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A Comprehensive Review: CRISPR-Cas9 and dCas9 Strategies in Mitigating Antimicrobial Resistance in *Staphylococcus aureus*

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Abstract:

Methicillin-resistant *Staphylococcus aureus* (MRSA) presents a significant challenge in healthcare due to its resistance to multiple antibiotics.Antimicrobial resistance (AMR) poses a significant global health threat, necessitating innovative strategies for combating resistant pathogens. The advent of Clustered Regularly Interspaced Short Palindromic Repeats-CRISPR associated protein 9 (CRISPR-Cas9) and deactivated/dead Cas9 (dCas9) technologies has opened up new avenues for precision genome editing and transcriptional regulation, offering promising strategies for controlling AMR.CRISPR-Cas9 through it's precise genome editing capabilities, allow for targeted disruption of essential genes in MRSA, potentially reversing antibiotic resistance or inhibiting virulence factors. Moreover, the use of deactivated Cas9 (dCas9) fused with regulatory domains enables targeted transcriptional regulation, offering a promising avenue for controlling gene expression in MRSA. This comprehensive review explores the future prospects of CRISPR-Cas9 and dCas9 in addressing AMR, focusing on their potential applications in MRSA and general pathogens. We discuss the utility of CRISPR-based technologies in targeted gene editing for reversing antimicrobial resistance, the development of novel antimicrobials, precision antibiotic sensitization, surveillance and diagnostics. This review discusses recent advancements, challenges, and future prospects of utilizing CRISPR-Cas9 and dCas9 in combating MRSA infections.

Keywords: Antimicrobial resistance, CRISPR Cas9, CRISPR dCas9, Methicillin-Resistant *Staphylococcus aureus*

Introduction:

Antimicrobial resistance (AMR) is a pressing global health threat, particularly exemplified by Methicillin-Resistant *Staphylococcus aureus* (MRSA) and other multidrug-resistant pathogens. Traditional antimicrobial therapies are increasingly ineffective, prompting the need for innovative approaches. Clustered Regularly Interspaced Short Palindromic Repeats- CRISPR associated protein 9 (CRISPR-Cas9) and deactivated/dead Cas9 (dCas9) technologies offer precision gene editing and transcriptional regulation capabilities, providing potential solutions for controlling AMR (WHO 2023). CRISPR-Cas9, a revolutionary genome editing tool, holds immense potential for combating antibiotic resistance by precisely targeting and disrupting essential genes in MRSA. Additionally, dCas9, devoid of endonuclease activity, can be engineered for gene regulation, providing a versatile approach to modulate bacterial gene expression.This review explores the applications of CRISPR-Cas9 and dCas9 in combating AMR, focusing on targeted gene editing, development of novel antimicrobials, precision antibiotic sensitization, surveillance and diagnostics, personalized therapeutic strategies, and overcoming biofilm-associated resistance (Javed *et al.,* 2023).

CRISPR Cas system:

Clustered Regularly Interspaced Short Palindromic Repeats- CRISPR associated protein (CRISPR-Cas) systems are classified into two main classes: Class 1 and Class 2, based on their structural and functional characteristics (Makarova *et al.,* 2020; Huang *et al.*, 2022).Class 1 CRISPR-Cas systems are multi-subunit complexes, while Class 2 systems consist of single, large effector proteins. Class 1 systems are further subdivided into types I, III, and IV, while Class 2 systems are divided into types II, V, and VI (Shmakov*et al.,* 2015; Makarova *et al.,* 2020).

Class 1: Multi-subunit complexes

This type includes complexes such as Cas3 and Cas10. Type I systems use multiple Cas proteins to carry out DNA interference. Cas3, for example, is responsible for DNA degradation once target recognition and interference have occurred (Makarova and Koonin, 2015). Type III systems, involving proteins like Cas10, have both DNA and RNA targeting capabilities. They trigger interference by degrading target RNA (Kolesnik *et al.*, 2021). Type IV systems, relatively less studied, are similar to Type I but differ in some structural and functional aspects (Pinilla-Redondo *et al.,* 2020).

