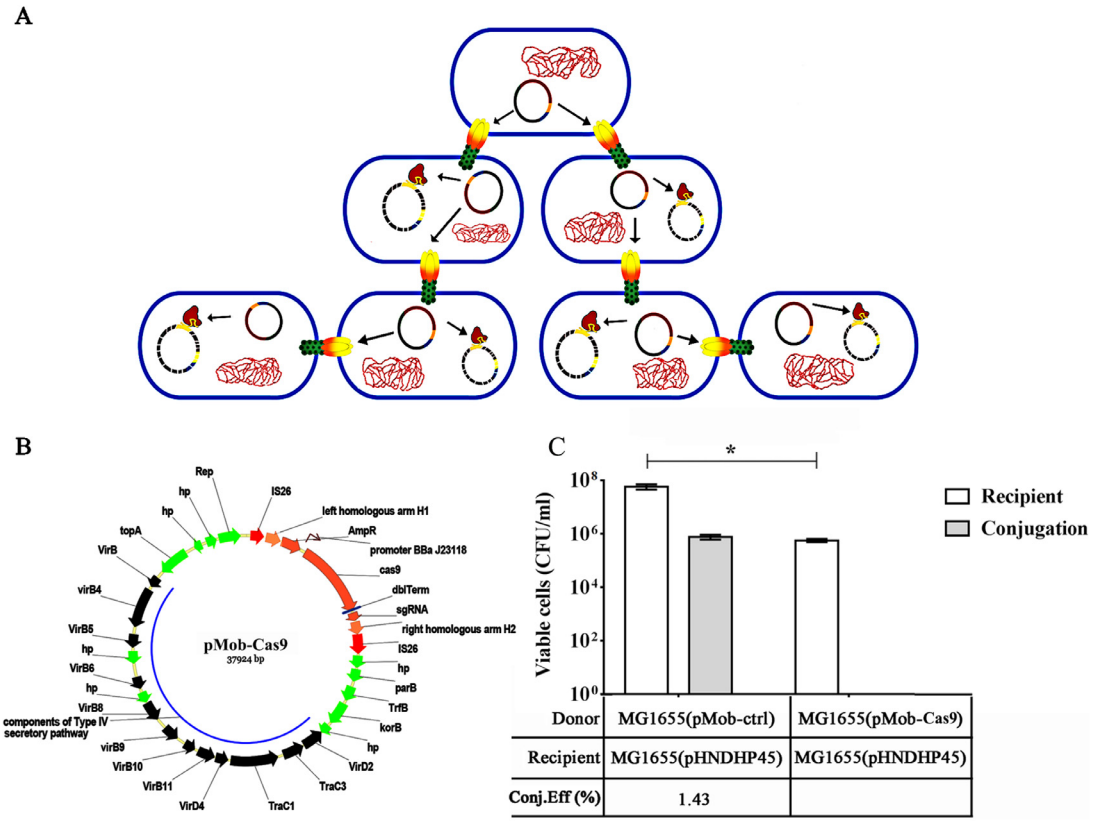

Fig. 3. CRISPR/Cas constructs are delivered into target bacteria by the host-independent conjugation for curing plasmid harbouring target sequences. (A) Cartoon representation of host-independent conjugative plasmid-mediated plasmid curing. The host-independent conjugative plasmid harbouring CRISPR/Cas constructs can be delivered into recipient cells which contain target plasmids, and the recipient strains accepting the plasmid can work as donors and then consecutively disseminate plasmids in microflora. (B) Circular representation of the host-independent conjugative plasmid pMob-Cas9. Genes are colour coded, depending on functional annotations, as follows: red, transposition; black, type IV secretory components; green, transposition/recombination/replication and other putative functions/hypothetical proteins; and yellow, homologous arms for recombination, the ampicillin resistance cassette and CRISPR/Cas construct. (C) The host-independent conjugative plasmid causes specific clearance of plasmid pHNSHP45. *Escherichia coli* MG1655 donor cells containing pMob-cas9 or pMob-ctrl were mated at a donor:recipient ratio of 1:1 for 8 h with *E. coli* MG1655 recipient cells that contain pHNSHP45. Cultures were plated on Luria-Bertani (LB) + colistin B to select for surviving recipient cells or LB broth + colistin B + ampicillin to select for transconjugants (ampicillin resistance is encoded by the host-independent conjugative plasmid). The colony-forming units (CFU) per millilitre values of recipient strains and transconjugants were calculated. Conjugation efficiency (Conj. Eff.) was calculated by counting the number of transconjugant cells divided by the number of recipient cells. Error bars indicate mean ± standard error of the mean of three independent experiments.

