Design Brief

Revolutionizing MRSA treatment: CRISPR-mediated disruption of VanS/VanR system to overcome vancomycin resistance*

Emily Packer ▫ Andover High School, Andover, MA

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Methicillin-resistant Staphylococcus aureus (MRSA) is an infection that presents a challenge in clinical settings due to its resistance to multiple antibiotics, most notably vancomycin. Conventional approaches, such as combinations with β-lactamase inhibitors or other antibiotics, offer only temporary effects but fail to address the underlying cause of resistance. This approach includes the use of a CRISPR-based strategy that targets the VanS/VanR system, a signaling pathway critical to vancomycin resistance. In MRSA, this VanS/VanR signaling pathway enables the bacteria to detect the presence of vancomycin and initiate a response that leads to the activation of genes involved in resistance. This proposed methodology involves using CRISPR to disrupt this protein interaction, ultimately leading to the vancomycin resistance genes remaining turned off. This design involves generating specific guide RNAs (gRNAs) to disrupt key components of the VanS/VanR signaling pathway, thereby sensitizing MRSA to vancomycin treatment. This CRISPR-mediated strategy offers a direct and targeted approach to disrupt the VanS/VanR system, addressing the root cause of vancomycin resistance in MRSA. By specifically targeting the mechanism of resistance, this treatment provides a promising solution to combating this challenge and addressing the root cause of antibiotic resistance as a whole.

Keywords: VanS/VanR, methicillin-resistant *Staphylococcus aureus* (MRSA), antibiotic resistance, CRISPR

Methicillin-resistant *Staphylococcus aureus* (MRSA) is a bacterial infection that has become a major public health concern. Unlike its non-resistant counterparts, MRSA has evolved mechanisms to evade the effects of various antibiotics, including methicillin, penicillin, amoxicillin, and oxacillin, among others (Mayo Clinic Staff, 2022). One of the defining characteristics of MRSA is its ability to cause a wide range of infections, both in healthcare settings and in the community. In healthcare facilities, MRSA infections often manifest as skin and soft tissue infections, such as abscesses, cellulitis, and surgical wound infections. These infections can be particularly challenging to treat because of many complications that can occur, such as sepsis. Outside of healthcare settings, MRSA can also spread among otherwise healthy individuals, leading to community-acquired MRSA infections. These infections can present as skin infections, pneumonia, or more serious

* *The authors were mentored by Lindsey L'Ecuyer from Andover High School. Please direct correspondence to: lindsey.lecuyer@andoverma.us.*

invasive infections, posing a significant risk to public health. Factors contributing to the transmission of MRSA in the community include close skin-to-skin contact, sharing contaminated items or surfaces, and living in crowded or unsanitary conditions (Kluytmans-Vandenbergh & Kluytmans, 2023).

Vancomycin is a drug used to treat MRSA, as it interferes with the bacteria's cell wall synthesis (*Figure 1*). Vancomycin is typically administered via various routes, including intravenous (IV) infusion, oral tablets, or intramuscular (IM) injection, depending on the severity and location of the MRSA infection (Wong et al., 2017). In particular, vancomycin targets a specific step in cell wall synthesis called peptidoglycan synthesis. Peptidoglycan is a crucial component of bacterial cell walls, providing structural support and protection to the cell. The mechanism of action of vancomycin involves binding to the D-Ala-D-Ala terminus of peptidoglycan precursors, which are essential building blocks for cell wall synthesis. By binding to these precursors, vancomycin prevents their incorporation into the growing peptidoglycan chain. This then inhibits the cross-linking of peptidoglycan molecules, weakening the cell wall structure and ultimately leading to cell lysis and death (Marshall et al., 1997).

One key mechanism behind vancomycin resistance in MRSA was the horizontal gene transfer of the VanA gene cluster. Enterococci bacteria, commonly found in the gastrointestinal tract, are known for their intrinsic resistance to various antibiotics. They possess genetic elements, such as the VanA gene cluster, that confer resistance to glycopeptide antibiotics like vancomycin. Horizontal gene transfer refers to the transfer of genetic material between different species, often facilitated by mobile genetic elements like plasmids. In the case of MRSA, the acquisition of the VanA gene cluster through horizontal gene transfer enables the bacteria to modify their cell wall structure, making them less susceptible to vancomycin (Cong et al., 2019). For instance, the VanA gene cluster originally inherited from Enterococci bacteriawas then transferred to MRSA,