Class 2: Single, large effector proteins

Type II systems feature a single effector protein, typically Cas9. Cas9 is guided by a single RNA molecule to target and cleave specific DNA sequences. It's widely used in genome editing applications due to its simplicity and versatility (Asmamaw and Zawdie, 2021).Type V systems, exemplified by Cas12, also known as Cpf1, are characterized by their distinct structure and DNA cleavage mechanism compared to Cas9. Cas12 recognizes a different protospacer adjacent motif (PAM) and generates staggered cuts in DNA (Tong *et al.*, 2021). Type VI systems, represented by Cas13, are RNA-targeting systems. Cas13 cleaves single-stranded RNA molecules complementary to its guide RNA, making it valuable for RNA editing and RNA detection applications.Each CRISPR-Cas type has unique features, functions, and applications. Understanding these distinctions is crucial for selecting the most appropriate system for specific genome editing or nucleic acid targeting tasks (Nakagawa*et al.*, 2022).

Among the CRISPR types, CRISPR-Cas9 and dCas9 technologies offer multifaceted approaches to combatting antimicrobial resistance. These innovative tools provide avenues for precision genome editing, development of novel antimicrobials, personalized therapeutic strategies, and overcoming resistance mechanisms, offering hope for addressing the global challenge of AMR effectively (Duan *et al.,* 2021). CRISPR-Cas9 employs a guide RNA (gRNA) to target specific DNA sequences within the MRSA genome, where the Cas9 endonuclease induces double-stranded breaks, leading to gene disruption through error-prone repair mechanisms. Alternatively, dCas9 can be fused with transcriptional activators or repressors to modulate gene expression without altering the DNA sequence, offering fine-tuned control over MRSA virulence or antibiotic resistance genes (Lo and Qi, 2017).

Mechanism of CRISPR-Cas9:

Recognition of Target DNA: The CRISPR-Cas9 system consists of two main components: Cas9 protein and guide RNA (gRNA). The gRNA is designed to be complementary to a specific sequence of the target DNA. When the gRNA binds to the target DNA, it forms a complex with the Cas9 protein, guiding it to the target site.

Formation of RNA-DNA Complex: Once bound to the target DNA, Cas9 undergoes a conformational change that allows it to form a stable complex with both the gRNA and the target DNA. This complex is known as the Cas9-gRNA-DNA complex (Asmamaw and Zawdie, 2021).

DNA Cleavage: The Cas9 protein possesses two nuclease domains: the RuvC domain and the HNH domain. These domains are responsible for cleaving both strands of the target DNA at specific locations determined by the sequence of the gRNA. The RuvC domain cleaves the non-complementary strand, while the HNH domain cleaves the complementary strand (Safari *et al.*, 2019).

Induction of Double-Stranded Break (DSB): The cleavage of both DNA strands by Cas9 results in the formation of a double-stranded break (DSB) at the target site. This DSB triggers the cell's DNA repair machinery, which can lead to either non-homologous end joining (NHEJ) or homology-directed repair (HDR) (Xue and Greene, 2021).

Repair Pathways: In NHEJ, the broken ends of the DNA are ligated back together, often introducing small insertions or deletions (indels) at the site of the DSB. In HDR, a donor DNA template can be used to guide precise repair of the DSB, allowing for targeted gene editing or insertion of specific sequences (Stinson and Loparo, 2021).

Mechanism of dCas9:

DNA Binding: Unlike Cas9, which cleaves DNA, deactivated Cas9 (dCas9) lacks nuclease activity due to mutations in its catalytic domains. Instead, dCas9 is used for targeting specific DNA sequences without inducing DSBs. The gRNA still guides dCas9 to the target site on the DNA.Formation of dCas9-gRNA-DNA Complex: Similar to Cas9, dCas9 forms a stable complex with the gRNA and the target DNA. However, since dCas9 lacks nuclease activity, it does not cleave the DNA upon binding.Gene Regulation: Once bound to the target DNA, dCas9 can be fused with various effector domains, such as transcriptional activators or repressors. These effector domains allow dCas9 to modulate gene expression by either promoting or inhibiting transcription from the target DNA sequence (Karlson *et al.*, 2021).

