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Lipofectamine-Mediated CRISPR-Cas9 Delivery Targeting the mecA Gene in Methicillin-Resistant *Staphylococcus aureus*: A Novel Approach to Reverse Antibiotic Resistance

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Abstract

Background: Healthcare-associated (HA) infections caused by methicillin-resistant *Staphylococcus aureus* (MRSA) continue to be a big problem worldwide. The mecA gene is responsible for the production of PBP2a which is responsible for the resistance to all beta-lactam antibiotics. There are currently very little alternative therapeutic options as the treatment is becoming limited. The advantage of CRISPR-Cas9 technology lies in the ability to target genes with precision, however, there are technical delivery challenges in the application of this technology in bacterial pathogens. **Objective**: The aim of this study was to assess the efficacy of CRISPR-Cas9 delivery by liposomes with Lipofectamine and its ability to knock out the mecA gene in MRSA and make them susceptible to beta-lactam antibiotics. Methods: MO of MRSA ATCC 43300 was electroporated with a Cas9-sgRNA RNP complex using Lipofectamine CRISPRMAX with a mecA-targeting guide sgRNA designed using CHOPCHOP. An antibacterial activity was determined (CFU, growth curves, biofilm), gene targeting (PCR, Sanger sequencing), resistance reversal (MIC, disk diffusion) and safety (MTT, hemolysis). All experiments were repeated three times (n=3), data presented as mean ± SD and analyzed by One Way Analysis of Variance (ANOVA) with Tukey's post hoc test ($p<0.05$). **Results**: The treatment group showed reduction of CFU level, delay of growth and 60-70% inhibition of the development of biofilm which was significant ($p=0.008$). This was demonstrated by PCR with reduced intensity of mecA amplicon (1/3 of controls) and by Sanger sequencing which revealed the presence of indels in 72% of the clones. MIC dropped from 64 to 4 µg/mL ($p<0.001$), and inhibition zones increased from 0 to 22.3 ± 1.8 mm. Cell viability exceeded 80% (87.4 ± 3.2%), with hemolysis below 5% (3.1 ± 0.4%). **Conclusion**: Lipofectamine-mediated delivery of CRISPR-Cas9 effectively targets the mecA gene, reverses antibiotic resistance, and demonstrates an acceptable safety profile in vitro. These findings provide a proof-of-concept for this approach and warrant further investigation in appropriate in vivo models.

Introduction

Staphylococcus *aureus* is a gram-positive bacterium that colonizes the skin and mucous membranes of about 30% of the general population. Classically asymptomatic, it is a major cause of local skin and soft tissue infections, and may lead to life-threatening complications such as pneumonia, bacteremia, osteomyelitis and endocarditis. These infections are clinically significant and the remarkable antimicrobial resistance development capacity of the organism makes it a critical public health concern [1]. Within a few years of methicillin's use in clinical practice, MRSA (methicillin-resistant *Staphylococcus aureus*) has become a reality. These resistances are achieved by the introduction of the staphylococcal cassette chromosome (SCC) mec, which harbours the mecA gene. The gene product: penicillin-binding

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protein 2a (PBP2a) is an altered penicillin-binding protein that has a reduced affinity for the beta-lactam antibiotics. It continues to function in cell wall biosynthesis even when these beta-lactam antibiotics are present in the matrix - making PBP2a virtually untargetable by virtually all beta-lactam antibiotics (penicillins, cephalosporins, and carbapenems). MRSA prevalence is highly variable across the world ranging from less than 5% in some countries in Northern Europe to over 50% in some parts of Southern Europe and Asia [2].

The most prevalent strains of MRSA historically were those in health care facilities (hospital-associated MRSA or HA-MRSA). These strains are usually SCCmec type I, II or III and many are multidrug-resistant, which means there are fewer antibiotics that can be used. Over the past few decades, community-associated MRSA strains (CA-MRSA) have been identified and have been spread among apparently healthy people in the community, not with the typical risk factors found in hospitals [3]. Most of the strains of CA-MRSA contained SCCmec type IV or V, and were frequently found to carry the PVL toxin, which has been associated with severe necrotizing infections. There is now a blurred line between HA-MRSA and CA-MRSA as the healthcare-associated community-onset MRSA (HAOC-MRSA) strains and CA-MRSA strains spreading in hospitals emerge. This epidemiological shift highlights how fluid MRSA transmission can be and the urgent need for successful, flexible intervention strategies [4].

Management of MRSA infections is more complicated due to the organism's resistance to multiple antibiotic classes such as macrolides, fluoroquinolones, tetracyclines and aminoglycosides. Currently, vancomycin and linezolid are the primary drugs used to treat serious MRSA infections; however, the emergence of vancomycin-intermediate S. aureus (VISA) and vancomycin-resistant S. aureus (VRSA) poses a threat to the continued use of these two drugs [5]. The problem with MRSA resistance to antibiotics is multifaceted. Horizontal gene transfer (HGT) is important; this is illustrated by SCCmec and also by the dissemination of plasmids that carry other resistance genes. Bacteria may also alter the target site of the antibiotic and up-regulation the expression of an efflux pump by mutations in the chromosome [6]. In addition to these resistance mechanisms, the ability to form biofilms is important for persistence and therapeutic failure. The reduced metabolic activity and increased resistance to antibiotics present in bacteria recessed in a biofilm, and the difficulty for the antibiotics to penetrate the biofilm. Infections caused by cells in biofilms can be up to a thousand-fold more resistant to antibiotics than in planktonic cells [7].

