How can the CRISPR system be utilized as an innovative approach to combat antibiotic resistance by specifically targeting efflux pumps in bacteria?

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Abstract

Antibiotic resistance has become a significant threat to global public health, rendering conventional treatments ineffective against bacterial infections. Efflux pumps, which actively expel antibiotics from bacterial cells, play a pivotal role in mediating this resistance. This review paper aims to explore the viability of utilizing CRISPR/Cas9 to disrupt efflux pumps in bacterial pathogens, potentially controlling antibiotic resistance. In our paper, we critically evaluate the current state of knowledge and future prospects of antibiotic resistance. We seek to shed light on the promising avenue of precision gene editing in the fight against antibiotic-resistant bacteria. We will go over three different papers written about the use of CRISPR in bacteria and its relation to efflux pumps. 2 of the papers will investigate how CRISPR/cas9 or CRISPRi can be used to lessen the effects of efflux pumps, and one of the papers will look at the natural CRISPR system in a bacteria and how it affects antibiotic resistance. Ultimately, this research paper aims to offer valuable insights and promote innovative strategies to effectively mitigate the global prevalent threat of antibiotic resistance.

Section 1: Introduction

The antibiotic resistance crisis is a growing global concern that arises from the overuse and misuse of antibiotics, leading to the development of bacteria that are resistant to these drugs. (McAdam et al. 2012) Antibiotics are essential in treating bacterial infections and have been pivotal in modern medicine, saving countless lives since their discovery. However, the misuse of antibiotics in both human and animal health and agriculture has accelerated the emergence of antibiotic-resistant bacteria, often called "superbugs." The emergence of superbugs, limited treatment options available, the global health impact, economic consequences, and the ineffectiveness of current antibiotics all make this problem very prevalent and dangerous to society. Proteins called efflux pumps in bacteria are a significant contributor to the increasing problem of antibiotic resistance. (Sharma et al., 2019) Efflux pumps are specialized proteins located in the cell membranes of various organisms that actively transport a diverse range of substances, including toxins, drugs, and potentially harmful compounds, from inside the cell to the external environment. More resistant bacteria are usually correlated with an increase in efflux activity in the cells. (Du et al., 2018). Research on efflux pumps has been conducted across various bacterial species, each focusing on distinct aspects of antibiotic resistance. However, a comprehensive examination of the potential applications of CRISPR/Cas-9 in addressing efflux pump-related challenges in many bacterial species is absent from existing studies. This paper aims to review recent case studies that employ CRISPR/Cas9 technology to target efflux pumps as a means of combating antibiotic-resistant bacteria. This paper will encompass a diverse range of bacterial species to offer a holistic perspective on this innovative approach.