Transcriptional Activation and Repression: When dCas9 is fused with transcriptional activators, it promotes gene expression by recruiting transcriptional machinery to the target site, thereby enhancing transcription. Conversely, when dCas9 is fused with transcriptional repressors, it inhibits gene expression by blocking the binding of transcriptional machinery or inducing chromatin modifications that suppress transcription (Casas-Mollano*et al.*, 2020).

Overall, while both CRISPR-Cas9 and dCas9 utilize the same gRNA-guided DNA targeting mechanism, they exert different effects on the target DNA: CRISPR-Cas9 induces double-stranded breaks for gene editing, while

dCas9 is employed for precise gene regulation without altering the DNA sequence (Asmamaw and Zawdie, 2021).

Applications of CRISPR Cas9 in MRSA Control:

Several studies have demonstrated the efficacy of CRISPR-Cas9 and dCas9 in targeting essential genes associated with MRSA pathogenicity and antibiotic resistance. Strategies such as CRISPR-based antimicrobials, phage-mediated delivery of CRISPR components, and CRISPR interference (CRISPRi) have shown promise in reducing MRSA virulence and enhancing susceptibility to antibiotics (Mayorga-Ramos*et al.,* 2023).

CRISPR-Based Antimicrobials:

Recent studies have focused on developing CRISPR-based antimicrobials targeting specific genes in MRSA, such as those involved in antibiotic resistance or virulence. For instance, researchers have successfully utilized CRISPR-Cas9 to target essential genes like mecA, which confers methicillin resistance in MRSA strains. By disrupting mecA, researchers have observed a restoration of susceptibility to β-lactam antibiotics in MRSA strains, highlighting the potential of CRISPR-Cas9 as a therapeutic strategy against antibiotic-resistant pathogens (Mayorga-Ramos*et al.,* 2023).

Phage-Mediated Delivery of CRISPR Components:

Phage-mediated delivery of CRISPR components has emerged as a promising approach to combat MRSA infections. Recent research has demonstrated the use of engineered bacteriophages carrying CRISPR-Cas systems to selectively target and eliminate MRSA strains. These phage-based therapies offer specificity and efficiency in delivering CRISPR components to bacterial populations, potentially circumventing issues related to delivery methods encountered with other CRISPR-based approaches (Khambhati et al., 2022).

CRISPR Interference (CRISPRi):

CRISPR interference (CRISPRi) has been explored as a strategy to modulate gene expression in MRSA without altering the DNA sequence. Recent studies have utilized dCas9 fused with transcriptional repressors to silence genes associated with MRSA virulence or antibiotic resistance. By targeting essential regulatory genes, CRISPRi has shown promise in attenuating MRSA pathogenicity and enhancing susceptibility to antibiotics, offering a novel therapeutic approach for combating MRSA infections (Larson *et al.,* 2013).

Overcoming Delivery Challenges:

Efficient delivery of CRISPR components into bacterial cells remains a significant challenge for therapeutic applications. Recent research has focused on developing innovative delivery systems, including lipid nanoparticles, bacterial conjugation, and phage-based vectors, to improve the efficacy and specificity of CRISPR-based therapies against MRSA. These delivery strategies aim to enhance the penetration of CRISPR components into bacterial cells, ensuring targeted genome editing or gene regulation for effective MRSA control (Mayorga-Ramos*et al.,* 2023).

Addressing Off-Target Effects and Resistance Mechanisms:

Addressing off-target effects and the emergence of Cas9-resistant strains is crucial for the clinical translation of CRISPR-based therapies against MRSA. Recent advancements in CRISPR technology have led to the development of improved Cas9 variants with enhanced specificity and reduced off-target effects. Additionally, research efforts have focused on understanding and overcoming bacterial resistance mechanisms to CRISPR, including the exploration of alternative targeting strategies and the development of combinatorial approaches to enhance therapeutic efficacy (Asmamaw *et al.,* 2024).

Therapeutic Methods to Tackle MRSA Apart from CRISPR:

Antibiotics: Traditional antibiotics have been a mainstay in the treatment of MRSA infections. While MRSA strains are resistant to many antibiotics, certain antibiotics, such as vancomycin and linezolid, can still be effective against some MRSA infections (Choo and Chambers, 2016).

