Development of CRISPR Antimicrobial for Specific Antibiotic Resistant Gene Targetingin *Acinetobacter baumannii*

การพัฒนาระบบ CRISPR ต้านจุลชีพแบบจำเพาะต่อยีนดื้อยาปฏิชีวนะในเชื้อ *Acinetobacter baumannii*

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ABSTRACT

Multi-drugs resistant *Acinetobecter baumannii* infection in hospitals is a serious public health problem. Previous works presented CRISPR/Cas systems for target elimination of antibiotics resistant bacteria including *E. coli, S. aureus, Z. mobilis* and *S. enterica*. We first demonstrated, in *E.coli*, CRISPR antimicrobials for specifically eliminating a plasmid encoding a fragment of New Delhi metallo-beta-lactamase 1 (NDM1) gene from clinically isolated *A. baumannii*. Then, we developed a conjugation system for delivering CRISPR antimicrobials from *E.coli* to *A. baumannii*. The number of viable *A. baumannii* after mating with *E. coli* donors decreased by 90% compared to control experiment. Future work should include the study of CRISPR/Cas activity and consequence of CRISPR antimicrobial induced DNA break in *A. baumannii*.

บทคัดย่อ

ปัจจุบันมีรายงานว่า *Acinetobecter baumannii* ซึ่งจัดอยู่กลุ่มแบคทีเรียดื้อยาปฏิชีวนะหลายชนิดเป็นจุลินทรีย์ก่อโรคที่เป็นปัญหาทางสาธารณสุข ที่ไม่สามารถใช้ยาปฏิชีวนะทั่วไปในโรงพยาบาลได้ ผู้วิจัยจึงสนใจนำระบบ CRISPR/Cas ที่สามารถตัดทำลายดีเอ็นเอเป้าหมายได้อย่างจำเพาะมาประยุกต์ใช้กำจัด *A. baumannii* ซึ่งงานวิจัยก่อนหน้าใช้ระบบ CRISPR/Cas กำจัดแบคทีเรียอย่างจำเพาะใน *E. coli, S. aureus, Z. mobilis* และ *S. enterica* ดังนั้นผู้วิจัยจึงนำส่งระบบ CRISPR/Cas ด้วยวิธี conjugation เพื่อกำจัดพลาสมิดที่มียีน NDM1 ใน *E.coli* จากนั้นนำส่งระบบ CRISPR/Cas ไปยัง *A. baumannii* ที่มียีน NDM1 พบว่าสามารถพบอัตราการรอดชีวิตของ *A. baumannii* ลดลง 90% เทียบกับชุดควบคุม ในอนาคตควรมีการศึกษาประสิทธิภาพของระบบ CRISPR/Cas และศึกษาผลกระทบจากการตัดดีเอ็นเอใน *A. baumannii*

Keywords: CRISPR antimicrobial, Conjugation, Antibiotics resistance

คำสำคัญ: การต้านจุลชีพด้วย CRISPR/cas การส่งถ่ายดีเอ็นเอระหว่างแบคทีเรีย การดื้อยาปฏิชีวนะ

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Introduction

CRISPR antimicrobial offers a new possibility for sequence specific elimination of targeted bacteria. The CRISPR/cas antimicrobial consists of two components: cas endonuclease that cuts DNA and guide RNA (gRNA) that specifies DNA target site for cas endonuclease. Cas and gRNA complex can specifically cut target DNA site on genome or plasmid, leading to cell death or plasmid loss. Two key advantages of CRISPR/cas antimicrobials over conventional antibiotics are specificity and programmability. Due to specificity of gRNA, CRISPR antimicrobials can eliminate targeted bacteria or plasmids in population without disrupting other non-targeted bacteria. Moreover, CRISPR antimicrobials can be re-designed for different DNA target sequences simply by changing a spacer region of a gRNA gene. Previous works showed that CRISPR antimicrobials could be transferred and specifically eliminates a targeted *Escherichia coli* (Citorik et al 2014; Cui et al 2016; Dong et al 2019), *Staphylococcus aureus* (Bikard et al 2014), *Zymomonas mobilis* (Cao et al 2017) and *Salmonella enterica* (Hamilton et al 2019).