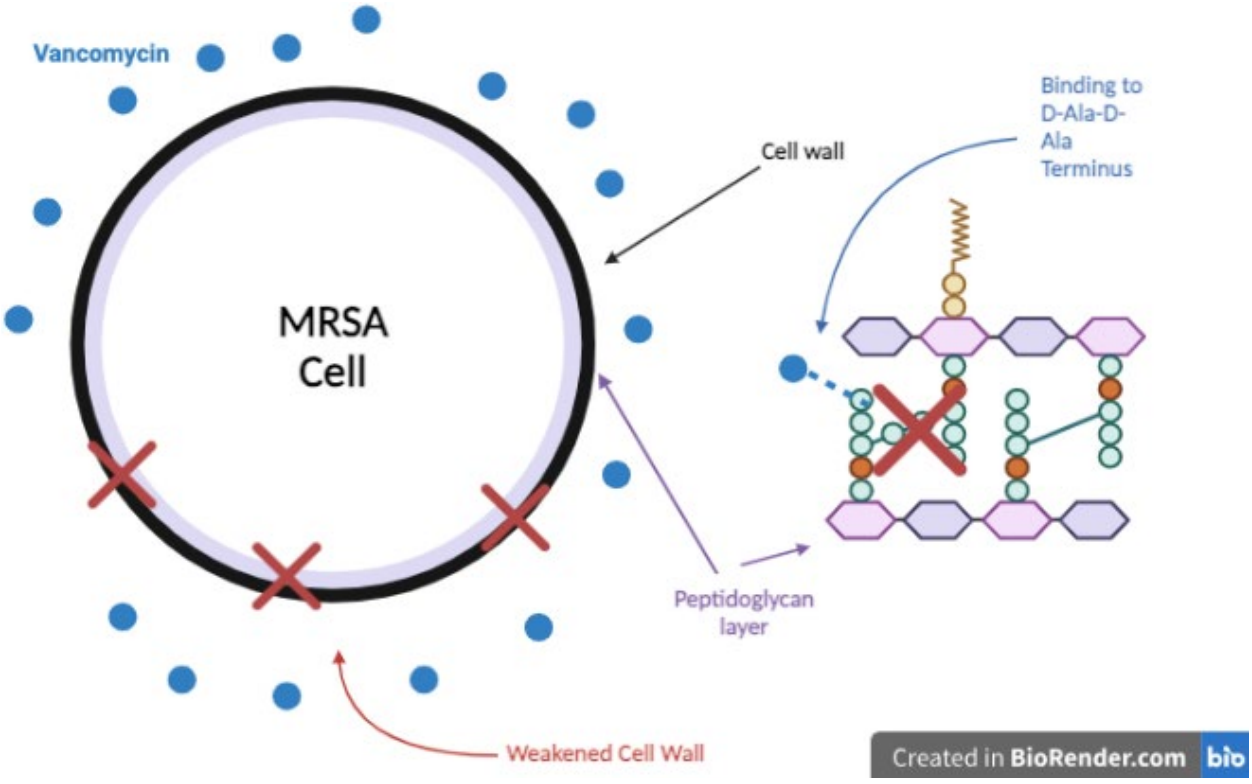

Figure 1. Mechanism of Action of Vancomycin on Bacterial Cell Wall Synthesis. Vancomycin binds to peptidoglycan precursors, inhibiting their incorporation into the cell wall and leading to weakened structural integrity and cell death.

where the bacteria continued to transfer the gene cluster to other MRSA bacteria (Périchon & Courvalin, 2009). The VanA gene cluster contained genes responsible for modifying the bacterial cell wall's peptidoglycan structure, which is the target of vancomycin's antimicrobial activity. By altering this structure, MRSA with the VanA gene cluster became resistant to the effects of vancomycin. This genetic adaptation has significantly complicated treatment options for MRSA infections, as vancomycin was traditionally relied upon as a potent antibiotic against Gram-positive bacteria, including MRSA strains (Dhungel et al., 2021).

Further, within the MRSA plasmid, it is important to distinguish that genes such as mecA and mecC confer resistance to methicillin, a β-lactam antibiotic, while blaZ mediates resistance to other β-lactam antibiotics (*Figure 2*). This genetic evolution has equipped MRSA with a strong defense against antibiotics, necessitating a shift in treatment approaches. The emergence of vancomycin resistance, particularly through the evolution of the vanA group, represents the continuing evolution of antibiotic resistance mechanisms, necessitating proactive strategies to address this growing threat (Dhungel et al., 2021).

In the evolution of modern-day MRSA, vancomycin resistance and β-lactam resistance overlapped. Therefore, the duality of vancomycin resistance and β-lactam resistance demonstrates the complexity in combating MRSA infections. β-lactam antibiotics, such as penicillins and cephalosporins, target bacterial cell wall synthesis. However, MRSA's ability to produce β-lactamase enzymes, including blaZ, enables it to evade the effects of β-lactam antibiotics (Okiki et al., 2020). The blaZ gene encodes for the β-lactamase enzyme, which breaks down β-lactam antibiotics such as vancomycin by cleaving their β-lactam ring structure, rendering antibiotics ineffective against the bacterium and leading to further resistance (Arêde et al., 2013).

The resistance mechanisms in MRSA are intricate, involving various molecular components that work together to confer resistance to these antibiotics. Among these components, the vancomycin resistance

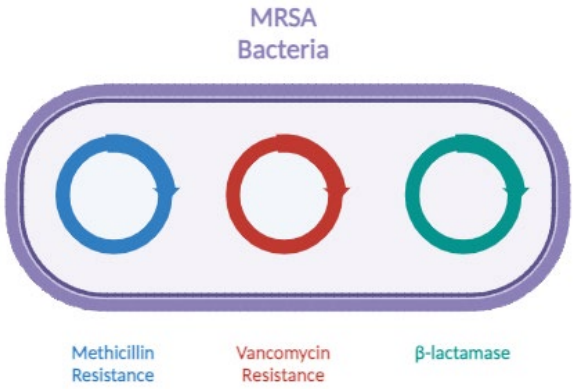

Figure 2. Components Contributing to Antibiotic Resistance in MRSA - Including Methicillin, BlaZ, and VanA

system encompassing the VanS and VanR proteins, as well as several other vancomycin resistance genes, play a pivotal role (Hong et al., 2008).