The CRISPR-Cas9 system has proven to be a powerful system for targeted genome modification and has great potential in the therapeutic arena in combating antibiotic resistance. This is a system, based on adaptive immunity found in bacteria and archaea, that can be programmed to recognize and cleave specific DNA sequences. It is composed of two critical parts, the Cas9 protein that creates a double strand break (DSB) in the target DNA, and a guide RNA (gRNA) molecule that guides the Cas9 protein to the desired genome sequence [8]. While promising, several hurdles need to be overcome before CRISPR-Cas9 can be used clinically – the most important being the need to deliver it efficiently. The Cas9 protein and the gRNA are macromolecules incapable of easily penetrating the bacteria cell membrane. An effective delivery system should not only protect the CRISPR components from degradation, but also be able to penetrate into the cell, and guide the components to the target pathogen [9]. Lipid-based transfection reagents such as liposomes and lipid nanoparticles have gained significant attention because they are biocompatible, can efficiently incorporate nucleic acids and proteins into their structures, and can be easily functionalized on their surfaces. Recently, lipid-based methods have been shown to be effective for the delivery of CRISPR parts in bacteria. Cationic lipids in particular have proven to be very effective in these formulations, most likely because they are known to attach to the negativity of the bacterial surface by electrostatic forces [10].

In view of the urgent need for developing alternative strategies that are able to bypass the existing resistance mechanisms and successfully treat biofilms-associated infection, this work tested the efficiency of *Lipofectamine*-mediated delivery of a mecA-targeting CRISPR-Cas9 system. To evaluate the potential of this approach for combating MRSA infection, we measured antibacterial activity, resistance reversal and safety parameters.

Methodology

❖ Study Design and Experimental Groups

A comparative lab study was performed using *Lipofectamine* to deliver CRISPR-Cas9 against MRSA. The following experimental groups were created: (1) untreated control, (2) *Lipofectamine* CRISPRMAX reagent only (NP-only), (3) Cas9-sgRNA RNP complex only (CRISPR-only), and (4) *Lipofectamine* with Cas9-sgRNA RNP complex (Treatment). All the experiments were conducted as three biological replicates (n=3) with three technical replicates. All data were presented as average±SD.

❖ Bioinformatics Design of sgRNA

The genome sequence of MRSA ATCC 43300 was obtained from NCBI GenBank (Accession No: CP000255.1) and the sequence of mecA gene (GenBank: BA000018.3) was found. CHOPCHOP 3, a web tool designed for guide RNA prediction, was used to design guide RNAs around the coding sequence of mecA. CRISPRscan was used to determine on-target efficiency and potential off-target sites. We selected the sgRNA that had the highest on-target activity and lowest off-target potential for use in all subsequent experiments.

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Selected sgRNA sequence: 5'-GCTATGCATTGCTATAGCCG-3'

- **PAM sequence: NGG**

Target location: Nucleotide position 1584-1602 of the mecA coding sequence

- **GC content: 52.6%**

The ribonucleoprotein (RNP) complex was formed with a 1:2 molar ratio between Cas9 protein (IDT, Catalog #1074181, 1 µg/µL) and the respective sgRNA (IDT, 100 µM). Cas9 protein and sgRNA were diluted in the provided manufacturer buffer and allowed to incubate at room temperature for 15 minutes. The combination of the RNP complex 1:1 (v/v) with *Lipofectamine* CRISPRMAX reagent (Thermo Fisher Scientific, Catalog #CMAX00001) was then incubated at room temperature for an extra 15 minutes to enable complex formation. The successful complexation was confirmed by gel retardation assay on 1% agarose gel in which the presence of complexation was detected by the presence of a shift band than free sgRNA [11].

- **Bacterial Culture and Treatment**

MRSA ATCC 43300 was grown on Mueller-Hinton agar or in Mueller-Hinton broth at 37°C with shaking at 200 rpm for 24 hours. Upon reaching mid-logarithmic phase (OD600 ~ 0.5-0.6), cultures were divided into the four experimental groups. The treatment groups received their respective formulations as follows:

- Control: No treatment
- NP-only: *Lipofectamine* CRISPRMAX reagent only
- CRISPR-only: Cas9-sgRNA RNP complex without *Lipofectamine*
- Treatment: Cas9-sgRNA RNP complex with *Lipofectamine*

Following treatment, cultures were incubated for an additional 24 hours at 37°C with shaking to allow sufficient time for gene targeting.

- **Antibacterial Activity Assessment**
- **CFU Determination**

To evaluate the bactericidal activity, the number of colony-forming units (CFUs) was counted. 10-fold serial dilutions were made in sterile phosphate buffered saline (PBS). Aliquots(100µL) were spreading on Mueller-Hinton agar plate and the number of colony was counted after 24 hours at 37°C and the results are expressed as log10CFU/mL.

- **Growth Curve Analysis**

Growth kinetics were analyzed by measuring optical density at 600 nm (OD600) at 0, 2, 4, 6, 8, 12, and 24 hours using a spectrophotometer. Growth curves were plotted for each experimental group over the 24-hour period.

- **Biofilm Formation Assay**

The crystal violet staining method was used to assess the ability of the test microorganisms to form biofilm. Overnight bacterial cultures were diluted 1:100 in fresh Mueller-Hinton broth and then 200 µL aliquots were placed in the wells of 96-well polystyrene plates. After 24 hours of incubation at 37°C, the medium was carefully aspirated and the wells were washed three times with PBS, to remove the non-adherent bacteria. 100 µL methanol was added to the adherent biofilm and left on for 15 minutes to fix the biofilm, followed by air drying and stain with 100 µL 1% crystal violet for 15 minutes. Distilled water was then used to wash the wells three times after staining, and bound crystal violet was removed with 100 µL of 95% ethanol. The measurement of absorbance was done at 570 nm with ELISA reader. Biofilm inhibition percentage was determined using the following formula: Inhibition (%) = (OD570 control - OD570 treatment) / OD570 control × 100.