In 2019, the US alone has over 2.8 million patients and over 35000 deaths due to antibiotic resistant bacteria infection. *Acinetobacter baumannii* is an importance pathogenic bacteria that is commonly found in the environment, in soil and water (CDC, 2019). *A. baumannii* can cause infections in the blood, urinary tract, and lungs (pneumonia), or in wounds in other parts of the body (Sebeny et al 2008). In 2017, carbapenem-resistant Acinetobacter caused approximately 8,500 infections in hospitalized patients and 700 deaths in the United States (AR Threats Report, 2019). While *A. baumannii* infection could be treated with antibiotics, a growing number of hospital isolated strains of *A. baumannii* were classified as multi-drug resistance (MDR) bacteria (Abbo et al 2005). Thus, there is a need for developing a new strategy for combating *A.baumannii* infection.

Here, we applied CRISPR antimicrobials for targeted elimination of antibiotic resistant *A. baumannii*. We delivered CRISPR antimicrobials via a conjugative mobilizable plasmid from *E.coli* donors to *A.baumannii* recipients. For our demonstrated system, CRISPR antimicrobial targeting New Delhi metallo-beta-lactamase 1 (NDM1) can reduced survivability of *A. baumannii* recipient by 90%. We confirmed that surviving clones still had NDM1 targets but point mutations or deletion base were detected in some clones. This project opened a possibility for practical uses of CRISPR antimicrobials in eliminating pathogenic bacterial populations via conjugation.

Objectives of the study

To test the possibility of applying CRISPR antimicrobials against antibiotics resistant gene in *A. baumannii.*

Methodology

Bacteria strain, plasmid and culture condition

Donor host cells in all experiments were *E.coli* DH5a (New England Biolab, USA). Recipient host cells in all experiments were E.coli SAR08 provided by Dr. Ellen Zechner, University of Graz, Austria (Reisner et al 2012). *A. baumannii* AB166 and AB377 were provided by Dr. Sutthirat Sitthisak, Naresuan University, Thailand. pHELP is the same as plasmid pRL443 provided by C. Peter Wolk, Michigan State University, USA (Elhai et al 1997). pgNDM1 is the same as plasmid pMM441 (Addgene #61271) provided by Dr. Timothy K. Lu, MIT, USA (Citorik et al 2014). pTarget_crtl is the same as pHH100 provided by Rahmi Lale, NTNU, Norway. pTarget_NDM1 was modified from pHH100 by inserting a NDM1 fragment between XbaI and PstI restriction sites. The NDM1 fragment was amplified from *A. baumannii* AB377 using primer ts37 (5' GGCCGCTCTAGAGACCAACGGTTTGGCGATCTG 3') and ts38 (5' GAATTCCTGCAGCCAAAGTTGGGCGCGGTTG 3'). Both E. coli and A. baumannii were grown in Luria-Bertani (LB) broth or agar plates (LB with 15 g agar/L) at 37°C. Where indicated, antibiotics were added to the selection medium to the following final concentrations: 25 ug/ml of chloramphenicol (Cm) 25 ug/ml of kanamycin (Km) and 10 ug/ml of tetracycline (Tc).