Current treatment protocols often rely on combining vancomycin with β-lactamase inhibitors (Tran & Rybak, 2018). However, this approach is not without limitations. β-Lactamase inhibitors, while useful in addressing β-lactamase production in MRSA, have significant limitations in treating MRSA's resistance to vancomycin. MRSA employs multiple mechanisms to resist antibiotics, including β-lactamase production and the alteration of target sites for antibiotics like vancomycin. This dual resistance makes β-lactamase inhibitors insufficient on their own (Alghamdi et al., 2023). Additionally, the use of these inhibitors can lead to the development of further resistance, as MRSA can mutate or acquire new resistance genes (Périchon & Courvalin, 2009). The inhibitors also pose risks of adverse reactions and can disrupt the natural microbiome, potentially causing secondary infections. These limitations highlight the need for more targeted and comprehensive treatments for MRSA beyond β-lactamase inhibition alone. The potential for MRSA to develop resistance to these combination therapies provides evidence for the urgency of exploring alternative strategies. Innovative approaches, such as gene-editing interventions, hold promise in overcoming the challenges posed by antibiotic-resistant MRSA strains (Tao et al., 2023). These strategies offer targeted and

precise mechanisms to disrupt resistance pathways, paving the way for more effective and sustainable treatment options.

The CRISPR-Cas9 system has shown promise in eliminating the VanA gene, which is responsible for vancomycin resistance in MRSA. In a study, single guide RNAs (sgRNAs) were designed to target the VanA gene, and these were cloned into a CRISPR-Cas9 plasmid. This system was introduced into bacterial cells via chemical transformation and conjugation methods. The results demonstrated that the CRISPR-Cas9 system effectively cleared VanA-harboring plasmids, significantly reducing bacterial resistance to vancomycin. However, despite its potential, there are limitations to using CRISPR for this purpose. One significant challenge is the delivery efficiency of the CRISPR-Cas9 system into bacterial cells, which is not spontaneous and may limit clinical application. Additionally, the potential for off-target effects and the need for precise sgRNA design to ensure high specificity and activity remain concerns. Finally, the method's efficacy depends on preventing the horizontal transfer of resistance genes, a complex task given the dynamic nature of bacterial gene exchange (Périchon & Courvalin, 2009).

Systems level

The VanS protein is a critical component of the vancomycin resistance system, acting as a transmembrane sensor histidine kinase. It detects vancomycin in the environment, triggering autophosphorylation upon binding to the drug. This activates VanS before phosphorylatingVanR, a response regulator protein, which in turn acts as a transcription factor. Phosphorylated VanR initiates the expression of several vancomycin resistance genes, such as VanA, VanX, VanH, and many more (*Figure 3*). These genes are critical in vancomycin resistance. For instance, the VanA encodes the ligase enzyme that modifies peptidoglycan precursors. This modification alters the binding site for vancomycin, rendering the antibiotic ineffective in inhibiting cell wall synthesis. Additionally, the VanX gene hydrolyzes the D-Ala-D-Ala dipeptide and

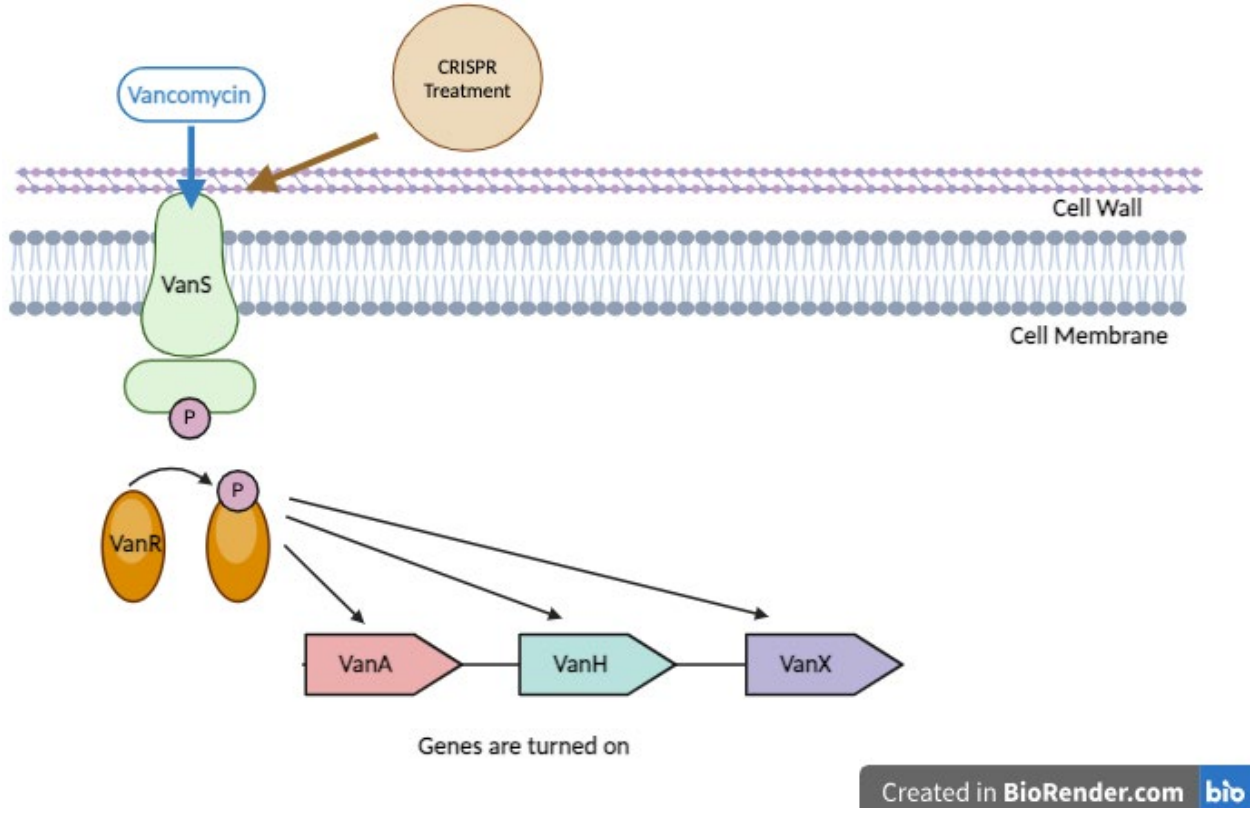

Figure 3. The VanS/VanR Signaling Pathway in MRSA: A Mechanism of Vancomycin Resistance.