Section 2: Efflux Pumps

At its core, antibiotic resistance refers to the ability of bacteria to withstand the effects of antibiotics, rendering these drugs ineffective. Bacterial antibiotics work by blocking crucial cellular processes in bacteria and triggering cellular responses that lead to bacterial cell death (Kohanksi et al., 2010). This phenomenon arises due to one pivotal mechanism: the utilization of efflux pumps. Efflux pumps are crucial defense mechanisms employed by bacteria, enabling them to expel not only antibiotics but also a diverse array of other compounds from within the cell. This general defense mechanism makes efflux pumps a robust strategy for bacteria to resist multiple antibiotics, posing a significant challenge in treating infections. Efflux pumps are very complex, resulting in a wide variety of these pumps with distinct functions and substrate specificities. Efflux pumps are categorized into families like the Major Facilitator Superfamily (MFS), Multidrug And Toxic compound Extrusion (MATE), Small Multidrug Resistance (SMR), and ATP-Binding Cassette (ABC) based on their structure and substrate specificity. Some, like MFS and MAR, share topological similarities, while others are distinguished by sequence homology or phylogenetic differences (Chetri, 2023). Many of these efflux pumps, especially in the MFS and ABC families, confer multidrug resistance. They are capable of expelling a broad range of substances, including different classes of antibiotics and non-antibiotic drugs. These pumps are present across a range of organisms, from bacteria and fungi to higher eukaryotes. The presence of efflux pumps in both prokaryotes and eukaryotes suggests an ancient evolutionary origin. Some pumps, like SMR, are exclusive to bacteria, while MET pumps are specific to eukaryotes. (Van Bambeke et al. 2000) Efflux pumps consist of ABC transporters, which are a diverse group of proteins responsible for the ATP-dependent import or export of various solutes. They consist of transmembrane domains (TMDs), with substrate-binding pockets and nucleotide-binding domains (NBDs) for ATP binding and hydrolysis, which drive the transport process. (Abdi et al. 2020) The two main categories of ABC exporters are homodimeric and heterodimeric exporters. Heterodimeric ABC exporters, such as PatAB, LmrCD, BmrCD, and EfrCD, are particularly relevant for antibiotic resistance in Gram-positive bacteria. While homodimeric exporters typically have two equivalent ATP-binding sites, heterodimeric exporters possess a degenerate binding site that doesn't support ATP hydrolysis. This reduced ATP hydrolysis capacity can alter the dynamics of the transport cycle, potentially enhancing resistance to antibiotics by impeding drug expulsion (Van Bambeke et al., 2000). It is essential to be able to lessen or completely eliminate the effects of efflux pumps in order to eradicate the problem of antibiotic resistance. One approach to mitigating the influence of efflux pumps involves the utilization of CRISPR-Cas9 genetic engineering technology.

Section 3: CRISPR CAS-9

CRISPR/Cas9 is a groundbreaking gene-editing technology that is transforming biomedical research. It allows for the correction of genetic errors, gene regulation, and rapid gene manipulation in cells and organisms. In the laboratory, it has been successfully used to correct genetic defects in mice and human embryos, offering potential clinical applications such as gene therapy, treating diseases like HIV, and customizing treatments for cancer. In the field of pediatrics, CRISPR/Cas9 holds promise for clinical applications, but there are ethical and safety concerns that need to be addressed. For example, there are concerns about the long-term effects of genetic modifications and the potential for unintended consequences in children. Another form of CRISPR is CRISPRi. CRISPRi and CRISPR/cas9 have quite a few differences.

In the CRISPR/Cas9 system, Cas9 is an endonuclease enzyme that can cut DNA at specific target sequences guided by a customizable RNA molecule called guide RNA (sgRNA or gRNA). It is used for DNA editing and modification (Vidyasagar, 2018). In contrast, CRISPRi uses a catalytically inactive or "dead" Cas9 (dCas9) protein. dCas9 cannot cut DNA like the active Cas9 but is used to interfere with gene expression by blocking or repressing the transcription of specific genes. The guide RNA in CRISPRi also directs dCas9 to the target gene's promoter region to achieve this repression (Jensen et al., 2021). CRISPRi is derived from the CRISPR/Cas9 system; it uses a modified version of the Cas9 protein called dCas9 (dead Cas9) to interfere with gene expression without making changes to the underlying DNA sequence. Both CRISPR/cas9 and CRISPRi are effective tools with distinct applications in genetic research and clinical potential, and they are both useful techniques to target efflux pumps.

Section 4: Case studies

In the following section, I will conduct a comprehensive literature review encompassing all relevant cases of CRISPR-Cas9 in conjunction with efflux pumps. It will cover CRISPRi in Targeting AcrAB-TolC Efflux Pump in E. coli, CRISPR-Cas in A. baumannii, and Antibiotic Resistance where researchers investigated the role of CRISPR-Cas systems in Acinetobacter baumannii, and lastly, CRISPR-Cas9 Gene Editing in Candida albicans, which focuses on using CRISPR-Cas9 gene editing to target and disrupt genes associated with efflux pumps in the fungal pathogen Candida albicans.