Transformation

pHELP, pgNDM1, pTarget_crtl and pTarget_NDM1 were transformed to *E.coli* donors and recipients by TSS transformation (Chung et al 1989), respectively. A single colony was regrown into 2 ml LB broth and incubated at 200 rpm 37°C overnight. 100 ul of overnight cultures was inoculated into 10 ml LB broth and incubated to exponential phase (OD600=0.3-0.7). Bacteria cells were put on ice for 10 min. Cells were collected by centrifugation at 5000 rpm 5 min 4°C. Supernatant was discarded. Cell pellet was resuspended gently in 1ml TSS buffer (5g PEG 8000, 1.5 mL 1M $MgCl_2$, 2.5 ml DMSO and adjust to 50 ml by LB; pH 6.5). The 1 ul of plasmid was mixed into 50 ul competent cells and incubated on ice for 30 min. Competent cells with plasmid were heat-shocked at 42°C for 45 s and immediately incubated on ice for 2 min. The 450 ul of fresh LB were added into cells and cells were incubated at 37°C for 1 h. Cells were plated on LB agar with antibiotics for selecting transformants.

Conjugation assay

Donor and recipient were each inoculated from a single colony into LB broth with antibiotics and incubated overnight in a 37°C 200 rpm shaker. Overnight cultures were regrown at 1% dilution fold in fresh LB broth with appropriate antibiotics until OD600 reached 0.3-0.7. Cells were spun down at 5000 g for 5 min and resuspended with 1 ml of LB broth three times in order to wash off residue antibiotics. The volumes of LB broth used in the final resuspension were adjusted so that both donor and recipient cells had OD600 ~ 0.3. Donor cells and recipient cells were mixed at 1:1 ratio by volume and 10 ul of mixed cells were dropped on nitrocellulose membrane on LB agar. Then, cells were incubated at 37°C 16 h. Following conjugation, each membrane was placed in a 1.5 tube containing 1 ml of PBS buffer and

vortexed for 10 s. The supernatant was serially diluted and plated on LB agar with appropriate antibiotic selection and incubated overnight at 37°C for 16–24 h. Colonies were counted manually.

Colony PCR and sequencing analysis

A single colony was picked with a sterile pipette tip, resuspended in 10 ul of sterile water and used as a PCR template. The amplifications of a CRISPR and a NDM1 region were carried out using the primer pair ts18 (5' GAAACACGCATTGATTTGAGTCAGC 3') and ts19 (5' GAAGTCGCTTGATATCTAGTATGACGTCTG 3') and NDM F(5' GGTTTGGCGATCTGGTTTTC 3') and NDM R (5' CGGAATGGCTCATCACGATC 3') provided by Dr. Sutthirat Sitthisak, Naresuan University, Thailand. PCR was conducted using a MyTaq™ Red Mix (Meridian Bioscience Asia Pte Ltd, Singapore). PCR was conducted by thermal cycler in a 10 µL volume reaction containing 5 ul of MyTaq Red Mix, 20 uM of each primer and 1 ul of template. The amplification conditions were 10 min of initial denaturation at 95°C, followed by 30 cycles of 95°C for 1 min, 60°C for 15 s, and 72°C for 10 s, and a final extension step of 72°C for 5 min. The 3 µL of PCR product from each reaction was loaded and electrophoresed in a 1% (w/v) agarose gel with 1 ul RedSafe™ Nucleic Acid Staining Solution (iNtRON Biotechnolory, Sound Korea) per 25 ml agarose gel solution. PCR products were purified from agarose gel using using NucleoSpin® Gel and PCR clean-up Kit (MACHEREY-NAGEL GmbH & Co.KG, Germany). The sequencing of purified PCR products was performed by Macrogen (Seoul, Sound Korea) with primer NDM F or NDM R. The sequencing results were analyzed using BLAST (Altschul et al 1990).