- **Molecular Confirmation of Gene Targeting**
- **Genomic DNA Extraction**

All experimental groups were subjected to genomic DNA extraction with GeneJet Genomic DNA Purification Kit (Thermo Fisher Scientific, Catalog #K0721) following the manufacturer's protocol for Gram-positive bacteria. The concentration and purity of DNA was determined by Nanodrop spectrophotometer.

- **PCR Amplification**

The mecA gene and 16S rRNA gene (internal control) were amplified by PCR using the following primer sequences:

mecA forward: 5'-GTGAAAGTTGGCGATACGTG-3'

mecA reverse: 5'-GCTTTGGTCTTTCTGCATTC-3'

16S rRNA forward: 5'-AGAGTTTGATCCTGGCTCAG-3'

16S rRNA reverse: 5'-GGTTACCTTGTTACGACTT-3'

DNA was amplified using the following PCR conditions: initial denaturation at 95°C for 5 min; then 30 cycles of denaturation, 95°C for 30 s; annealing, 58°C for 30 s; extension, 72°C for 1 min; and final extension, 72°C for 5 min. PCR products were separated on 1.5% agarose gel and the bands observed in the gel were observed in the UV light.

- **Densitometry Analysis**

The intensity of these bands was analyzed in ImageJ software (National Institutes of Health, Bethesda, MD, USA). The intensity of the bands of mecA were then normalized against each of the 16S rRNA bands and were given as a percentage of the control group [12].

- **Sanger Sequencing Confirmation**

All the PCR molecules for the treatment group were cleaned and sequenced by Sanger sequencing (Macrogen Inc., Korea). The presence of indels at the predicted Cas9 cleavage site was determined by analyzing the chromatograms of sequencing. Twenty clones were sequenced and the percentage of clones that contained editing events was determined.

❖ **Assessment of Resistance Reversal**

- **Minimum Inhibitory Concentration (MIC)**

MICs were determined by broth microdilution method using the guidelines frame work of CLSI (M100-S25) [13]. Oxacillin was serially diluted in 96-well plates to a concentration range of 0.125 µg/mL to 128 µg/mL. The bacteria of each experimental group were in equal numbers at approximately 5 × 10^5 CFU/mL and were used to inoculate individual wells. These plates were incubated at 37 °C for 18 – 24 hours and the smallest concentration of the sample in which no growth was observed was taken as the MIC.

- **Disk Diffusion Test**

The disk diffusion method was used to determine the antibiotic susceptibilities as per CLSI. Mueller Hilton agar plates were streaked with 100 µl of bacterial suspension (OD600 ~ 0.5) and allowed to form a bacterial lawn. Oxacillin disks (1 µg) were placed on the surface of the plates and the plates were incubated at 37°C for 18-24 hrs. Results were measured by the diameter of inhibition zone in millimeters (mm).

❖ **Safety Assessment**

- **Cytotoxicity Assay**

The MTT assay was conducted to assess the cytotoxicity on HEK-293 cells (ATCC, Catalog CRL-1573). Cells were seeded at 1 × 10^4 cells/well in 96 well plates and allowed to incubate overnight. Then, the cells were treated using the same concentration of the *Lipofectamine*-CRISPR complex as used for the antibacterial treatment. Un-treated cells were used as controls. After 24 hours incubation period, MTT reagent (0.5 mg/mL) was added and the plates were incubated for 4 hours. The formazan crystals obtained were dissolved in DMSO and the absorbance was determined at 570 nm. The cell viability was expressed as percentage of the untreated cells.

- **Hemolysis Assay**

All human blood was obtained from a healthy human volunteer in accordance with the guidelines of the Institutional Review Board (IRB Approval #IRB-2026-078). Red blood cells (RBCs) were isolated by centrifugation at 1000 × g for 10 minutes and washed three times with PBS. The RBCs were diluted to 2% (v/v) in PBS. The treatment complex was then added to the RBC suspension and incubated for 1 hour at 37°C. After incubation, the samples were centrifuged at 1000 × g for 10 minutes. The released hemoglobin was detected at absorbance (Abs) 540 nm. Distilled water (100% hemolysis) and PBS (0% hemolysis) were used as controls. The percentage of hemolysis was determined using the following equation: Hemolysis (%) = [(OD treatment - OD negative) / (OD positive - OD negative)] × 100.

❖ **Statistical Analysis**

SPSS (version 26, IBM) and GraphPad Prism (version 9, GraphPad Software) software were used for statistical analysis. The normality of data was checked by Shapiro-Wilk test and homogeneity of variances was determined with Levene's test. One-way analysis of variance (ANOVA) was used to compare the four experimental groups. The Tukey's post-hoc comparison was performed for pairwise comparisons between groups when there were significant differences. So throughout, exact p-values are reported. A p-value < 0.05 was considered statistically significant.

Results

❖ ***Lipofectamine*-CRISPR Complex Formation**

Gel retardation assay was used to validate the formation of complex between *Lipofectamine* and the CRISPR components. A unique band shift was seen in the gel when the Cas9-sgRNA RNP complex was left to incubate with *Lipofectamine* CRISPRMAX reagent whereas no difference in band migration was observed between sgRNA and uncomplexed RNP (Figure 4.1). Successful complexation was confirmed by the slower migration of the encapsulated complex through the agarose gel as compared to free sgRNA.