In the research paper, "Engineering a CRISPR interference system targeting AcrAB-TolC efflux pump to prevent multidrug resistance development in Escherichia coli," the researchers use the bacteria Escherichia coli (E. coli), specifically the E. coli strain HB101. This strain is commonly used in laboratory research and is a well-known model organism for various genetic and molecular biology studies. The researchers use a CRISPR interference (CRISPRi) system to target the AcrAB-TolC efflux pump. They designed single-guide RNAs (sgRNAs) to target components of the AcrAB-TolC efflux pump, including AcrA, AcrB, and TolC. These sgRNAs are designed to bind to the non-template strand of the genes and interfere with their transcription, reducing the expression of the efflux pump components. In their experiments, the researchers primarily use a form of gene silencing with the CRISPRi system. Gene silencing involves reducing the expression of specific genes without making permanent changes to the DNA sequence (Wan et al., 2022). In this case, the sgRNAs are guiding the Cas9 protein to bind to the target genes' promoter regions, preventing their transcription and thereby reducing the expression of the AcrAB-TolC efflux pump components. This is a reversible and temporary method of gene regulation, as it does not alter the underlying DNA sequence. The protein-coding regions of the genome are primarily being targeted with the researchers' CRISPRi system. Specifically, they designed sgRNAs to bind to the non-template strands of the coding sequences (CDs) of the AcrA, AcrB, and TolC genes. By targeting the CDs, they aim to interfere with the transcription of these genes and reduce the expression of the corresponding proteins that make up the AcrAB-TolC efflux pump. The effectiveness of the CRISPRi method varied depending on the specific sgRNAs used and the target genes. The sgRNAs designed to target AcrA, AcrB, and TolC were effective in reducing the transcription of these genes, with varying degrees of inhibition. The most effective sgRNA cluster, acrB1tolC2, resulted in a significant reduction in the transcription of all three genes (AcrA, AcrB, and TolC). They found that the CRISPRi system, which used sgRNAs, is much more effective in regulating the

AcrAB-TolC efflux pump by targeting acrA, acrB, and tolC than a previous study that used sRNAs (small regulatory RNAs)(Wan et al., 2022). This reduction in gene expression correlated with increased susceptibility to multiple antibiotics, such as rifampicin, erythromycin, and tetracycline. Additionally, the CRISPRi system reduced biofilm formation in E. coli HB101. Overall, the study demonstrated that the CRISPRi system could be a promising tool for preventing the development of multidrug resistance in E. coli by targeting, specifically, the AcrAB-TolC efflux pump.

The next study I reviewed was "CRISPR-Cas in Acinetobacter baumannii Contributes to Antibiotic Susceptibility by Targeting Endogenous AbaI ."In this paper, the researchers highlight the connection between the CRISPR-Cas system and antibiotic resistance in A. baumannii. They found that deleting any component of the CRISPR-Cas system led to increased antibiotic resistance because the CRISPR-Cas system was found to repress multidrug efflux pumps. Deletion of the system led to increased efflux pump activity, which is associated with antibiotic resistance. The researchers chose to study Acinetobacter baumannii, which is a multidrug-resistant bacterium that poses challenges in clinical anti-infective treatments. This bacterium can acquire multidrug resistance through various mechanisms, including horizontal gene transfer and mutations in its biology (Wang et al., 2022). It can survive in various environments and is a significant public health concern. CRISPR-Cas systems are classified into various types (I-VI) and subtypes. Type I-Fb CRISPR-Cas system is prevalent in A. baumannii and consists of specific Cas proteins and CRISPR arrays. The researchers investigate how the Type I-Fb CRISPR-Cas system in A. baumannii affects quorum sensing (which is how the bacteria communicate with each other via signaling molecules) and antibiotic resistance. The study focused on a clinical isolate called AB43, which possesses a complete I-Fb CRISPR-Cas system. They found that the Cas3 nuclease of the Type I-Fb CRISPR-Cas system in AB43 regulates quorum sensing. Specifically, it reduces the synthesis of the quorum sensing regulator abaI mRNA.This reduction leads to decreased efflux pump activity, weaker biofilm formation, increased production of reactive oxygen species (ROS), and decreased drug resistance (Wang et al., 2022). The study analyzed a collection of clinical A. baumannii isolates and found that most isolates with incomplete or no CRISPR-Cas systems were multidrug-resistant. This study highlights the crucial role of the CRISPR-Cas system in mediating antibiotic susceptibility in *Acinetobacter baumannii*. By regulating quorum sensing and efflux pump activity, the CRISPR-Cas system inadvertently affects the bacterium's ability to resist antibiotics. These findings offer new insights into the complex mechanisms behind antibiotic resistance in *A. baumannii*. Targeting or manipulating the CRISPR-Cas system could become a viable approach to combat the growing issue of multidrug resistance in this clinically significant bacterium.