Results

Our initial model system for testing CRISPR antimicrobials consisted of three plasmids: pHELP, point mutations or deletion base and pTarget (Fig 1A). point mutations or deletion base was a mobilizable plasmid encoding a R1162 broad-host-range origin of replication/transfer, a chloramphenicol resistant marker, a cas9 endonuclease gene, a tracrRNA gene and a CRISPR cassette. This CRISPR cassette had a spacer target in New Delhi metallo-beta-lactamase 1 (NDM1) gene. pHELP was self-transmissible conjugative plasmid encoding RK2 origin of replication/transfer, a tetracycline resistance gene and a RK2 conjugation machinery. pTarget was a plasmid encoding a RK2 origin of replication/transfer, a kanamycin resistance gene, mCherry gene and a fragment of NDM1 gene taked from clinically isolated multi drug resistant *A. baumannii*. We transferred both pHELP and point mutations or deletion base to *E.coli* DH5a (donors) and transferred pTarget to *E.coli* SAR08 (recipients) by TSS chemical transformation. We tested whether this CRISPR antimicrobial system was functional by conjugating donors (harboring point mutations or deletion base and pHELP) to recipients (harboring pTarget). We expected point mutations or deletion base to be transferred to recipients by conjugation machinery of pHELP. Once in the recipients, a cas9 endonuclease, crRNA and tracrRNA expressed from point mutations or deletion base should cut and eliminated pTarget (Fig 1B)

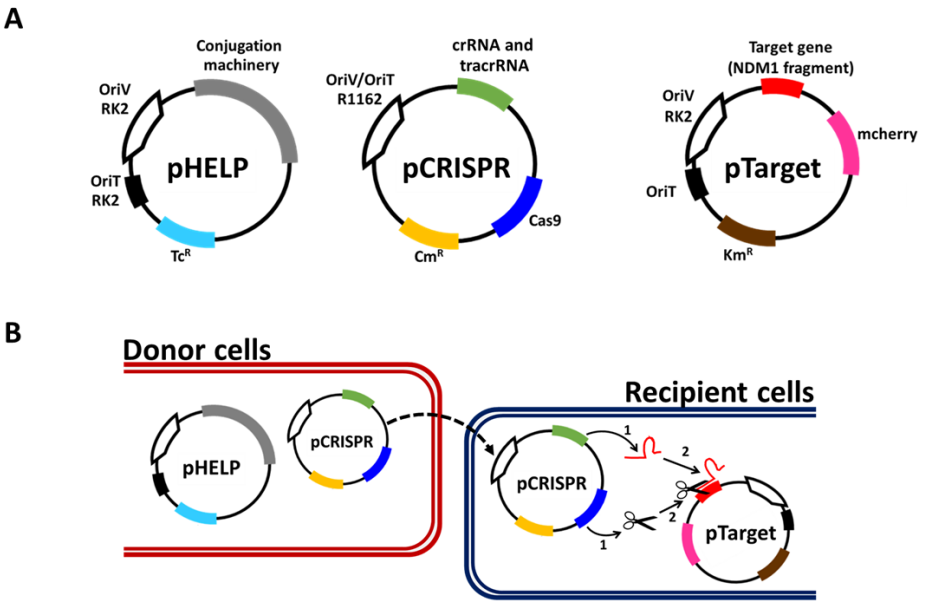

Figure 1 A schematics of CRISPR antimicrobial delivery and elimination of target plasmid. (A) A schematics of RK2-based conjugative helper plasmid (pHELP), a CRISPR/cas mobilizable plasmid (point mutations or deletion base) and a target plasmid (pTarget). **(B)** A schematic of point mutations or deletion base mobilized from donors to recipients and eliminating pTarget.

For conjugation experiment in *E.coli*, donors containing both pHELP and point mutations or deletion base were mated with the *E.coli* SAR08 recipients that had pTarget at 1:1 donor-to-recipient ratio for 16 h. After conjugation, mated cells were plated on Luria-Bertani (LB) agar media with kanamycin (Km) or kanamycin and chloramphenicol (Km and Cm) to quantify the number of total recipients and the number of transconjugants with pTarget or pControl, respectively (Fig 2A). We conducted two conjugation experiments using pTarget with NDM1 fragment (pTarget_NDM1) and, as a negative control, using pTarget without NDM1 (pTarget_ctrl). When using pTarget_NDM1, the number of surviving transconjugant (i.e. recipient cells with both pTarget_NDM1 and point mutations or deletion base) was almost four order of magnitude lower than the number of total recipients. On the other hand, when point mutations or deletion base was mobilized to control recipients (having pTarget_ctrl), the number of all recipients and surviving transconjugants were not significantly different (Fig 2B). Therefore, we confirmed that the point mutations or deletion base can be conjugatively transferred to *E. coli* and mediated sequence specific elimination of a target sequence taken from *A. baumannii* (AB377) antibiotics resistant gene.