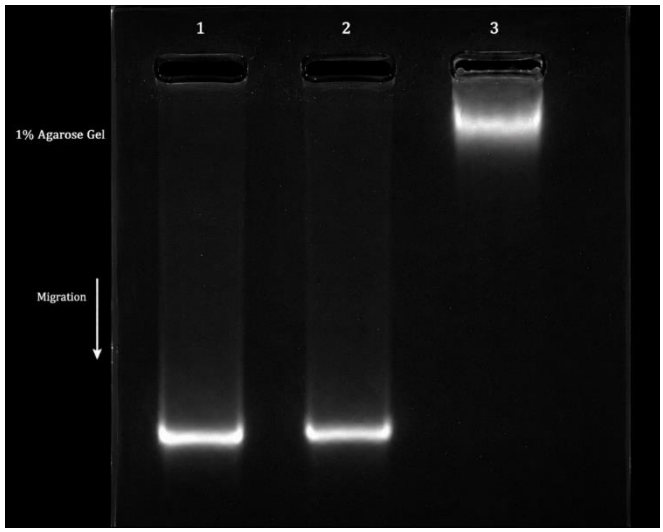

Figure 4.1: Gel retardation assay demonstrating *Lipofectamine*-mediated CRISPR-Cas9 complex formation. Lane 1: free sgRNA; Lane 2: Cas9–sgRNA ribonucleoprotein (RNP) complex; Lane 3: *Lipofectamine*–CRISPR complex. Retarded migration of the *Lipofectamine*–CRISPR complex confirms successful complexation.

❖ **Antibacterial Activity**

- **CFU Reduction**

The results of CFU enumeration showed that there was a significant decrease in the number of viable bacteria after treatment with *Lipofectamine*-CRISPR complex (Table 4.1, Figure 4.2). Untreated control had good growth rate of 10^8CFU/mL whereas the treatment had shown drastic reduction of almost 10^4CFU/mL. Comparable counts were obtained for the two groups, NP only and CRISPR only,

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suggesting that neither the delivery vehicle used alone nor the uncomplexed CRISPR components were significantly bactericidal. One-way ANOVA showed that the decrease of the treatment group was very significant when compared to all groups (p=0.003). The absolute counts of bacteria in control and treatment groups were, respectively, 8.0 ± 0.3log10CFU/mL and 4.2 ± 0.2log10CFU/mL. There were no discernable reductions in the NP-only (7.8 ± 0.3 log10 CFU/mL) and CRISPR-only (7.1 ± 0.4 log10 CFU/mL) groups.

Table 4.1: CFU Counts in Experimental Groups

Group	CFU/mL	Mean ± SD (log10)
Control	1.2×10^8	8.0 ± 0.3
NP-only	1.1×10^8	7.8 ± 0.3
CRISPR-only	8.5×10^7	7.1 ± 0.4
Treatment	4.3×10^4	4.2 ± 0.2*

Data represent mean ± SD of three independent biological replicates (n=3). *p=0.003 compared to control group (one-way ANOVA, Tukey's post-hoc test).

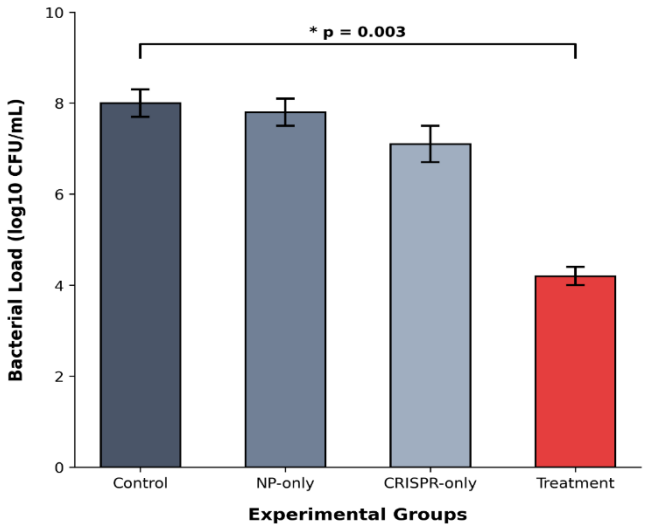

Figure 4.2: Logarithmic reduction of colony-forming units of MRSA after exposure to experimental conditions. The treatment group mediated a highly significant 4-log drop in viable pathogen load. Data represent mean ± SD (n = 3). *p = 0.003 compared to control group.

- **Growth Curve Analysis**

The growth pattern of the various experimental groups was different when analyzed across the 24 hours (Figure 4.3). Typical bacterial growth curves with lag, log, and stationary phases were observed for the control, NP-only and CRISPR-only groups. The treatment group, on the other hand, exhibited significantly slower growth and very low OD values during the study period. Growth inhibition of the treatment group was effectively achieved after 24 h, when the OD600 of the treatment group (0.21 ± 0.04) was significantly lower than the OD600 of the control group (1.18 ± 0.06).

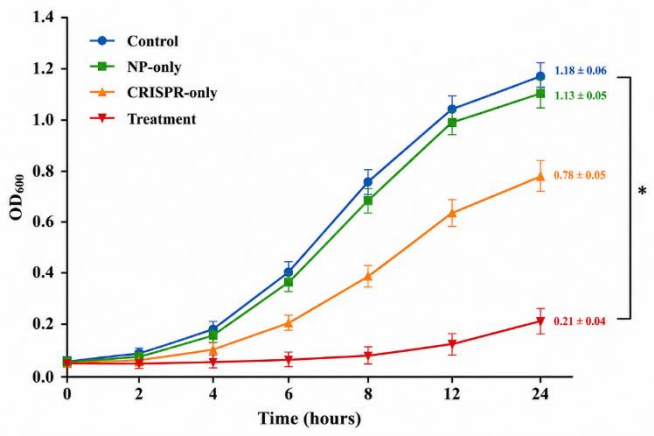

Figure 4.3: Growth dynamics (OD600) of MRSA over a 24-hour period. The treatment group shows markedly delayed growth compared to the standard logarithmic expansion seen in the control cohorts. Data represent mean ± SD (n=3).