The last paper I reviewed was "A CRISPR–Cas9-based gene drive platform for genetic interaction analysis in Candida albicans".The paper focuses on the genetic manipulation of the fungal pathogen Candida albicans, a leading cause of infection in immunocompromised individuals. It presents treatment challenges due to its close evolutionary relationship with humans, leading to limited non-toxic antifungal agents. Their study investigates efflux pump mutants in *C. albicans* and their role in mediating susceptibility to antifungal stress, particularly to fluconazole. Fluconazole holds significant importance in the treatment of fungal infections, particularly those caused by *Candida albicans*. They discovered that mutations in efflux pump genes, notably CDR11, significantly affected the organism's sensitivity to fluconazole. (Shapiro et al., 2018). The researchers then developed a GDA platform using CRISPR-Cas9 technology.

Candida albicans is a diploid organism, meaning it has two copies of each gene. So, traditional methods of genetic manipulation can be inefficient in diploids since altering both gene copies to study gene function is complex and time-consuming. The GDA platform simplifies this process. Also, In the context of antifungal and antibiotic resistance, the GDA platform allows researchers to swiftly create and study mutants lacking specific efflux pumps. Understanding how these pumps contribute to drug resistance can inform the development of more effective antifungal treatments. In their GDA system, a specially designed DNA donor molecule acts as a 'selfish' genetic element. When introduced into the organism, it not only replaces the targeted gene but also propagates itself to replace corresponding genes at other loci. Their research uncovered synthetic lethal interactions between transporters, providing insights into cellular ion homeostasis and potential therapeutic targets (Shapiro et al., 2018). The principles established in this study could be applied to other fungal pathogens, like *Candida auris*, known for multidrug resistance. Their research transforms the ability to perform genetic interaction analysis in *C. albicans*, offering insights into virulence factors and antibiotic resistance. In addition, the principles and methodologies developed could be applied to other fungal pathogens, potentially aiding in the treatment and understanding of various fungal infections.

Section 5: Discussion/Conclusion

In comparing the results of these three research papers, it is evident that they all employ CRISPR technology to target specific genetic elements associated with antibiotic resistance in different bacterial species. However, there are notable differences in their approaches and outcomes.

The first paper, which focuses on E. coli, demonstrates the successful use of CRISPR interference (CRISPRi) to target the AcrAB-TolC efflux pump components. The effectiveness of this approach varies depending on the specific single-guide RNAs (sgRNAs) used, with the most effective sgRNA cluster (acrB1tolC2) resulting in a significant reduction in the transcription of the targeted genes. This reduction in gene expression is correlated with increased susceptibility to multiple antibiotics, highlighting the potential for CRISPRi to combat multidrug resistance in E. coli.