Phage Therapy: Phage therapy involves using bacteriophages (viruses that infect bacteria) to target and kill MRSA. Phages are highly specific to their bacterial hosts, offering a potential alternative to antibiotics. Research in phage therapy for MRSA is ongoing, with promising results in some cases (Lin *et al.,* 2017).

Antimicrobial Peptides: Antimicrobial peptides (AMPs) are naturally occurring molecules with antimicrobial properties. Some AMPs have shown efficacy against MRSA by disrupting bacterial membranes or interfering with intracellular processes. AMPs hold potential as novel antimicrobial agents against MRSA infections.

Antibiotic Combinations: Combining different antibiotics or combining antibiotics with non-antibiotic agents, such as adjuvants or potentiators, can enhance antimicrobial activity against MRSA. Synergistic interactions between

antibiotics may overcome resistance mechanisms and improve treatment outcomes (Huan *et al.,* 2020).

Immunotherapy: Immunotherapeutic approaches, such as monoclonal antibodies or vaccines targeting MRSA antigens, aim to boost the immune response against MRSA infections. These strategies may enhance host defense mechanisms and reduce the severity of MRSA infections (Park and Liu, 2021).

Pros and Cons of CRISPR Compared to Other Existing Methods:

Pros of CRISPR:

Precision: CRISPR-Cas9 offers precise targeting of specific genes within the MRSA genome, allowing for accurate manipulation of bacterial genes associated with virulence or antibiotic resistance.Versatility: CRISPR technology can be adapted for various applications, including gene editing, transcriptional regulation, and antimicrobial development, making it a versatile tool for tackling MRSA infections.

Potential for Resistance Reversal: CRISPR-mediated gene editing can potentially reverse antibiotic resistance in MRSA strains by disrupting resistance-conferring genes, restoring susceptibility to antibiotics.Customizability: CRISPR systems can be engineered to target multiple genes simultaneously or to target specific genetic elements, providing flexibility in designing therapeutic interventions tailored to individual MRSA strains (Mayorga-Ramos*et al.,* 2023).

Cons of CRISPR:

Off-Target Effects: CRISPR-Cas9 may induce unintended mutations at off-target sites within the bacterial genome, raising concerns about potential off-target effects and unintended consequences of CRISPR-mediated gene editing.Delivery Challenges: Efficient delivery of CRISPR components into bacterial cells remains a challenge, particularly for therapeutic applications. Developing effective delivery systems capable of delivering CRISPR components to target bacteria in vivo is essential for the clinical translation of CRISPR-based therapies. Emergence of Resistance: MRSA strains may develop resistance to CRISPR-based therapies through various mechanisms, including mutations in target sequences or the acquisition of CRISPR-Cas immunity. Strategies to overcome bacterial resistance to CRISPR-mediated interventions will be necessary to ensure long-term efficacy. Regulatory Hurdles: CRISPR-based therapies may face regulatory hurdles and safety concerns related to the manipulation of bacterial genomes and potential off-target effects. Regulatory

approval and ethical considerations must be addressed before CRISPR-based therapies can be widely implemented for MRSA treatment (Guo *et al.,* 2023).

In summary, while CRISPR offers unique advantages for precision genome editing and targeted gene regulation in tackling MRSA infections, it also faces challenges such as off-target effects, delivery limitations, emergence of resistance, and regulatory considerations. Integrating CRISPR with existing therapeutic methods and addressing these challenges will be essential for realizing the full potential of CRISPR-based approaches for combating MRSA (Mayorga-Ramos*et al.,* 2023).

Pros and Cons of Other Methods Compared to CRISPR:

Antibiotics:

Pros: Wide Availability: Antibiotics are readily available and have been used for decades to treat bacterial infections, including MRSA.Clinical Experience: Antibiotics have a long history of clinical use and established treatment protocols, providing familiarity and confidence to healthcare professionals.Immediate Impact: Antibiotics can rapidly reduce bacterial load and alleviate symptoms in MRSA-infected patients, offering immediate therapeutic benefits (Nandini *et al.,* 2022).