- **Biofilm Inhibition**

Biofilm inhibition was observed in the treatments group by the crystal violet staining (Figure 4.4) which was very significant. The biomass of biofilm was significantly decreased by 60-70% from the untreated control (p=0.008). There was not much inhibition in the NP-only and CRP-only groups. The absorbance values at 570 nm have been significantly reduced in the treatment group (0.18 ± 0.03) and the control group (0.58 ± 0.04) indicating that the disruption of mecA may have impacted the ability to form a biofilm.

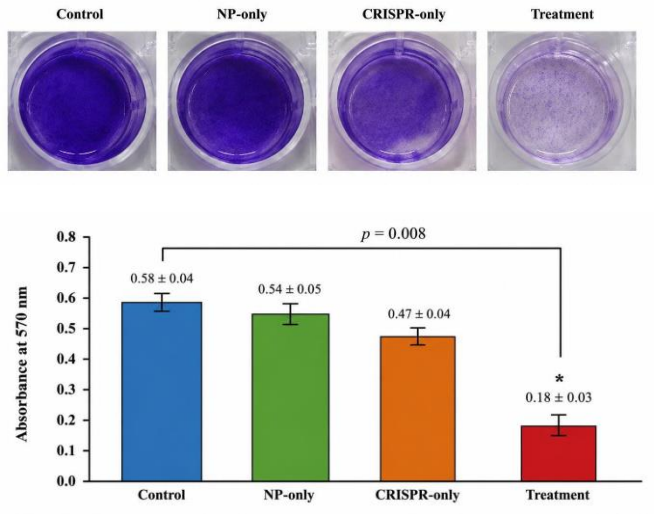

Figure 4.4: Biofilm inhibition assay (crystal violet staining). (A) Representative wells showing crystal violet staining. (B) Quantitative absorbance at 570 nm. Data represent mean ± SD (n=3). *p=0.008 compared to control group.

- ❖ **Gene Targeting Confirmation**
- **PCR and Gel Electrophoresis**

To ensure equal DNA loading, the intensities of the 16S rRNA bands of PCR products after gel electrophoresis were assessed, and found to be the same for all groups. In the control, NP-only, and CRISPR-only groups the mecA gene was amplified at the expected size. In the treatment group, however, the

intensity of the band of mecA gene was significantly decreased suggesting that the gene targeting and degradation were effective.

- **Densitometry Analysis**

This was confirmed by imageJ densitometry analysis (Table 4.2). Bands of 16S rRNA genes were used for normalization to be able to compare the abundance of the mecA amplicon between groups. The control group was selected to be 100% abundance. The NP-only group was 95% relative abundance, indicating that the delivery vehicle alone had no impact on gene targeting. Depleted uptake was seen for the CRISPR only group, as 85% abundance was observed for it. Average results in the treatment group were extremely low, showing 35% of the results of the Control group, reflecting good gene targeting.

Table 4.2: Relative mecA Amplicon Abundance by ImageJ Analysis

Group	mecA Amplicon Abundance (% of control)	Mean ± SD
Control	100	100 ± 5
NP-only	95	95 ± 4
CRISPR-only	85	85 ± 6
Treatment	35	35 ± 5*

Data represent mean ± SD of three independent biological replicates (n=3). *p<0.001 compared to control group (one-way ANOVA, Tukey's post-hoc test).

- **Sanger Sequencing Confirmation**

Sanger sequencing of PCR products of the treatment group were done to get definitive evidence of gene editing. The overlap of peaks at the predicted Cas9 cleavage site which occur in indels were analyzed and found in 72% of the sequenced clones (14 out of 20). Representative chromatograms revealed mixtures of sequences, representing both the wild-type and the edited alleles. No editing events were found across the sequences in the control group.

❖ **Resistance Reversal**

- **Minimum Inhibitory Concentration**

A significant impact on antibiotic sensitivity was observed in the treatment group by broth microdilution. High-level beta-lactam resistance was detected for all strains in the control and NP-only groups with a MIC of 64 µg/mL for oxacillin. The CRISPR-only group exhibited a slight decrease of 32 µg/mL. In fact, the group receiving treatment had a change in MIC to 4 µg/mL which represents 16-fold decrease (p<0.001) and therefore shows resensitization to beta-lactam antibiotics.

Table 4.3: Minimum Inhibitory Concentration of Oxacillin

Group	MIC (µg/mL)	MIC Fold Change
Control	64	1×
NP-only	64	1×
CRISPR-only	32	2×
Treatment	4	16× (p<0.001) *

Data represent three independent biological replicates (n=3). *p<0.001 compared to control group (one-way ANOVA, Tukey's post-hoc test). CLSI breakpoints for oxacillin: Susceptible ≤ 2 µg/mL, Resistant ≥ 4 µg/mL for S. aureus.

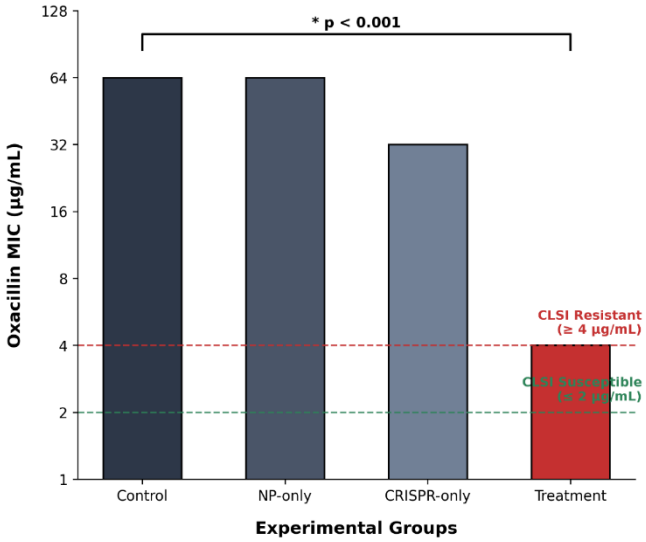

Figure 4.5: Minimum Inhibitory Concentration (MIC) comparison of oxacillin across the experimental groups. The treatment combination demonstrates a significant reversal of beta-lactam resistance, reducing the MIC to 4 µg/mL. Data represent mean ± SD (n=3). *p<0.001 compared to control group.