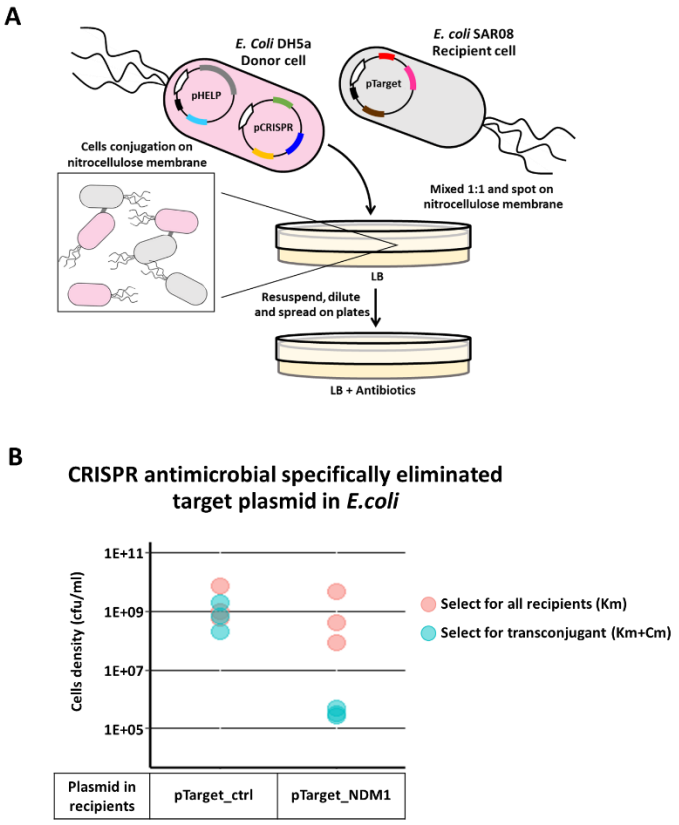

Figure 2 CRISPR antimicrobial can eliminate a specific target plasmid without disrupting other plasmids. (A) A schematic of an experiment for testing CRISPR antimicrobial in *E.coli.* (B) Estimated numbers of recipients or transconjugants after donor-recipient mating. Each point represents the number of recipients (selected with Km, Red) or transconjugant that retained pTarget ctrl or pTarget_NDM1 (selected with Km and Cm, Blue) from each independent experiment.

Next, we attempted to demonstrate CRISPR antimicrobial in multidrug resistant *A.baumannii* strains (AB166 and AB377) isolated from clinical samples. Both *A.baumannii* strains had confirmed NDM1 genes in their genomes or plasmids. Donors (*E. coli* with pHELP and point mutations or deletion base) and recipients (AB166 or AB377) were mixed at 1:1 donor-to-recipient ratio. As negative control experiments, we used donors harboring pHELP alone without point mutations or deletion base. After 16 h conjugation on nitrocellulose membranes, cells were resuspended and plated on selective media to quantify the number of surviving recipients. We found that, when the donors had both point mutations or deletion base and pHELP, the numbers of surviving recipients were about 90% lower than when the donor had only pHELP (negative control) (Fig 3). This results implied that point mutations or deletion base (which has a spacer targeting NDM1 gene) could be transferred into and mediated elimination of *A.baumannii* strains that had NDM1 target. Nonetheless, we found that some *A.baumannii* cells that received pCRISRP (select by using LB agar with Km and Cm) still survived. Thus, we planned to check

whether point mutations or deletion base and NDM1 target did co-exist in these surviving cell and whether the NDM1 target was mutagenized.