the VanH gene participates in the biosynthesis of altered peptidoglycan precursors, respectively (Grasty et al., 2023). A tailored biological system is designed to address vancomycin resistance mechanisms in MRSA. This system centers on leveraging CRISPR-Cas9 technology, which utilizes guide RNAs (gRNAs) to direct the Cas9 enzyme to the specific genomic locations, where it introduces precise alterations. In this case, the focus is on deactivating the VanS and VanR genes, which are pivotal components of the vancomycin resistance mechanism in MRSA. By strategically targeting these genes using CRISPR-Cas9, the aim is to prevent the action of the other vancomycin resistance genes downstream in the pathway. The goal is to disrupt this signaling pathway at its inception. This disruption would prevent the activation of the vancomycin resistance genes, effectively "switching off" the mechanism responsible for vancomycin resistance in MRSA (*Figure 4*).

Focusing on the vancomycin resistance mechanism, particularly the VanS/VanR system, is crucial due to several reasons. Understanding and directly targeting the vancomycin resistance mechanism hold the potential to revitalize treatment strategies for MRSA infections, which are notorious for their resistance to multiple antibiotics.

The specificity of targeting the vancomycin resistance mechanism sets it apart from more generalized resistance mechanisms like mecA or blaZ (Okiki et al., 2020). By homing in on the VanS/VanR system, interventions can be tailored with greater precision, potentially minimizing off-target effects and risks of exacerbating antibiotic resistance (Tao et al., 2023), which is a growing concern in healthcare settings.

Device level

Combining this CRISPR-Cas9 technology with a traditional antibiotic treatment like vancomycin presents a promising strategy for combating antibiotic resistance in MRSA. This approach targets and overcomes the specific resistance mechanisms within

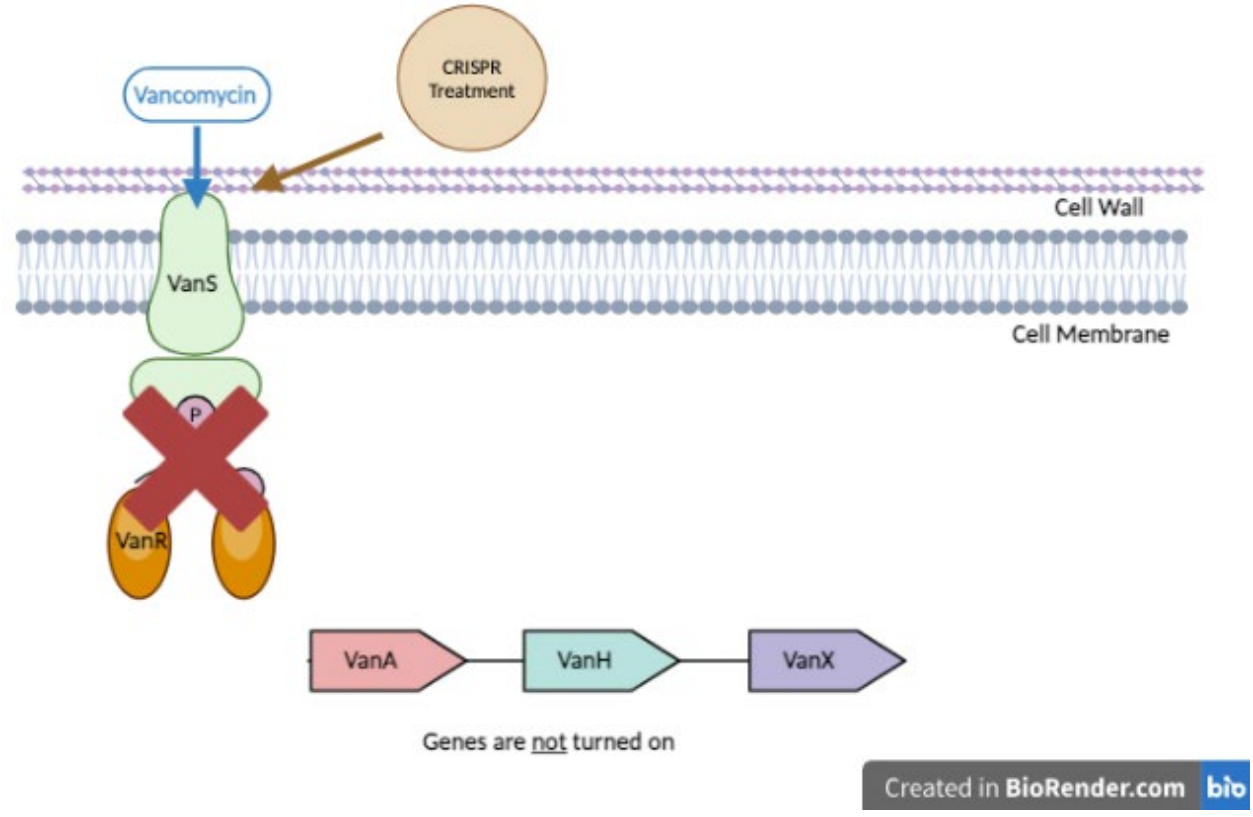

Figure 4. Illustrates the CRISPR-Mediated Disruption of the VanS/VanR signaling pathway in MRSA.