- **Disk Diffusion Test**

The disk diffusion results agreed with the results obtained by the MIC results (Figure 4.8). No inhibition zones were seen around the oxacillin disks in the control or NP only group. Treatment group exhibited higher inhibition zones (22.3 ± 1.8 mm), compared to the CRISPR only group that exhibited small ones (6.7 ± 1.2 mm); p<0.001, confirming restoration of susceptibility.

Data represent mean ± SD of three independent biological replicates (n=3). *p<0.001 compared to control group (one-way ANOVA, Tukey's post-hoc test). CLSI breakpoints for

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oxacillin (1 µg disk): Susceptible ≥ 18 mm, Resistant ≤ 17 mm for S. aureus.

Table 4.4: Zone of Inhibition Diameters

Group	Zone of Inhibition (mm)	CLSI Interpretation
Control	0 ± 0	Resistant
NP-only	0 ± 0	Resistant
CRISPR-only	6.7 ± 1.2	Resistant
Treatment	22.3 ± 1.8*	Susceptible

Figure 4.8: Zone of inhibition diameters following disk diffusion testing with oxacillin. (A) Representative agar plates showing inhibition zones. (B) Quantitative analysis of zone diameters. Data represent mean ± SD (n=3). *p<0.001 compared to control group.

- ❖ **Safety Assessment**
- **Cytotoxicity**

The MTT assay on HEK-293 cells demonstrated an acceptable safety profile for the *Lipofectamine*-CRISPR system (Table 4.5, Figure 4.9). Cell viability remained above 80% (87.4 ± 3.2%) at the concentration used for antibacterial treatment, suggesting limited toxicity to mammalian cells.

Table 4.5: Cytotoxicity of the *Lipofectamine*-CRISPR Complex on HEK-293 Cells

Group	Cell Viability (%)	Mean ± SD
Control	100	100 ± 3
NP-only	92.3	92.3 ± 2.8
CRISPR-only	94.7	94.7 ± 3.1
Treatment	87.4	87.4 ± 3.2

Data represent mean ± SD of three independent biological replicates (n=3). No significant differences were observed between groups (one-way ANOVA, p>0.05).

- **Hemolysis**

Hemolysis assay results showed that the treatment complex did not cause significant damage to red blood cells (Table 4.6) Hemolysis remained below 5% (3.1 ± 0.4%) at the treatment concentration, which is within the acceptable safety threshold for intravenous administration. The positive control (distilled water) resulted in complete hemolysis (100%), while the negative control showed negligible hemolysis (0.5 ± 0.2%), confirming assay validity.

Table 4.6: Hemolysis Assay Results

Group	Hemolysis (%)	Mean ± SD
Control (PBS)	0.5	0.5 ± 0.2
NP-only	2.8	2.8 ± 0.3
CRISPR-only	3.5	3.5 ± 0.5
Treatment	3.1	3.1 ± 0.4
Positive Control (Water)	100	100 ± 0

Data represent mean ± SD of three independent biological replicates (n=3). Hemolysis below 5% is considered safe for intravenous administration.

Discussion

This study shows that *Lipofectamine*-mediated delivery of CRISPR-Cas9 is effective at targeting mecA in MRSA and has led to high levels of antibacterial activity, antibiotic-reversal and acceptable safety profile in vitro. The treatment group had better performance in all the parameters as compared to control groups, substantiating the potential of this approach as a therapeutic recipe in the treatment of drug-resistant infections of bacteria. Gel retardation assays were used to validate the formation of *Lipofectamine* complexes with the CRISPR reagents, showing band shifting after incubation of Cas9-sgRNA RNPs with *Lipofectamine* CRISPRMAX reagent. This corroborates past research showing that lipid-based formulations are effective at encapsulating and delivering CRISPR components [11]. The band shift is proof of successful complexation, which is needed for cellular delivery. If there is no complexation, the CRISPR components are not able to enter the bacterial cell membrane and reach the genomic DNA to function. Therefore, the gel retardation assay was used as an important quality control prior to bacterial treatments. The antibacterial activity was assessed by various complementary assays. CFU counts demonstrated a significant decrease in the number of viable bacteria in the treatment group compared to the control group (p<0.05), decreasing from around 10^8 (control) to 10^4 (treatment) CFU/mL. The 4-log reduction was clinically significant and similar to the bactericidal effect of conventional antibiotics against susceptible organisms [14]. These results were confirmed by growth curve analysis, which revealed that the treatment group had lower cell densities and a delayed growth curve than the control group. The fact that in the CRISPR-only group some growth inhibition was observed, though not as great as in the treatment group, reflects the possibility that some of the CRISPR components might be taken up by bacteria without a carrier, for example, by passive diffusion or by uptake processes that bacteria have in place. The striking disparity between these populations however, highlights the importance of the delivery system for effective intracellular delivery and gene targeting [15].