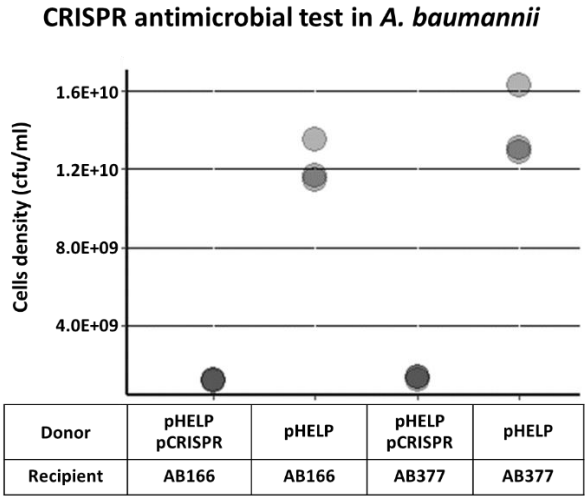

Figure 3 CRISPR antimicrobials against NDM1 reduced viability of *A.baumannii* recipients (Km^R) with NDM1 target(s). Donor cells (DH5a), containing both pHELP and point mutations or deletion base or only pHELP, were mated with *A.baumannii* (AB166 or AB377). Mated cells were plated on LB agar with Km to quantify the number of recipients. Each point represents the estimated number of surviving recipients from each independent experimental replicates.

We randomly selected ten surviving AB166 and AB377 transconjugant clones for colony PCR (Fig 4A). We demonstrated that the NDM1 gene were found in all selected AB166 and AB377 transconjugants while point mutations or deletion base could be confirmed only in AB377 transconjugants. We then selected for sequencing three PCR products from AB166 and from AB377 colonies with the most visible NDM1 PCR bands. The results showed that two PCR samples of AB377 and one PCR sample of AB166 had point mutations or single base deletion in the NDM1 gene (Fig 4B). These mutations in NDM1 gene may allow these surviving *A. baumannii* transconjugants to harbor both point mutations or deletion base and NDM1 gene.