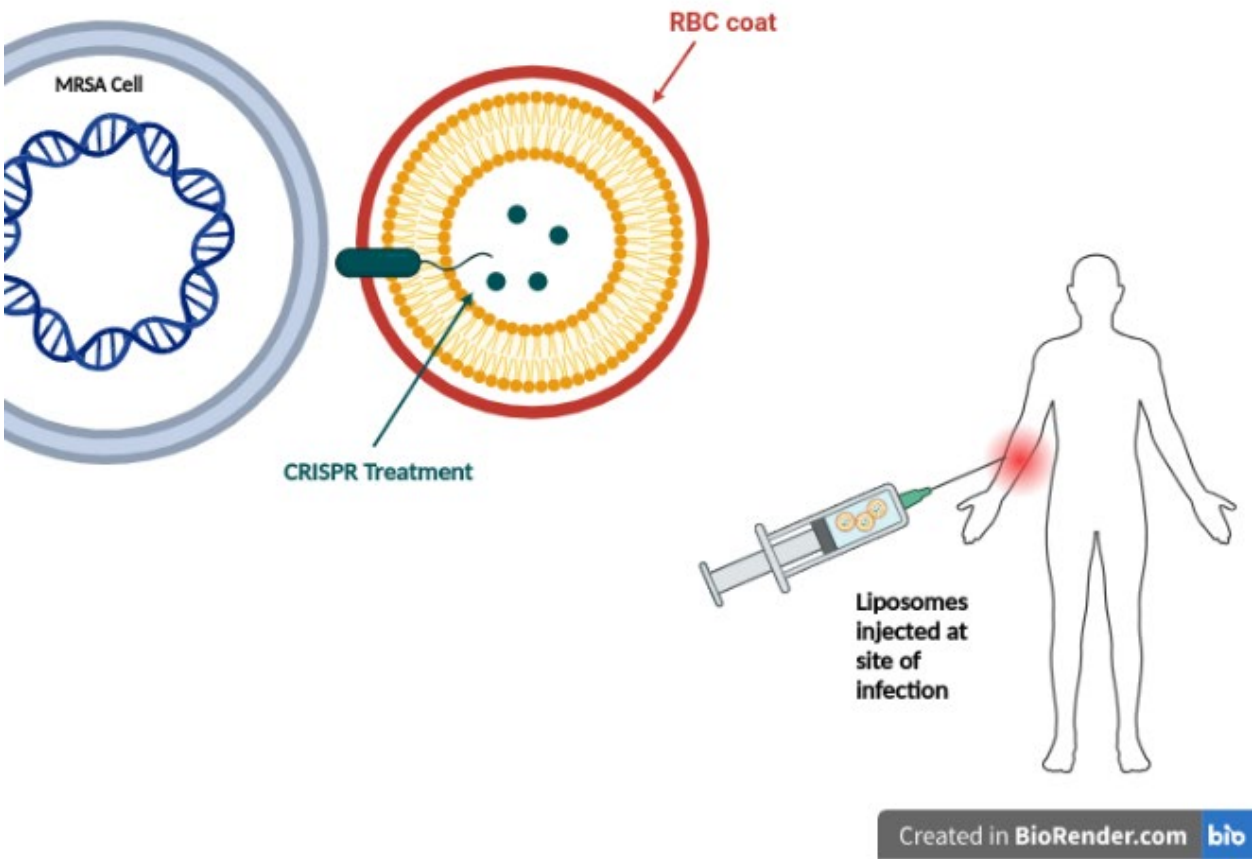

Figure 5. Liposomes used for targeted delivery of CRISPR treatment to site of MRSA infection.

MRSA strains, offering a multifaceted solution to a complex problem (Tao et al., 2023). The main component of this approach is the CRISPR-Cas9 system, consisting of several key components that work together to achieve targeted genome editing and sensitization of MRSA to vancomycin.

Functionally, this approach aims to disrupt the vancomycin resistance mechanism mediated by MRSA's VanS/VanR system. VanS, a sensor protein, detects vancomycin and activates downstream resistance genes through VanR. By designing gRNAs to target critical regions within VanS and VanR, the signaling pathway responsible for vancomycin resistance can be disrupted effectively. Upon delivery into MRSA cells, Cas9 guided by gRNAs recognizes and cleaves target DNA sequences, initiating DNA repair mechanisms such as non-homologous end joining (NHEJ) or homology-directed repair (HDR) (Xue & Greene, 2021). This targeted genetic modification renders the vancomycin resistance mechanism non-functional, thereby sensitizing MRSA to vancomycin treatment.

The treatment would be delivered through a sequential approach. The first step is the delivery of the CRISPR-Cas9 system.This can be achieved using a viral vector, such as the adeno-associated virus (AAV), engineered to carry the Cas9 gene and the guide RNA sequences to the specific VanS/VanR genes (Xue & Greene, 2021). Once delivered, the Cas9 protein guided by the designed gRNAs initiates precise DNA cleavage at the VanS and VanR genes, disrupting the resistance mechanism.

Several strategies can be employed to ensure specificity and efficacy for precise delivery and targeting of the CRISPR-Cas9 system to the site of the MRSA infection. One approach is to use localized delivery systems that target the CRISPR-Cas9 components directly to the infected area. This can be achieved through a liposome formulation to encapsulate and inject the

CRISPR-Cas9 components specifically at the site of the MRSA infection (Rani et al., 2022). They are also coated with red blood cells (RBCs) to act as a biomimetic agent, preventing immune cells from recognizing and attacking them (*Figure 5*). These localized delivery systems minimize systematic exposure and enhance the concentration of CRISPR-Cas9 near the target cells, increasing the likelihood of effective gene editing.