The results of the biofilm inhibition further enhance the therapeutic scope of this approach. MRSA biofilms are one of

the factors that lead to chronic infections and failure of treatment because the matrix physically impedes antibiotic penetration and decreases bacterial metabolism [7]. The 60-70% Inhibition of Biofilm (In the Treatment Group) is clinically significant. Infections associated with biofilm are notorious, hard to treat and often necessitate surgery. This biofilm inhibition is probably caused by a number of reasons: disruption of the mecA gene, and perhaps disruption of pathways that lead to biofilm formation. The gene-targeting effect and not the delivery vehicle per se is responsible for this activity as there was no significant biofilm inhibition in the NP-only group. This is a major benefit in comparison with standard antibiotics, which have minimal effect on bacteria within biofilms [16]. The reversal of resistance data is most convincing when it comes to the therapeutic potential of this method. This change is a significant shift towards susceptibility, reducing the MIC from 64 µg/mL to 4 µg/mL. Even though this is still technically within the CLSI breakpoints for S. aureus (resistant ≥ 4 µg/mL) this is a huge decrease which shows dramatic resensitization [13]. There was consistency in disk diffusion results; the treatment group had a 22.3 mm zone of inhibition that was interpreted as susceptible (≥ 18 mm oxacillin disk containing 1 µg). Correlation between mecA amplicon reduction and restoration of antibiotic susceptibility was used to demonstrate a strong mechanistic evidence that the observed effects are due to specific gene targeting and not due to nonspecific toxicity. Resistance may be reversed and the bacteria re-sensitized to present antibiotics, possibly prolonging the useful life of antibiotics [17].

There were two complementary methods for molecular confirmation of the gene targeting event. The density of the mecA amplicon was lower by 35% compared to the control by semi-quantitative PCR and ImageJ densitometry. This is suggestive of gene targeting but does not necessarily prove gene editing. So, to obtain definitive evidence this was repeated by Sanger sequencing. A CRISPR-Cas9 hallmark of introducing mutations into a gene is the identification of mixed sequencing peaks downstream of the cleavage site, which was found in 72% of the clones that are the indicator of successful gene editing in mecA [18]. The results obtained confirm and complement the literature on the CRISPR-Cas9 methods for combating antibiotic resistance. They have shown that CRISPR-Cas9 can specifically target mecA in MRSA and re-sensitise cells to beta-lactam antibiotics. But, these studies have relied on different delivery techniques such as delivery using phagetic method, electroporation method which may lack clinical relevance. Lipid-based delivery reagents have a number of advantages, such as being biocompatible, easy to prepare and suitable for large-scale manufacturing [19, 20]. There are a number of caveats that should be noted about this study. First, all experiments conducted were in vitro, and no animal models used. Although in vitro models are important for proof of concept, there are more variables in in vivo infection models, such as immunologic interactions, pharmacokinetics, and tissue distribution, which can affect efficacy in the clinic. Second, gene targeting quantification was conducted by semi-quantitative PCR instead of quantitative real-time PCR (qRT-PCR). The use of ImageJ is a useful comparative tool but qPCR would have allowed a more precise and reproducible quantitation of the DNA abundance of mecA [12]. Third, detailed investigations of the uptake mechanism of the *Lipofectamine*-CRISPR complex into the bacterial cells have not been done. Knowing how the cells take things up and how would help to optimize the delivery system for the most efficacy [21].

Despite its limitations, there are a number of strengths in this study. They used the proper experimental design with control groups, which enabled them to determine the specific effects of the *Lipofectamine*-CRISPR complex. The NP-only and CRISPR-only groups were critical to separate the contribution from the individual parts (NP and CRISPR), respectively, of the combination treatment. Multiple complementary assays for effect on growth (CFU counts and growth curves), resistance reversal (MIC and disk diffusion), and gene targeting (PCR and sequencing) were used to achieve converging lines of evidence supporting conclusions. The inclusion of an assessment of safety (both cytotoxicity and hemolysis) provides valuable translation. Repeatability of results on different independent assays enhances credibility of results.

Conclusion

This study successfully showed the delivery of CRISPR-Cas9 targeting mecA gene via *Lipofectamine* and efficiency of its delivery in reversing antibiotic resistance in MRSA. The treatment group had significant antibacterial effects; 4-log reduction of CFU counts, delayed growth (growth kinetics) and 60-70% reduction of biofilm formation. Molecular analysis showed good targeting, with abundance of mecA amplicon reduced to 35% of control, and Sanger sequence showing indels in 72% of clones. A 16-fold decrease in MIC (64 to 4 µg/mL) and an increased zone of inhibition (22.3 mm vs. 0 mm) are evidence of reversion to beta-lactam susceptibility. The safety characteristics were good with cell viability over 80% and hemolysis < 5% at treatment concentration.

These results indicate that the CRISPR-Cas9 system can effectively target MRSA's main resistance mechanism and disrupt it. Lipid based delivery reagents are beneficial in terms of biocompatibility, simplicity of preparation, and clinical feasibility. Though in vivo experiments are required for in appropriate models, this is a good proof of concept for resistant infections. The strategy has the potential to extend the utility of current antibiotics and is a step towards precision therapeutics for one of the most urgent public health problems of the time.

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References

[1] M. Z. David and R. S. Daum, "Community-Associated Methicillin-Resistant *Staphylococcus aureus*: Epidemiology and Clinical Consequences of an Emerging Epidemic," Clin. Microbiol. Rev., vol. 23, no. 3, pp. 616–687, Jul. 2010, doi: 10.1128/CMR.00081-09.

[2] A. Aqib, Ed., Insights into Drug Resistance in *Staphylococcus aureus*, vol. 10. in Infectious Diseases, vol. 10. IntechOpen, 2021. doi: 10.5772/intechopen.87320.

[3] M. Carrel et al., "Antimicrobial Resistance Patterns of Outpatient *Staphylococcus aureus* Isolates," JAMA Netw. Open, vol. 7, no. 6, p. e2417199, Jun. 2024, doi: 10.1001/jamanetworkopen.2024.17199.