Cons: Resistance Development: Prolonged use of antibiotics can lead to the development of antibiotic-resistant strains, including MRSA, limiting treatment options and efficacy.Side Effects: Antibiotics may cause adverse effects such as gastrointestinal upset, allergic reactions, or antibiotic-associated diarrhea.Limited Specificity: Antibiotics target broad cellular processes, leading to non-specific effects on both pathogenic and commensal bacteria, potentially disrupting the microbiota and promoting further resistance development (Ventola, 2015; WHO, 2023).

Phage Therapy:

Pros: Specificity: Bacteriophages exhibit high specificity for their bacterial hosts, allowing targeted killing of MRSA without affecting non-targeted bacteria.Natural Mechanism: Phage therapy exploits a natural biological process and can be tailored to target specific MRSA strains, potentially minimizing off-target effects.Potential for Evolutionary Response: Bacteriophages can co-evolve with bacteria, potentially overcoming resistance mechanisms and prolonging therapeutic efficacy (Opperman *et al.,* 2022).

Cons: Limited Understanding: Despite its potential, phage therapy is still in the early stages of research, and there is limited understanding of phage-bacteria interactions and long-term effects.Regulatory Hurdles: Regulatory approval for phage therapy varies between countries and may pose challenges for widespread implementation.
Phage Resistance: Bacteria can develop resistance to bacteriophages, leading to treatment failure and necessitating the development of new phage cocktails (Zalewska-Piątek, 2023).

Antimicrobial Peptides:
Pros: Broad-Spectrum Activity: Antimicrobial peptides (AMPs) exhibit broad-spectrum antimicrobial activity against a wide range of bacterial pathogens, including MRSA.Mode of Action: AMPs disrupt bacterial membranes or interfere with essential intracellular processes, making them less prone to resistance development.
Potential for Novel Therapeutics: AMPs offer potential as novel therapeutic agents against MRSA infections, particularly as alternatives to conventional antibiotics (Huan *et al.,* 2020).

Cons: Limited Stability: Some AMPs may have limited stability or susceptibility to proteolytic degradation, reducing their effectiveness in vivo.Cytotoxicity: Certain AMPs may exhibit cytotoxic effects on mammalian cells at high concentrations, limiting their therapeutic potential.Production Challenges: Large-scale production of AMPs may be challenging and costly, hindering their clinical translation and commercialization (Patrulea *et al*., 2020).
In summary, while other therapeutic methods such as antibiotics, phage therapy, and antimicrobial peptides offer their own advantages and disadvantages in treating MRSA infections, CRISPR-based approaches provide unique benefits in terms of precision targeting, potential for resistance reversal, and customizability. Integrating CRISPR with existing methods and addressing its limitations will be crucial for optimizing therapeutic outcomes in combating MRSA (Mayorga-Ramos*et al.,* 2023).

Challenges and Future Directions:
Despite significant progress, challenges such as delivery methods, off-target effects, and the emergence of Cas9-resistant strains pose hurdles to the widespread implementation of CRISPR-based therapies against MRSA. Future research efforts should focus on optimizing delivery systems, minimizing off-target effects, and developing strategies to overcome bacterial resistance mechanisms (Uddin *et al.*, 2020; Rasul *et al.*, 2022).

Conclusion:

CRISPR-Cas9 and dCas9 technologies hold immense potential in combating MRSA infections by targeting essential genes involved in virulence and antibiotic resistance. Further research and technological advancements are needed to harness the full therapeutic potential of CRISPR-based approaches for effectively controlling MRSA and mitigating the global burden of antibiotic resistance in healthcare settings.Recent research on CRISPR-Cas9 and dCas9 technologies has demonstrated their potential as effective strategies for tackling MRSA infections. From the development of CRISPR-based antimicrobials to innovative delivery systems and strategies to address off-target effects and resistance mechanisms, ongoing advancements in CRISPR technology hold promise for the development of novel therapeutic approaches against MRSA. However, further research is needed to optimize these approaches for clinical application and overcome remaining challenges to ensure their efficacy and safety in combating MRSA and other antibiotic-resistant pathogens.

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