Following the gene editing process, vancomycin treatment should be initiated. The timing of vancomycin administration is crucial, typically after allowing sufficient time for the CRISPR-Cas9 system to induce gene editing and sensitize MRSA cells to vancomycin's effects.

The combined therapy will allow for a synergistic effect. The gene-edited MRSA cells will become more susceptible to vancomycin, enhancing the effect of the antibiotic that will be later given. This synergy will improve treatment outcomes and reduce the changes of antibiotic resistance development. Continuous monitoring of the patient's response to the therapy will be needed, including evaluating bacterial load, assessing clinical symptoms, and monitoring for adverse effects or resistance emergence during treatment (Wong et al., 2017).

Parts level

The CRISPR-Cas9 system, when designed to target the VanS and VanR genes in MRSA, utilizes specific genetic components to disrupt the signaling pathways associated with vancomycin resistance. The key components involved in this targeted gene editing include guide RNAs (gRNAs), promoters, terminators, and the Cas9 enzyme (*Figure 6*).

The first critical component is the gRNA that leads the Cas9 to the VanS protein. It is designed to target the VanS gene within MRSA specifically. The VanS gene encodes a sensor kinase crucial for detecting vancomycin presence, a pivotal antibiotic in MRSA treatment. The VanS gRNA guides the Cas9 enzyme to the VanS gene, inducing double-strand breaks at precise genomic

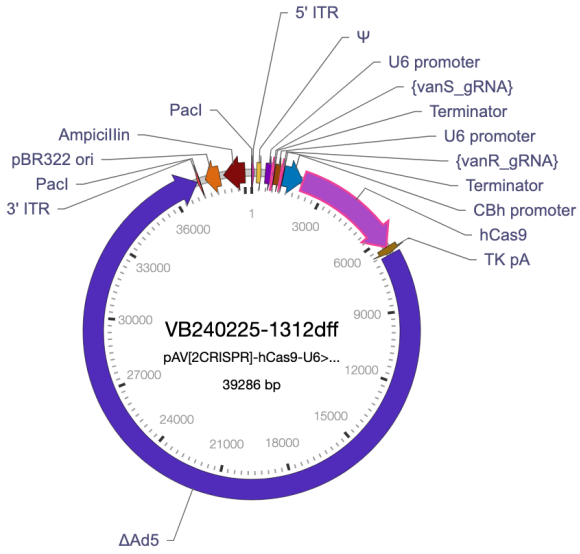

Figure 6. CRISPR-Cas9 Plasmid with VanS and VanR gRNAs.

locations. This disruption interferes with the sensor kinase's function, hindering its ability to activate the vancomycin resistance pathway (Périchon & Courvalin, 2009).

Similarly, the VanR gRNA targets the VanR gene, which encodes a response regulator protein involved in regulating the transcription of vancomycin resistance genes. By guiding Cas9 to VanR, the VanR gRNA initiates double strand breaks within VanR, disrupting its regulatory role. This disruption prevents the activation of downstream resistance genes in response to vancomycin exposure, further sensitizing MRSA to antibiotic treatment (Périchon & Courvalin, 2009).

The U6 promoter sequences drive the expression of these gRNAs, ensuring their production within the CRISPR-Cas9 system. These promoters are essential for initiating the transcription of gRNAs targeting VanS and VanR, respectively. Following transcription, the gRNAs guide Cas9 to these specific sites for targeted gene editing (Ma et al., 2014).

The Cas9 enzyme, encoded by the hCas9 gene within the CRISPR-Cas9 system, plays a role in executing the targeted gene editing. Cas9 generates double-stranded breaks at the locations guided by the gRNAs, initiating the cell's DNA repair mechanisms. This repair process often leads to mutations or disruptions in the VanS and VanR genes,

impairing their function and effectively sensitizing MRSA to vancomycin treatment. This is because this disruption prevents MRSA from sensing vancomycin accurately and activating its resistance mechanisms, ultimately enhancing the antibiotic's effectiveness against this pathogen (Redman et al., 2016).

Terminators located downstream to the gRNA sequences facilitate transcription termination, ensuring precise control over gRNA expression levels. This controlled expression is crucial for maintaining the balance between effective gene editing and minimizing off-target effects (*Vectorbuilder*, n.d.).

Safety

The design of CRISPR and gRNAs is essential to precise targeting of the intended sequences within the MRSA genome, guiding the Cas9 enzyme to induce double strand breaks at these locations. This targeted approach minimizes the risk of off-target effects, where unintended genetic modifications could occur elsewhere in the genome (Tao et al., 2023). This CRISPR design is meticulously engineered to minimize off-target effects, ensuring precision in genetic editing. Through careful selection of guide RNAs (gRNAs) and thorough computational analysis, we have identified sequences with minimal homology to unintended genomic regions. Additionally, employing advanced Cas proteins with enhanced specificity further enhances the precision of our editing. Rigorous validation protocols, including deep sequencing and functional assays, validate the accuracy of our CRISPR system, providing confidence in its ability to target the desired loci with minimal off-target effects. This approach not only enhances the safety of genetic modifications but also underscores our commitment to ethical and responsible genome editing practices (Sentmanat et al., 2018). In vitro assays can be used to assess the specificity, efficiency, and potential off-target effects before progressing to *in vivo* studies. Firstly, the designated gRNAs would be synthesized and purified to ensure their integrity and functionality. Next, they are introduced into cell cultures containing MRSA strains to evaluate their ability to guide the Cas9 enzyme to the target genes, VanS and VanR.The cells are then analyzed to determine if the gRNAs effectively induce the double-stranded breaks they need to at the intended genomic locations within the MRSA genome (Pavlou et al., 2022). Furthermore, *in vitro* assays would also involve functional assays to assess the impact of CRISPR-mediated gene editing on MRSA's vancomycin resistance. These assays may include measuring challenges in gene expression, protein levels, or phenotypic characteristics related to vancomycin sensitivity.