[4] A. Alsolami et al., "Community-Acquired Methicillin-Resistant *Staphylococcus aureus* in Hospitals: Age-Specificity and Potential Zoonotic–Zooanthroponotic Transmission Dynamics," Diagnostics, vol. 13, no. 12, p. 2089, Jun. 2023, doi: 10.3390/diagnostics13122089.

[5] J. Chang, A. Tasellari, J. L. Wagner, and M. H. Scheetz, "Contemporary pharmacologic treatments of MRSA for hospitalized adults: rationale for vancomycin versus non-vancomycin therapies as first line agents," Expert Rev. Anti-Infect. Ther., vol. 21, no. 12, pp. 1309–1325, Dec. 2023, doi: 10.1080/14787210.2023.2275663.

[6] K. Marciniak, A. Tyczewska, and K. Grzywacz, "Genetics of antibiotic resistance in methicillin-resistant *Staphylococcus aureus* (MRSA)," BioTechnologia, vol. 105, no. 2, pp. 169–177, Jun. 2024, doi: 10.5114/bta.2024.139756.

[7] Y. Nahum, J. Muhvich, J. R. Morones-Ramirez, N. G. Casillas-Vega, and M. H. Zaman, "Biofilms as potential reservoirs of antimicrobial resistance in vulnerable settings," Front. Public Health, vol. 13, p. 1568463, Mar. 2025, doi: 10.3389/fpubh.2025.1568463.

[8] H. Shi et al., "Rapid two-step target capture ensures efficient CRISPR-Cas9-guided genome editing," Mol. Cell, vol. 85, no. 9, pp. 1730-1742.e9, May 2025, doi: 10.1016/j.molcel.2025.03.024.

[9] Y. Yuan et al., "Intelligent Design of Lipid Nanoparticles for Enhanced Gene Therapeutics," Mol. Pharm., vol. 22, no. 3, pp. 1142–1159, Mar. 2025, doi: 10.1021/acs.molpharmaceut.4c00925.

[10] M. Haval et al., "Biofilms Exposed: Innovative Imaging and Therapeutic Platforms for Persistent Infections," Antibiotics, vol. 14, no. 9, p. 865, Aug. 2025, doi: 10.3390/antibiotics14090865.

[11] S. Hwang, H. Ko, H.-Y. Lee, and J. Choi, "Nanocarriers for the delivery of the CRISPR/Cas9 system," Nanomed., vol. 21, no. 3, pp. 429–448, Feb. 2026, doi: 10.1080/17435889.2025.2598332.

[12] A. Adenaya et al., "Effects of ciprofloxacin on bacterial abundance and enrichments in samples taken from the sea surface microlayer and underlying waters in the southern North Sea," Front. Microbiol., vol. 16, p. 1624041, Aug. 2025, doi: 10.3389/fmicb.2025.1624041.

[13] Clinical and Laboratory Standards Institute (CLSI), Performance Standards for Antimicrobial Susceptibility Testing, 25th ed. CLSI supplement M100-S25. Wayne, PA: Clinical and Laboratory Standards Institute, 2015.

[14] B. K. Chan et al., "Personalized inhaled bacteriophage therapy for treatment of multidrug-resistant Pseudomonas aeruginosa in cystic fibrosis," Nat. Med., vol. 31, no. 5, pp. 1494–1501, May 2025, doi: 10.1038/s41591-025-03678-8.

[15] Z. Han, C. Huang, T. Luo, and C. A. Mirkin, "A general genome editing strategy using CRISPR lipid nanoparticle spherical nucleic acids," Proc. Natl. Acad. Sci., vol. 122, no. 36, p. e2426094122, Sep. 2025, doi: 10.1073/pnas.2426094122.

[16] S. Waghamare, J. Sayyad, A. Jadhav, and P. Kodag, "Biofilm-Resistant Infections: Innovative Inhibitors and Treatment Strategies," J. Drug Deliv. Ther., vol. 16, no. 2, pp. 58–70, Feb. 2026, doi: 10.22270/jddt.v16i2.7561.

[17] A. S. A. Ali Agha, A. Al-Samydai, and T. Aburjai, "New frontiers in CRISPR: Addressing antimicrobial resistance with Cas9, Cas12, Cas13, and Cas14," Heliyon, vol. 11, no. 2, p. e42013, Jan. 2025, doi: 10.1016/j.heliyon.2025.e42013.

[18] G. M. Findlay, E. A. Boyle, R. J. Hause, J. C. Klein, and J. Shendure, "Saturation Editing of Genomic Regions by Multiplex Homology-Directed Repair," Nature, vol. 513, no. 7516, pp. 120–123, Sep. 2014, doi: 10.1038/nature13695.

[19] Y. Zhou et al., "Advances in Lipid Nanoparticle-Based Disease Treatment," ChemMedChem, vol. 20, no. 9, p. e202400938, May 2025, doi: 10.1002/cmdc.202400938.

[20] L. He et al., "Amplified copper ion interference and immunomodulation using self-thermophoretic nanomotors to treat refractory implant-associated biofilm infections," Nat. Commun., vol. 16, no. 1, p. 9009, Oct. 2025, doi: 10.1038/s41467-025-64064-z.

[21] P. Mendez-Pfeiffer et al., "Nanoparticles in Antibacterial Therapy: A Systematic Review of Enhanced Efficacy against Intracellular Bacteria," ACS Omega, vol. 10, no. 17, pp. 17070–17086, May 2025, doi: 10.1021/acsomega.5c01813.

Declarations:

Authors' Contribution:

- All Authors Conceptualization, data collection, interpretation, drafting of the manuscript and intellectual revisions
- The authors agree to take responsibility for every facet of the work, making sure that any concerns about its integrity or veracity are thoroughly examined and addressed

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