3.4. The engineered CRISPR/Cas system in cells blocks conjugation and transformation

The engineered CRISPR/Cas system can destroy plasmids in *E. coli* by transformation and conjugation, which are the two main mechanisms of HGT used by bacteria to acquire antibiotic resistance genes. This suggests that the engineered CRISPR/Cas system that exists in the host can also prevent plasmid DNA transformation and conjugation (Fig. 4A). To test whether the engineered CRISPR/Cas system in the *E. coli* strain MG1655 can prevent plasmid conjugation, a non-transmissible plasmid pCas9sgRNA-*mcr*-1, which contains sgRNA designed to target *mcr-1*, was constructed and transformed into *E. coli* strain MG1655, and used as a recipient for conjugation. The *E. coli* strain MG1655 containing the plasmid pCas9sgRNA-ctrl was used as a control. The *E. coli* strain MG1655, harbouring the conjugative plasmid pHNSHP45, was used as a donor. In conjugation experiments, under selection for transconjugants, the engineered CRISPR/Cas system in the recipient resulted in an 88-fold reduction (5.29 + E07 vs 6.0 + E05 CFU/mL; $P < 0.05$) in the number of target recipient cells compared with the control cells lacking the target sgRNA. The results indicated that the *E. coli* strain MG1655, which harbours an engineered CRISPR/Cas system, limited the HGT of the conjugative plasmid pHNSHP45 (Fig. 4B). To test whether the engineered CRISPR/Cas system in host cells can prevent plasmid transformation, plasmid pHNSHP45 was transformed into the competent MG1655 *E. coli* strain harbouring either pCas9sgRNA-*mcr*-1 or pCas9sgRNA-ctrl plasmids. The result showed that the presence of the engineered CRISPR/Cas system led to a 1076-fold (2.69 + E05 DNA vs 2.5 + E02 CFU/μg DNA) reduction in the number of transformants compared with the control cells lacking sgRNA (Fig. 4C). This indicates that the engineered CRISPR/Cas system in cells protects the host from plasmid transformation.

4. Discussion

The transfer of conjugative plasmid-mediated multi-drug resistance is largely responsible for the development of antibiotic-resistant pathogens and causes serious problems in the treatment of infectious diseases. It is widely accepted that conjugation is the most common mechanism of HGT in natural environments and the gastrointestinal tract. Since bacterial conjugation is the critical factor in the dispersion of antibiotic resistance to bacterial pathogens, the authors intended to 'fight fire with fire' (i.e. exploit conjugative plasmids to eliminate drug resistance plasmids in bacteria). The occurrence and dissemination of colistin resistance gene *mcr-1* harboured on plasmid poses a considerable threat to the treatment of clinical infections. Therefore, the prevention of distribution of *mcr-1* and restoration of bacterial sensitivity to polymyxins would be of important scientific significance.