Primarily, an optimal dose of the CRISPR treatment must be established. This refers to determining the most effective and safe amount of CRISPR therapy and vancomycin for treating MRSA infection and allows for the right balance to be found between therapeutic efficacy and minimizing potential side effects (Asher et al., 2023). Firstly, dose-response studies will be conducted, where varying concentrations of CRISPR components and vancomycin will be tested against MRSA strains *in vitro*. These studies assess how different doses impact bacterial growth, survival, and the development of resistance mechanisms. Additionally, *in vivo* studies using animal models infected with MRSA will be conducted to evaluate the therapeutic response to different doses of CRISPR therapy and vancomycin. They will assess parameters such as bacterial load reduction, tissue-specific distribution, and host immune responses. Based on the results from these preclinical studies, researchers will optimize the doses for further testing in clinical trials (Levison & Levison, 2013). This optimization process will consider factors such as pharmacokinetics, pharmacodynamics, tissue distribution, and potential drug interactions.

After establishing the optimal dosages and administration schedules in preliminary experiments, researchers will move on to comprehensive safety assessments. These assessments will involve testing for off-target effects of CRISPR therapy. Moreover, carefully selecting liposomes as the delivery method will ensure precise targeting and reduce the risk of unintended genomic

modifications (Pavlou et al., 2022). Similarly, the combination of CRISPR therapy with vancomycin will require compatibility studies. There will need to be an evaluation of the interaction between CRISPR components and vancomycin to ensure no adverse effects or interference with each other's mechanisms of action (Li et al., 2021). Safety assessments for CRISPR treatments can include off-target analysis using high-throughput sequencing, genomic stability assays like karyotyping, indel assessment through T7 endonuclease I cleavage assays, transcriptomic analysis via RNA sequencing, functional assays to evaluate cellular behavior, immunogenicity assessment, and long-term stability testing to ensure minimal off-target effects and sustained therapeutic efficacy (Sentmanat et al., 2018). These assessments will need to examine potential drug-drug interactions, determine optimal timing for combined administration, and assess any synergistic or antagonistic effects on bacterial growth and resistance development.

Next, once preclinical testing is complete, the treatment can move on to human trials. They will be divided into the typical three phases of human clinical trials that are recommended by the FDA. Phase I will involve testing the combination treatment on a small group of MRSA patients who are otherwise healthy. These patients must be medically stable, free from significant comorbidities, and able to provide informed consent. Phase II will involve a larger group of MRSA patients, and this phase aims to gather more comprehensive data on the therapy's efficacy and safety in a real-world clinical setting. The main focus of this phase is on safety and feasibility. The patients in this phase must have confirmed MRSA infections who have not responded adequately to conventional antibiotic treatments. Phase III involves an even larger group of patients to further safety and efficacy and compare to existing treatments, such as using β-lactamase inhibitors. These comparisons will be done through randomized controlled trials that are double-blind and placebo controlled. Several critical signs will indicate the treatment's efficacy. The primary objective is microbiological clearance, where the therapy aims to eliminate or significantly reduce MRSA bacterial presence (FDA, 2023). This will be evaluated through cultures and quantitative PCR, measuring MRSA colony counts or detecting a negative conversion in culture samples post-treatment. In addition, clinical improvement will be a key marker observed in patients undergoing the therapy. Positive changes in the MRSA-related symptoms such as skin infections, fever, pain, and inflammation signify treatment success. Furthermore, a notable aspect of this therapy is its intended reduction in antibiotic resistance. By sensitizing MRSA strains to vancomycin, this design aims to reverse resistance mechanisms (Minnesota Department of Health, 2022). This is monitored through antibiotic susceptibility testing, comparing MRSA strains' response to vancomycin before and after treatment, with increased sensitivity indicating therapeutic efficacy.

Discussion

One of the primary benefits of this project is the potential to overcome antibiotic resistance in MRSA strains by sensitizing these bacteria to vancomycin through CRISPR-mediated genetic modifications (Xue & Greene, 2021). Additionally, CRISPR technology's precision allows for results in highly targeted interventions. This minimizes damage to beneficial bacteria and reduces the risk of adverse effects in patients. This precision contributes to personalized treatment strategies (Dhungel et al., 2021). Further, targeting resistance mechanisms directly offers a significant advantage. Firstly, it circumvents the need to develop entirely new antibiotics. Traditional antibiotic development faces challenges such as the time-consuming process, high costs, and emergence of resistance against newly introduced antibiotics (Lee et al., 2013). By addressing the specific genetic components that confer resistance in MRSA, this approach leverages existing antibiotics like vancomycin more effectively, extending their clinical utility. Additionally, directly targeting resistance mechanisms can delay or even reverse the development of resistance in MRSA strains. By disrupting the genetic

pathways responsible for antibiotic resistance, the design aims to render MRSA susceptible to vancomycin once again. This strategy not only improves treatment outcomes for current infections but also contributes to long-term strategies for managing antibiotic resistance in clinical settings.

However, some challenges accompany this design. Safety concerns regarding CRISPR-mediated genome editing require a lot of evaluation and monitoring. Off-target effects, unintended genetic alterations, and immune responses to CRISPR components must be addressed to ensure patient safety and regulatory approval (Ayanoğlu et al., 2020). Another challenge is the complexity of delivering CRISPR components alongside conventional antibiotics like vancomycin. This is because optimizing delivery methods, ensuring efficient uptake by bacterial cells, and minimizing host immune responses are ongoing areas of research and development (Huang et al., 2022).