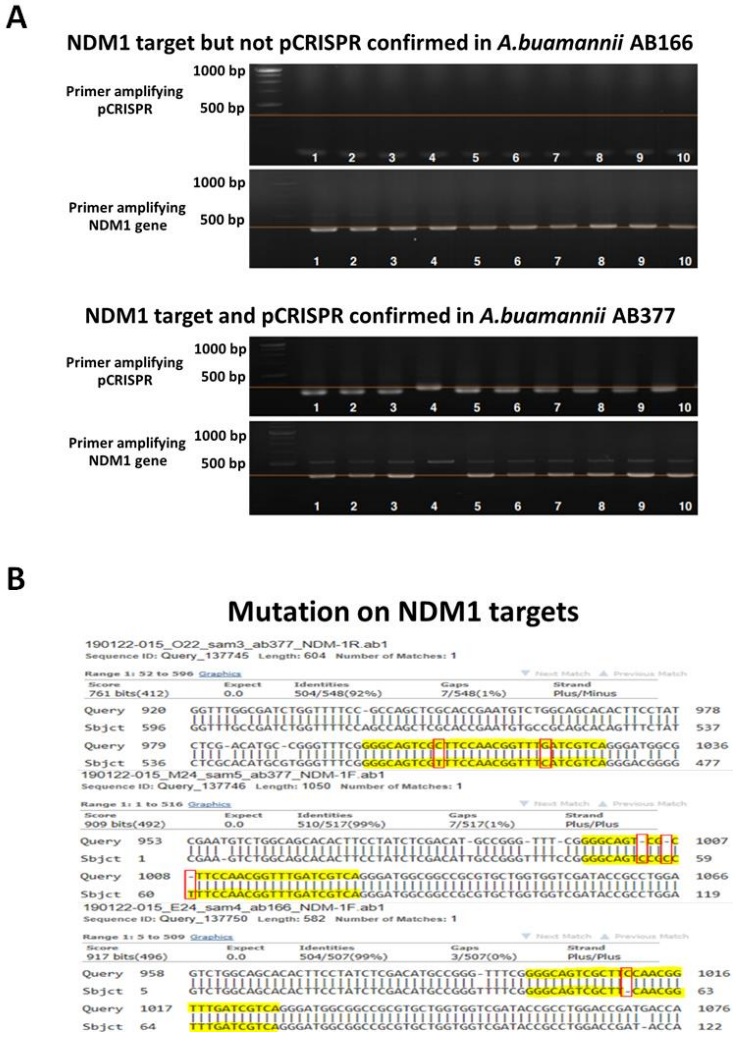

Figure 4 Confirmation of point mutations or deletion base delivery and mutation at the targeted gene. (A) After mating experiment with *A.baumannii* recipients (AB166 and AB377 in Fig 3), ten transconjugant colonies were randomly selected for colony PCR to confirm the presence of point mutations or deletion base and NDM1 target. (B) PCR produced from colony number 4,8 and 9 of AB166 and 3,5 and 9 of AB377 were sequenced. The sequencing results from colonies with mutations were shown here, aligned with the original NDM1 sequence near protospacer target of CRISPR highlighted in yellow. Red frames indicates mutated-locations.

Discussion and Conclusions

Our work presents a progress toward application of CRISPR antimicrobials in non-*E.coli* bacteria. Previously, CRISPR antimicrobials have been demonstrated in only few bacteria including *E.coli, S.enterica, S.aureus* and *Z.mobilis* (Citorik et al 2014; Cui et al 2016; Hamilton et al 2019; Bikard et al 2014; Cao et al 2017). Here, we applied the CRISPR antimicrobials for eliminating multidrug resistant *A. baumannii* isolated from clinical samples. We transferred CRISPR antimicrobial by conjugation machinery

encoding on a self-transmissible helper plasmid. We demonstrated that the CRISPR antimicrobials targeting NDM1 gene on a mobilizable plasmid can reduce survivability of *A. baumannii* transconjugants by ~ 90%. To the author knowledge, this work is the first demonstration of CRISPR antimicrobials in *A. baumannii*. We found that some *A. baumannii* clones could survive despite having both NDM1 target and a plasmid (point mutations or deletion base) with CRISPR/Cas system targeting NDM1. Sequencing results showed that some of these surviving clones had point mutation(s) or deletion on NDM1 targets. Previous work showed that the CRISPR antimicrobial can distinguish between bacterial target whose genomic DNA differed by as a little as a single base (Citorik et al 2014). Thus, it is possible that these mutation(s) prevented the target from getting cut by CRISPR/Cas systems expressed from our point mutations or deletion base.

Previous work showed that DNA repair system(s) in *A. baumannii* was too weak for the majority of cells to survive CRISPR/Cas mediated double-strand break (Wang et al 2019). In our experiment, we estimated that up to 10% of *A. baumannii* recipients could survive. A previous work in *E.coli*, using the same CRISPR antimicrobial delivery vector as ours, reported less than 1% surviving *E.coli* transconjugants (Citorik et al 2014). The apparently lower CRISPR antimicrobial efficiency in our study had several possible explanations. For example, the NDM1 target in our *A. baumannii* might be located on a plasmid or at a genomic location that was less sensitive to double-strand break (Cui et al 2016; Hamilton et al 2019). The delivered CRISPR/Cas cassette might be repressed, mutagenized or degraded by defense mechanism in *A. baumannii* (Vrancianu et al 2020). Alternatively, *A. baumannii* might have a higher mutation rate (compared to *E.coli*) which enable a sizable part of the population to escape from CRISPR/Cas targeting. Future work should systematically measure and compare efficiencies of CRISPR antimicrobials in *A. baumannii* and other medically relevant bacterial pathogens in order to explore their limitations and develop strategies for improvement.

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