In contrast, the second paper examines Acinetobacter baumannii, a multidrug-resistant bacterium. This paper is very different from the other two. While the other two papers use the CRISPR genetic engineering technology to go in and make edits, this paper investigates what happens if we turn off the natural CRISPR system in the bacteria, revealing the importance of CRISPR. The researchers found that deleting any component of the CRISPR-Cas system in A. baumannii leads to increased antibiotic resistance due to increased efflux pump activity. This finding emphasizes the role of the CRISPR-Cas system in repressing efflux pumps, and its deletion results in higher antibiotic resistance. The study also identifies the regulatory role of the Cas3 nuclease in quorum sensing and its impact on antibiotic resistance.

The third paper focuses on Candida albicans, a fungal pathogen, and its efflux pumps. This research paper introduces an innovative CRISPR-Cas9-based Gene Drive Array (GDA) platform specifically tailored for the genetic analysis of *Candida albicans*, which enables the efficient creation of homozygous double-deletion mutants by using a modified DNA donor molecule that acts as a 'selfish' genetic element. It replaces targeted genes and propagates to replace corresponding genes at other loci. The study focuses on dissecting the roles of efflux pump genes in antifungal resistance, especially towards fluconazole, revealing specific genes

like CDR11 that significantly impact the pathogen's drug sensitivity. Additionally, the research delves into adhesin genes critical for biofilm formation, a key factor in *C. albicans'* pathogenicity. Through high-throughput screening and comprehensive genetic interaction analysis, the researchers identify crucial virulence regulators and how these genetic networks adapt under various environmental stressors, including antifungal treatments. Their research marks a significant advancement in the field of fungal genetics, antibiotic resistance, and infectious disease research.

While CRISPR technology shows promise in combating antibiotic resistance, its effectiveness can vary due to many factors. The differences in the bacterial species themselves could contribute to variations in antibiotic resistance levels. A. baumannii, for instance, is notorious for its intrinsic and acquired antibiotic resistance mechanisms, often making it more resistant to antibiotics compared to E. coli or Candida albicans. The natural variation in antibiotic resistance among these species may explain some differences in the extent to which CRISPR-based interventions affected resistance levels. The variability in outcomes across these studies could also be attributed to the selection of specific genes or loci as targets for CRISPR-based interventions. Some genes may have a greater influence on antibiotic resistance, while others may have smaller or more nuanced effects. For example, In the E. coli study, the researchers designed single-guide RNAs (sgRNAs) to target components of the AcrAB-TolC efflux pump, including AcrA, AcrB, and TolC. The effectiveness of the CRISPR interference (CRISPRi) method varied depending on the specific sgRNAs used and the target genes. For instance, the sgRNA cluster acrB1tolC2 was highly effective in reducing the transcription of all three genes (AcrA, AcrB, and TolC), resulting in a significant reduction in antibiotic resistance (citation). On the other hand, some sgRNAs may have had lesser effects on specific target genes. In the A. baumannii research, the focus was on the Type I-Fb CRISPR-Cas system's impact on quorum sensing and antibiotic resistance. Deletion of its components boosted antibiotic resistance through increased efflux pump activity. This implies multiple CRISPR-Cas genes collectively influence antibiotic resistance

Overall, these papers showcase the versatility of CRISPR technology in addressing antibiotic resistance. They demonstrate its potential to reduce antibiotic resistance in different bacterial species through various strategies, including gene silencing (CRISPRi), regulatory role modulation (Cas3 nuclease), and precise gene editing (CRISPR-Cas9). The varying effectiveness of these methods across different organisms and targeted mechanisms emphasizes the need for tailored approaches in combating antibiotic resistance. The findings from these studies not only advance our understanding of the genetic foundations of antibiotic resistance but also open avenues for developing more effective treatments against resistant strains of bacteria and fungi. As we continue to explore the capabilities of CRISPR technology, it is important to consider the specificities of each organism and the ethical implications of genetic manipulation, ensuring that these powerful tools are used responsibly and effectively in the ongoing battle against antibiotic resistance.

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