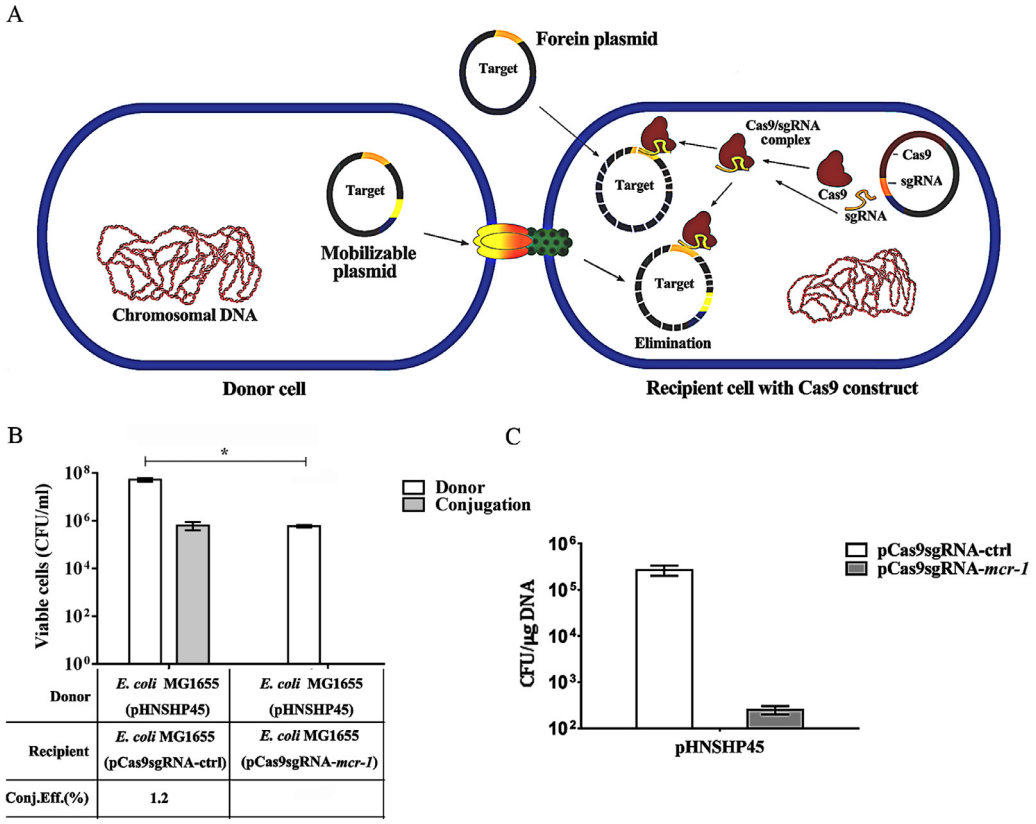

Fig. 4. The engineered CRISPR/Cas system in cells limits conjugation and transformation by targeting DNA. (A) Cartoon representation of CRISPR/Cas9-mediated horizontal gene transfer limitation in bacteria. The engineered CRISPR/Cas constructs in bacteria limit plasmid conjugation and transformation among bacteria. (B) *Escherichia coli* MG1655 donor cells containing conjugative plasmid pHNSHP45 were mated at a donor:recipient ratio of 1:1 for 8 h with *E. coli* MG1655 recipient cells that contain the non-conjugative plasmid pCas9sgRNA-*mcr-1* or pCas9sgRNA-ctrl. After conjugation, cells were plated on Luria-Bertani (LB) + colistin B to select for surviving donor cells or LB + colistin B + ampicillin to select for transconjugants (ampicillin resistance is conferred by plasmid pCas9sgRNA-*mcr-1* or pCas9sgRNA-ctrl). The colony-forming units (CFU) per millilitre values of recipient strains and transconjugants were calculated. Conjugation efficiency (Conj. Eff.) was calculated by counting the number of transconjugant cells divided by the number of recipient cells. (C) Plasmid pHNSHP45 was transformed into competent *E. coli* MG1655 containing either pCas9sgRNA-*mcr-1* or pCas9sgRNA-ctrl plasmids. Transformants were enumerated on LB + colistin B + ampicillin medium to calculate transformation efficiencies which are represented as CFU per microgram (μg) DNA. Error bars indicate mean ± standard error of the mean of three independent experiments.

This study presents a strategy that makes use of bacterial conjugation to deliver a programmable DNA nuclease, CRISPR/Cas9, into the antibiotic-resistant pathogen to eliminate plasmid harbouring the colistin resistance gene *mcr-1*, and obstruct the transfer of resistance between strains. The results demonstrate that the *mcr-1*-harbouring plasmids in the recipient bacteria can be eliminated, and the recipient bacteria can be sensitized to antibiotics following conjugative delivery of the CRISPR/Cas system targeting *mcr-1* from donor *E. coli* strain S17-1 (Fig. 2A). The conjugative plasmid pFSEC-01 isolated from the porcine *E. coli* strain FSEC-01 was modified by replacing the sequence between two IS26 elements of the plasmid pFSEC-01 with the CRISPR/Cas-encoding cassette. The type IV secretion system gene cluster, which plays an important role in the process of bacterial conjugation, was retained [28]. Conjugation experiments by co-cultivation of the *E. coli* strain MG1655:pMob-Cas9 with the *E. coli* strain SHP45 revealed that the plasmids carrying the CRISPR/Cas-encoding cassette were successfully transferred to the recipient *E. coli* strain, and the pHNSHP45 plasmids were eliminated successfully.

As the type II CRISPR system from *S. pyogenes* has been adapted to be a powerful gene-editing tool, it can work in cells in a host-independent way, and the Cas9 nuclease can be programmed to target any DNA sequence with the appropriately matched motif [29]. Therefore, in theory, this strategy could be utilized to eliminate any antibiotic resistance plasmid in bacteria, thus making this strategy more applicable. As the target specificity of Cas9 nuclease relies on sgRNAs, the conjugative plasmid may be expanded to include multiple sgRNA cassettes, which can be designed to target different positions within the same gene or target different genes. Targeting several positions of an antibiotic resistance gene on a single construct reduces the probability of a plasmid escaping elimination due to spontaneous mutations in the sgRNA target recognition sequence. Multiple sgRNA cassettes engineered in the conjugative plasmid could be used to target different drug resistance genes, thereby extending the range of targets and further reducing the dissemination of antibiotic resistance. With simple modifications, the application scope of this strategy can be further extended. For example, sgRNAs may be designed to specifically target antibiotic resistance genes on a chromosome rather than those on mobile plasmids; in such cases, the transferred CRISPR/Cas9 will kill the pathogen [30,31]. Furthermore, due to the modularity, accuracy and simplicity of CRISPR/Cas engineering, libraries of multiplexed conjugative CRISPR/Cas-harbouring plasmids targeting various antibiotic resistance genes could be constructed rapidly and used as a cocktail therapy.

To the authors' knowledge, this is the first report on delivery of engineered CRISPR/Cas9 by a host-independent conjugative plasmid into bacteria via conjugation to remove or block the spread of *mcr-1*-carrying plasmids. It provides an example of a means to counteract the worsening dissemination of colistin resistance among bacterial pathogens. Towards practical implementation and use of this system, the selective marker gene on the plasmid must

be removed to avoid spread of the antibiotic-resistant trait of the plasmid. Future research will be focusing on screening natural broad-host-range conjugative plasmids from bacteria, and developing vectors with high transconjugation efficiency. As bacterial conjugation is a universal, naturally occurring process among bacteria, it is envisaged that this approach could be optimized for therapeutic application, or for removing antibiotic resistance genes from the resistance reservoir in the human microbial flora and from bacteria in natural settings, such as wastewater systems, agriculture and fish factories, farms and hospitals.

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Competing interests

None declared.

Ethical approval

Not required.

Supplementary material

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ijantimicag.2018.09.017.

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