While effective in some cases, the current approach to targeting vancomycin resistance, the use of β-lactamase inhibitors, has limitations in its scope of action. β-lactamase inhibitors primarily target resistance mechanisms related to β-lactam antibiotics, such as penicillins and cephalosporins (Tran & Rybak, 2018). In contrast, this CRISPR design targets the vancomycin resistance mechanism specifically, addressing a critical aspect of MRSA infections in which vancomycin is the antibiotic of choice. In addition, β-lactamase inhibitors are often used in combination with β-lactam antibiotics (such as vancomycin), requiring patients to take multiple medications (Tran & Rybak, 2018). This can increase the complexity of treatment regimens, potentially leading to medication errors, reduced patient compliance, and increased healthcare costs (Jimmy & Jose, 2011). In contrast, this design aims to enhance the efficacy of vancomycin as a standalone antibiotic by preventing the activation of vancomycin resistance genes in MRSA strains. Moreover, β-lactamase inhibitors may face challenges related to bacterial resistance development over time (González-Bello et al., 2019). Bacteria can evolve mechanisms to bypass or counteract the effects of β-lactamase inhibitors, leading to treatment failures and the emergence of more resistant bacterial strains. By targeting vancomycin resistance mechanisms through CRISPR-based interventions, this design offers a targeted approach that reduces the likelihood of resistance development.

Further improvements to the system could involve refining the CRISPR editing techniques for enhanced specificity and efficiency (Dhungel et al., 2021). Advancements in delivery systems, such as nanoparticle-based carriers, could improve targeted delivery to MRSA strains while reducing off-target effects (Mizra, 2021). Moreover, integrating CRISPR with other therapies, such as immunotherapies or phage therapies, may further enhance treatment outcomes and reduce the risk of resistance development.

Next steps

The next steps could involve an in vitro experiment where MRSA strains are cultured and subjected to varying concentrations of vancomycin and CRISPR components, both individually and in combination (*Figure 7*). This would allow for an assessment of bacterial growth inhibition in the MRSA infections and determine whether or not synergistic effects are occurring. To model these effects, *Escherichia coli* (*E. coli*) would be used instead of MRSA in a high school lab. *E. coli* provides safety and ease of cultivation compared to MRSA, which proposes greater risks in a non-specialized lab environment (Yokoyama, 2020).

Nutrient agar plates would be prepared, and an *E. coli* culture would be inoculated onto a prepared nutrient agar plate using sterile techniques. The inoculated plates would then be incubated at around 37°C, the optimal temperature of *E. coli* cell growth. This step would allow the *E. coli* to proliferate and form visible colonies on the agar plates (Yokoyama, 2020).

Then, once the colonies are visible, individual colonies would be selected for genetic modification. They would be modified to mimic MRSA cells through the injection of a plasmid containing VanS, VanR, and several other vancomycin

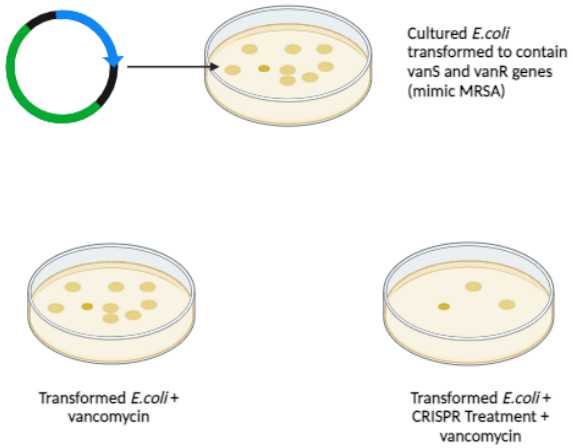

Figure 7. Experiment using transformed E. coli to assess bacterial growth inhibition with vancomycin + CRISPR treatment.

resistance genes that VanR turns on. Competent *E. coli* cells would be prepared and mixed with the plasmid containing the VanS, VanR, and other vancomycin resistance genes. The mixture would undergo a heat shock treatment to facilitate the uptake of the plasmid by the *E. coli* cells. After the heat shock, the cells would recover in LB broth before being plated on agar plates containing ampicillin to serve as a selective agent.

Following the successful transformation and selection of vancomycin-resistant *E. coli* colonies to mimic MRSA cells, the sensitivity of these colonies to vancomycin would be tested. This would be done by inoculating the colonies into LB broth and allowing them to grow. Then, aliquots of the culture would be placed onto agar plates with and without vancomycin. The plates would be incubated overnight, and the growth of *E. coli* colonies would be observed. This test would help determine whether the vancomycin resistance genes confer resistance to vancomycin in *E. coli*.

To assess the effectiveness of the treatment for the vancomycin resistance genes, the CRISPR-Cas9 system targeting the VanS and VanR genes could be introduced into the vancomycin-resistant *E. coli*. The CRISPR-Cas9 system would be delivered to the cells and subsequent vancomycin sensitivity testing would be performed to compare the growth of *E. coli* with vancomycin alone and with vancomycin in combination with CRISPR treatment. This would help evaluate the ability of CRISPR to enhance the efficacy of vancomycin treatment by targeting the resistance mechanisms of bacteria.

Author contributions

In developing this paper, E.P., was responsible for researching, planning, and drafting the content independently. Throughout the process, E.P. collaborated closely with her mentor, L.L., whose guidance and expertise significantly enriched the project. L.L. provided invaluable insights, offered critical feedback on the experimental design and methodology, and helped refine the presentation of results and discussions. Their mentorship played a pivotal role in ensuring the scientific rigor and clarity of the paper, contributing to its overall quality and coherence.

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